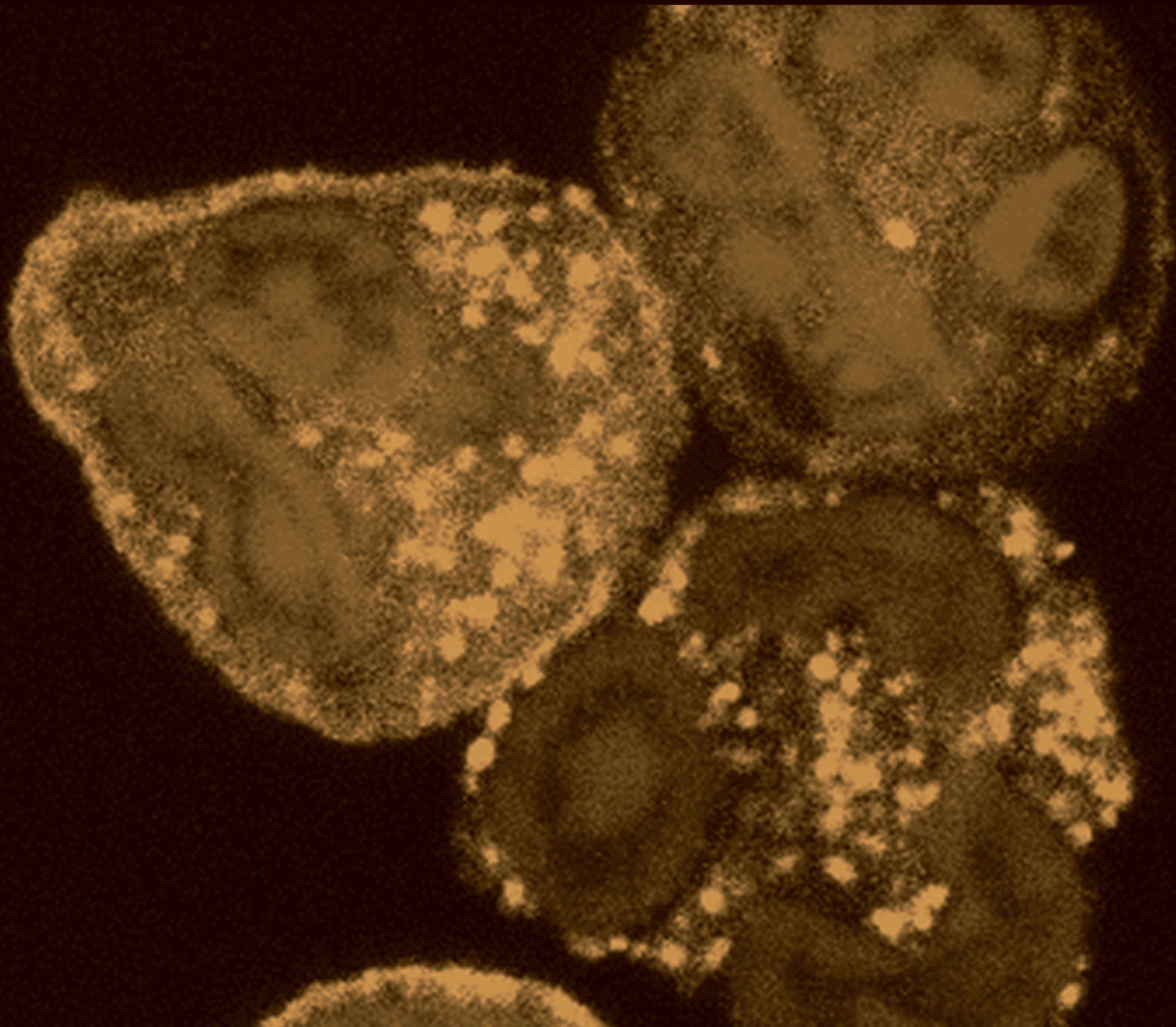
Mediators of Inflammation

Intravitreal Inflammation: From Benchside to Bedside

Guest Editors: Mario R. Romano, John Christoforidis, and Ahmed M. Abu El-Asrar



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Editorial

Intravitreal Inflammation: From Benchside to Bedside

Mario R. Romano,[1] John Christoforidis,[2] and Ahmed M. Abu El-Asrar[3]

[1] *Department of Ophthalmology, Istituto Clinico Humanitas, Rozzano, 20089 Milan, Italy*
[2] *Department of Ophthalmology, College of Medicine, The University of Arizona, Tucson, AZ 85711, USA*
[3] *Department of Ophthalmology, College of Medicine, King Saud University, Riyadh 11451, Saudi Arabia*

Correspondence should be addressed to Mario R. Romano; romanomario@email.it

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Inflammation plays a major role in the formation and in the progression of sight-threatening chorioretinal diseases such as diabetic retinopathy (DR), proliferative vitreoretinopathy (PVR), uveitis, and age-related macular degeneration (AMD). A greater understanding of the underlying pathological mechanisms is necessary for the development of better therapeutic agents and relies on the analysis of clinical specimens as well as on animal models. Contrary to the retina, the vitreous humour (VH) is a transparent gel that fills the posterior chamber of the eye and can be sampled without causing visual loss. In recent years, advances in the analysis of VH samples have highlighted new biological mechanisms of long-known diseases and have improved the accuracy of diagnostic procedures.

In this special issue, we report how the VH findings at the benchside can be translated to the bedside, and how this may help clinical practice. The papers have been contributed by a number of experts in the field and include both review articles that provide an overview of the work conducted to date, as well as original articles reporting recent discoveries and innovations. In order to highlight the translational relevance of VH analyses, most of the papers are focused on a specific disease entity. We hope that this series of manuscripts will be beneficial for clinicians in their diagnostic and therapeutic approaches towards intravitreal inflammatory conditions and for researchers in appreciating some of the recent innovations and their clinical implications in this field. Each of the manuscripts in this series is briefly highlighted as follows.

The paper by M. Angi et al. "*Proteomic analyses of the vitreous humour*" describes how to correctly collect and handle VH specimens and presents a clear workflow for proteomic analyses. This is significant since the VH is not a straightforward tissue to analyze due to its viscous consistency. Proteomic technologies have dramatically evolved over the past years, allowing identification of an increasing number of disease-specific proteins in the VH. Moreover, recent proteomic studies on the VH from animal models of autoimmune uveitis have highlighted new pathways associated to autoimmune triggers and intravitreal inflammation that could become the targets for much needed therapies.

Another example of the usefulness of proteomic analyses of the VH in translational research is presented by O. Simó-Servat et al. in "*Usefulness of the vitreous fluid analysis in the translational research of diabetic retinopathy*" who applied fluorescence-based difference gel electrophoresis (DIGE), as well as flow cytometry, to identify new candidates involved in the inflammatory process that occurs in DR. The authors provide evidence supporting the role of proinflammatory mediators such as cytokines (i.e., IL-1β, IL-6, IL-8, and TNFα), chemokines (i.e., MCP-1, SDF-1, and IP-10), and adhesion molecules (i.e., VCAM, ICAM-1, and VAP-1) in the pathogenesis of DR. Such persistent low-grade inflammation contributes to the damage of the internal blood-retinal barrier and to the development of proliferative diabetic retinopathy (PDR).

A. M. Abu El-Asrar et al. in "*Osteopontin and other regulators of angiogenesis and fibrogenesis in the vitreous from patients with proliferative vitreoretinal disorders*" and "*High-mobility group box-1 and endothelial cell angiogenic markers in the vitreous from patients with proliferative diabetic retinopathy*" investigate the role of osteopontin and other regulators of angiogenesis and fibrogenesis, such as high-mobility group box-1 (HMGB1) and connective tissue growth factor (CTGF) in the pathogenesis of proliferative

vitreoretinal disorders with a concomitant increase of antifibrogenic pigment epithelium-derived factor (PEDF) levels in the VH. Moreover, the authors report that HMGB1, soluble vascular endothelial-cadherin (sVE-cadherin), and soluble endoglin (sEng) regulate the angiogenesis of endothelial cells in PDR.

R. dell'Omo et al. in "*Vitreous mediators in retinal hypoxic disease*" describe that serum adiponectin (APN) levels correlate with blood inflammatory marker levels and with DR as response to endothelium dysfunction, indicating the role of APN as endogenous modulator of microvascular function and inflammation.

S. N. Moysidis et al. in "*Mechanisms of inflammation in proliferative vitreoretinopathy: from bench to bedside*" describe the indirect activation of PDGFRα by non-PDGFs as trigger that leads to development of PVR. In this pathway, the intracellular reactive oxygen species (ROS) plays a key role, leading to activation of Src family kinases (SFKs) that promote phosphorylation and activation of PDGFRα. The ROS could be one of the therapeutic targets of multimodal approach.

D. Gologorsky et al. in "*Therapeutic interventions against inflammatory and angiogenic mediators in proliferative diabetic retinopathy*" report the latest focus of targeted therapies for proliferative diseases through the block of vascular adhesion molecules such as ICAM-1, VCAM-1, inflammatory factors including the interleukins, tumor necrosis factor (TNF), insulin-like growth factor (IGF), and angiopoietins (Ang-2).

Analysis of the VH is a valuable adjunct also for the management of patients with uveitis and especially in the diagnosis of neoplastic diseases masquerading as chronic intraocular inflammation, as reported by E. M. Damato et al. in "*Vitreous analysis in the management of uveitis.*" For example, increased levels of T-cell cytokine, IL-6, in VH is characteristic of uveitis, whereas increased levels of IL-10 and in particular IL-10/IL-6 ratio greater than 1 should prompt cytological analysis for the diagnosis of vitreoretinal lymphoma. The involvement of VH in neoplastic diseases and the pros and cons of performing VH biopsies in the clinical practice are further discussed in the review article by M. Asencio-Duran et al. entitled "*Vitreous diagnosis in neoplastic diseases.*"

J. L. Vallejo-Garcia et al. in "*Role of inflammation in endophthalmitis*" discuss the role of inflammation in infective endophthalmitis, reporting that the damage to the retina in this rare but severe diseases is mediated by the host immune reaction through toll-like receptors, cytokines, HMGB1, and aB-crystallin. A better understanding of the host immune reaction and the cellular pathways leading to tissue damage is also essential to improve clinical outcomes. Corticosteroids are frequently administered with antibiotics but often do not fully control the host immune reaction with consequent visual loss. A novel TLR2 ligand, Pam3Cys, has demonstrated encouraging results when administrated before the onset of endophthalmitis and also when injected in combination with intravitreal antibiotics.

J. B. Christoforidis et al. in "*Intravitreal devices for the treatment of vitreous inflammation*" describe the importance of the modulation of pharmacokinetics in the treatment of chronic intraocular inflammation. Long-term treatments are currently provided by drug-delivery devices, which include nonbiodegradable and biodegradable devices. The therapeutic agents that can be delivered are ganciclovir, fluocinolone acetonide, triamcinolone acetonide, and dexamethasone. The next small-scale biodegradable devices already described are liposomes, microspheres, and nanoparticles from 0.01 to 1,000 μm in diameter.

J. B. Christoforidis et al. in "*Systemic treatment of vitreous inflammation*" also report that many classes of systemic drugs may be used alone or in combination to control intraocular inflammation while closely monitoring side effects. Many of these inflammatory disorders require long-term treatment, and hence steroid-sparing agents, including antimetabolites, alkylating agents, and biological agents, are being used.

The emerging topic of sterile endophthalmitis is presented by J. Marticorena et al. in "*Sterile endophthalmitis after intravitreal injections.*" It is an infrequent complication of intravitreal injections and seems to develop in the context of the off-label use of drugs that have not been conceived for intravitreous administration. Sterile inflammation secondary to IVTA and IVB share many characteristics, such as the acute and painless vision loss present in the vast majority of the cases.

Inflammation also plays a major role also in the aging retina, where free radicals and oxidized lipoproteins are considered to be major causes of tissue stress. F. Parmeggiani et al. in "*Mechanism of inflammation in age-related macular degeneration*" report that the consequence is a parainflammation, a chronic status which contributes to initiation and progression of neurodegenerative diseases such as age-related macular degeneration (AMD). The parainflammatory deregulation that is already present in the early stage of AMD may notionally support the preventive employment of agents directed against the immune-inflammatory response in combination with high-dose nutritional supplements.

We sincerely hope that the present special issue may provide useful information to understand the mechanisms, the clinical effects, and the novel treatments of inflammation in which the vitreous is involved.

Mario R. Romano
John Christoforidis
Ahmed M. Abu El-Asrar

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Review Article

Vitreous Mediators in Retinal Hypoxic Diseases

Roberto dell'Omo,[1] Francesco Semeraro,[2] Giulio Bamonte,[3] Francesco Cifariello,[1] Mario R. Romano,[1] and Ciro Costagliola[1]

[1] *Dipartimento di Scienze per la Salute, Università del Molise, Via Francesco de Sanctis, 86100 Campobasso, Italy*
[2] *Dipartimento di Oftalmologia, Università di Brescia, 25121 Brescia, Italy*
[3] *HagaZiekenhuis, 2545 Den Haag, The Netherlands*

Correspondence should be addressed to Ciro Costagliola; ciro.costagliola@unimol.it

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The causes of retinal hypoxia are many and varied. Under hypoxic conditions, a variety of soluble factors are secreted into the vitreous cavity including growth factors, cytokines, and chemokines. Cytokines, which usually serve as signals between neighboring cells, are involved in essentially every important biological process, including cell proliferation, inflammation, immunity, migration, fibrosis, tissue repair, and angiogenesis. Cytokines and chemokines are multifunctional mediators that can direct the recruitment of leukocytes to sites of inflammation, promote the process, enhance immune responses, and promote stem cell survival, development, and homeostasis. The modern particle-based flow cytometric analysis is more direct, stable and sensitive than the colorimetric readout of the conventional ELISA but, similar to ELISA, is influenced by vitreous hemorrhage, disruption of the blood-retina barrier, and high serum levels of a specific protein. Finding patterns in the expression of inflammatory cytokines specific to a particular disease can substantially contribute to the understanding of its basic mechanism and to the development of a targeted therapy.

1. Introduction

Oxygen supply of the retina is provided by a dual circulation. The photoreceptors and the greater portion of the outer plexiform layer receive nourishment from the choriocapillaris, whereas the inner retinal layers are supplied by the superficial and deep capillary plexuses formed by branches of the central artery of the retina. Inner retinal layers show highest sensitivity to hypoxic challenges [1], whereas outer retinal layers are more resistant to a hypoxic stress [2].

The causes of retinal hypoxia are many and varied. Systemic causes include the cardiovascular effects of chronic obstructive airways disease and the ocular ischemic syndrome associated with arterial obstructive conditions such as carotid artery stenosis [3], hyperviscosity syndromes, anemia, and trauma [4, 5]. Most common causes of local retinal hypoxia include retinal artery and vein occlusion, diabetic retinopathy (DR), retinal detachment, uveitis, and retinopathy of prematurity.

The retinal tissue is capable of inducing protective mechanisms such as glycolysis, angiogenesis, vasodilation, and erythropoiesis under hypoxic-ischemic conditions [6]. These mechanisms deemed of putative importance for limiting the damage are lost within hours of the hypoxic-ischemic insult following which cell death and tissue damage occur [7].

Under hypoxic conditions, a variety of soluble factors are secreted into the vitreous cavity including cytokines, chemokines, and growth factors.

Cytokines, which usually serve as signals between neighboring cells, are involved in essentially every important biological process, including cell proliferation, inflammation, immunity, migration, fibrosis, tissue repair, and angiogenesis [8].

Chemokines are multifunctional mediators that can direct the recruitment of leukocytes to sites of inflammation, enhance immune responses, and promote stem cell survival, development, and homeostasis [9].

Growth factors have been detected from ocular fluid of patients with diabetic retinopathy and other retinal disorders [10].

Finding patterns in the expression of inflammatory mediators specific to a particular disease can substantially contribute to the understanding of the basic mechanism of this disease and consequently to the development of a targeted therapy.

2. Dosage of Vitreous Mediators

Recently, a particle-based flow cytometric analysis method (PFCAM) has been established to overcome some of the intrinsic limitations of the conventional enzyme-linked immunosorbent assay (ELISA) and it has been used to analyze the vitreous inflammatory mediators by multiplex bead in patients with several vitreoretinal disorders [11–14].

The technology utilizes microspheres as the solid support for a conventional immunoassay, affinity assay, or DNA hybridization assay which are subsequently analyzed on a flow cytometer. Overall, the fluorescent readout of the flow cytometric assay is more direct, stable, and sensitive than the colorimetric readout of the ELISA. As the ELISA requires enzyme amplification, it is prone to variability and errors in the amount of amplification.

The sensitivity of the flow cytometric systems can be enhanced further by reducing the number of beads per test. This increases the ratio of cytokine to capture antibody in each test without reducing the potential signal strength of the assay (the number of capture antibodies per bead). PFCAMs are more reproducible than the ELISA which exhibits a significant variation between experiments and between plates within experiments. PFCAMs are also more accurate and reliable because the data are calculated from the mean of dozens of beads, each of which functions as an individual replicate. For many cytokines, the multiplexed and uniplexed PFCA assays are comparable suggesting that multiplexing does not significantly reduce the overall quality of the assay. In contrast, the conventional ELISA has limited capabilities. Finally, PFCAMs are much cheaper than ELISA when six or more cytokines are measured simultaneously.

Despite these advantages of PFCAM over ELISA, it should be kept in mind that, whatever the test used, there are some conditions which can alter the vitreous concentration of a certain protein, independently from the intraocular secretion of the protein itself.

For example, high serum levels of a specific protein could influence its intravitreous concentration. Similarly, the disruption of the blood-retina barrier produces an increase of proteins in the vitreous fluid. Finally, vitreous hemorrhage, which often occurs in conditions like proliferative diabetic retinopathy and vein occlusion, can produce an influx of serum proteins, such as growth factors, into the vitreous fluid.

3. Oxidative Stress

Oxidative stress, which may occur because of an imbalance between the production and the removal of reactive oxygen species (ROS), is considered to be a critical mediator in injury secondary to ischemic disorders.

Superoxide anion ($O_2^{\bullet-}$) is one of the major ROS. The release of $O_2^{\bullet-}$ in retinal ischemia was proven either directly by electron paramagnetic resonance or indirectly by showing diminished damage after the administration of antioxidant drugs such as EGB 761 extracted from *Ginkgo biloba*, vitamin E, mannitol, superoxide dismutase, and several other compounds [15–20].

The importance of $O_2^{\bullet-}$ is also indicated by the fact that a manganese superoxide dismutase mimetic and transgenic manganese superoxide dismutase gene inhibited ischemia/reperfusion-induced retinal injury and diabetes-induced oxidative stress [21, 22]. ROS formed during oxidative stress can directly attack polyunsaturated fatty acids and initiate ROS chain reactions that result in lipid peroxidation in cellular membranes and a variety of oxidized products, including aldehydes, which are extremely reactive and can damage biological macromolecules. Injury can occur distal to the initial site of ROS attack because aldehydes are relatively long-lived compared with free radicals [23].

The resultant end products are well-known peroxidation markers of polyunsaturated fatty acids and are capable of inducing apoptosis in neuronal cells [24].

3.1. Nitric Oxide. Nitric oxide is synthesized by the enzyme NO synthase (NOS) from L-arginine. NOS exists in three isoforms: neuronal (nNOS) and endothelial (eNOS) which are constitutively expressed and inducible (iNOS). Enhanced nNOS, eNOS, and iNOS expressions have been reported in the retina in response to hypoxia [25]. Glial cells have been suggested to be the major cell types producer [26] but infiltrating leukocytes may also be an important source of iNOS production.

NO has been described to have neuroprotective and neurotoxic roles [27]. For example, NO produced by the eNOS isoform represents a protective response, since it produces vasodilatation and increased blood flow, maintaining retinal perfusion in hypoxic-ischemic conditions [28, 29].

However, besides these beneficial effects, eNOS is also involved in vascular-endothelial-growth-factor (VEGF-) induced vascular hyperpermeability [30].

NO production from nNOS and iNOS contributes to cytotoxicity resulting in cell death and axonal damage. Other than the generation of free radicals, a number of pathways such as N-methyl-D-aspartate-(NMDA-) mediated intracellular Ca^{2+} influx and CREB-mediated transcription of apoptotic proteins such as Bax, Bad, and Bcl–xl are triggered by NO resulting in neuronal death [31–33].

In retinal ischemia, RGCs death has been reported to be due to the involvement of iNOS as it has been observed that iNOS-positive leukocytes enter the ganglion cell layer and surround the RGCs and cause their degeneration. NO induces the proapoptotic cascade in hypoxic neural tissues by increasing phosphorylation of Bcl-2 [31]. Other mechanisms by which NO contributes to cytotoxicity may be peroxynitrite-mediated oxidative damage, DNA damage, and energy failure [34–36].

It has been shown that NO can react with the superoxide anion (O2−) to form peroxynitrite (OONO−) [37] which is neurotoxic. NO alone, even at high levels, has been reported as nontoxic to cortical neurons, but becomes neurotoxic after its reaction with O2− to form ONOO− [38]. *In vitro* studies have shown that the formation of OONO− increases the VEGF-induced permeability of retinal microvascular endothelial cells [39] and tissue damage through DNA damage reduced cellular antioxidant defenses and lipid peroxidation [40, 41].

A common target for peroxidation is polyunsaturated fatty acids (PUFAs) present in membrane phospholipids. Lipid peroxidation of retinal membrane PUFAs results in the loss of membrane function and structural integrity [42, 43].

For reasons that remain unclear, retinal endothelial cells seem particularly susceptible to peroxidation-induced injury, whereas pericytes, smooth muscle cells, and perivascular astrocytes are relatively resistant [44–47].

The retina is highly susceptible to lipid peroxidation since 20% of its dry weight is composed of lipids containing a high level of different PUFAs including docosahexaenoic acid (DHA; 22:6ω−3), arachidonic acid (AA; 20:4ω−6), and choline phosphoglyceride. Retinal vessels, in contrast to parenchyma, contain saturated fatty acids like stearic acid as well as unsaturated ones including AA and DHA, but the important DHA precursor, eicosapentaenoic acid (20:5ω−3), is not detected in retinal vessels.

A large body of evidence supports the idea that the increase in oxidative stress in retinal microvasculature is a key factor for the development of diabetic retinopathy [48–50].

A large body of evidence has also demonstrated an increase in reactive oxygen species and NO production in different tissues and cell types during diabetes, or after the exposure to high glucose [50, 51], which have been claimed to contribute to the vascular alterations observed in diabetic retinopathy. In fact, oxidative and nitrosative stress have been associated to the increase of apoptosis in retinal endothelial cells exposed to hyperglycemic conditions [49, 52–55].

It has been demonstrated that elevated glucose per se induces an increase in the levels of ROS in retinal endothelial cells [49].

3.2. Excitotoxicity. Glutamate, the excitatory neurotransmitter in the retina, is released by photoreceptors, bipolar cells, and ganglion cells and mediates the transfer of visual signals from the retina to the brain [56].

Augmented release of glutamate and its accumulation in extracellular spaces in hypoxic-ischemic conditions, leading to the activation of glutamate receptors, has been implicated in hypoxic/ischemic neuronal death [57, 58].

Glutamate exerts its action through ionotropic (aminomethyl-propionic-acid (AMPA) *N*-methyl-D-aspartate (NMDA), and kainate glutamate receptors) and metabotropic receptors [59, 60]. Glutamate receptor-mediated damage has been reported to occur in glaucoma, central, and branch retinal arterial and retinal vein occlusions resulting in the loss of retinal ganglion cells [61].

Neurotoxic effects of glutamate are reported to occur predominantly through the activation of ionotropic glutamate receptors (GluR). NMDA receptors are highly permeable to Ca^{2+} [62–65], their activation resulting in an increase in the intracellular calcium levels [61, 65–67].

Ca^{2+} overloadd has been reported to be a central event in neuronal death during ischemia [68, 69].

In fact, abnormal higher concentrations of calcium lead to inappropriate activation of enzymes such as proteases, nucleases, and lipases which are harmful to the cellular constituents and generate free radicals as well as cause mitochondrial failure which results in energy depletion and further free radical production [70].

Depolarization of neuronal membranes due to energy failure results in Ca^{2+} influx through the voltage-dependent Ca^{2+} channels followed by Ca^{2+}-dependent glutamate release [71] which further increases the extracellular accumulation of glutamate. Activation of ionotropic glutamate receptors also results in influx of Na^{+} and Cl^{-} ions, inducing osmotic swelling. Glutamate acting via NMDA receptors activates nNOS [72] and the production of NO [73].

Glutamate-induced activation of AMPA and NMDA receptors has been shown to enhance the production of tumor ncrosis factor (TNF)-α [74–76] and interleukin-1β (IL-1β) [77] significantly.

Cooperation between glutamate receptors and inflammatory cytokines may be one of the mechanisms involved in cell damage.

Glutamate toxicity also results in glutathione depletion and oxidative stress [78]. Glutathione is a major cellular antioxidant which protects the cells against oxidative stress [79–81]. Increase in intracellular ROS in response to glutathione depletion has been reported in several studies [82]. Removal of excess glutamate from the extracellular space by glutamate transporters is crucial to terminate glutamate excitotoxicity. Glutamate transporters are responsible for the removal of glutamate from the extracellular fluid in the retina. It has been suggested that excess glutamate accumulation in the extracellular spaces may result from a failure of the glutamate transporters, such as GLAST, in the vicinity of RGCs [83]. Glutamate transporters have been described as necessary to prevent excitotoxic retinal damage and to synthesize glutathione and their deficiency has been reported to result in RGC degeneration [83].

3.3. Role of Inflammation. Hypoxia-ischemia is known to attract macrophages to hypoxic areas through expression of monocyte-chemoattractant-protein-(MCP-) 1. The hypoxia-activated macrophages and microglia, the immune effector cells in the retina, release TNF-α which has been reported as a triggering factor to activate the production of interleukin-(IL-) 8, VEGF, and MCP-1 in retinal vascular cells and/or glial cells adjacent to microvessels [84].

Several inflammatory molecules including intercellular-adhesion-molecule-(ICAM-) 1, TNF-α, IL-1, NOS, and COX-2 released by activated inflammatory cells and glial elements play a major role in the degeneration of retinal capillaries [85, 86]. Expression of adhesion molecules, intercellular adhesion molecule-(ICAM-) 1, and vascular-cellular-adhesion-molecule-(VCAM-) 1 on the endothelial

cells facilitating leukocyte adhesion and infiltration into the areas of damage, has been reported to be induced by TNF-α and IL-1 [87–90]. ICAM-1 is important for establishing adhesion of leukocytes before their movement across the endothelium into the tissue [91]. IL-1 and TNF-α may also be involved in transcriptional activation of the iNOS gene [92, 93].

The proliferative diabetic retinopathy (PDR) retinal environment is characterized by the upregulation of iNOS, COX-2, ICAM-1, caspase 1, VEGF, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and increased production of NO, prostaglandin E2, and IL-1β, as well as increased permeability and leukostasis. Localized inflammation is responsible for capillary occlusion and degeneration leading to the ischemia-induced vasculogenesis, which results in DR [94].

Increased leukocyte adhesion (via ICAM1-CD18) to retinal vascular endothelium with resulting endothelial damage, breakdown of the blood retina barrier, capillary nonperfusion, and ischemia contribute to neovascularization. Inhibition of integrin α-4, which forms a part of very late antigen-4 (VLA-4) that binds to VCAM-1, decreases TNF-α, VEGF, NF-κB and reduces leukocyte adhesion and vascular leakage [95]. Cytokines produced by inflammatory cells play a central role in the pathogenesis of PDR by promoting leucocyte-mediated damage to retinal vasculature [96].

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, produced by neutrophils, is associated with leukocyte adhesion and vascular leakage in diabetic maculopathy and neovascularization. NADPH oxidase is a mediator of DR possibly by reducing peroxisome proliferator-activated-receptor- (PPAR-) γ and activating the NF-κB pathway [97].

In vitro, apocynin and superoxide dismutase prevented suppression of PPAR-γ in bovine retinal endothelial cells treated with high glucose [97]. In this environment, the balance is shifted in favour of matrix metalloproteinases (MMPs) and away from their inhibitors, tissue inhibitor of matrix metalloproteinases (TIMPs). MMP-2 and MMP-9 actively degrade collagen IV, which is a major component of basement membranes, causing the extracellular matrix degradation needed for angiogenesis in DR.

4. Cytokines

4.1. Tumor Necrosis Factor α. Tumor necrosis factor α is an inflammatory mediator of neuronal death after ischemic injury in the brain and retina [98]. TNF-α is a member of the death-inducing ligand (DIL) family; it triggers the extrinsic pathway of apoptosis and acts through its two primary receptors, TNFR1 (p55) and TNFR2 (p75).

TNF-α was identified and isolated because of antiangiogenic activity; when injected into tumors, it causes tumor vessels to regress resulting in tumor necrosis [99].

So, it is quite clear that TNF-α has antiangiogenic effects, but it may also have proangiogenic effects in some situations. Despite its inhibition of endothelial cell proliferation *in vitro*, sustained release of TNF-α in cornea or injection of 105 units of recombinant TNF-α into the vitreous cavity of rabbits causes cellular infiltration and neovascularization (NV) in the cornea [100, 101], possibly by induced expression of other proangiogenic proteins such as interleukin-8, VEGF, and fibroblast-growth-factor- (FGF-) 2 [102]. In cultured vascular endothelial cells, TNF-α induces expression of VEGF receptor 2 and neuropilin-1 [103]. In mice, subcutaneous implantation of a pellet containing a low dose (0.01–1 ng) of murine recombinant TNF-α stimulated angiogenesis, while implantation of a pellet containing a high dose (1–5 μg) inhibited angiogenesis demonstrating opposite effects depending upon the concentration [104].

This paradox may be explained in part by the ability of TNF-α to activate 2 intracellular signaling pathways in endothelial cells, one leading to apoptosis [105] and one that promotes survival and proliferation through the activation of nuclear factor-kappa B (NF-κB) [106]. In addition, TNF-α recruits inflammatory cells, which stimulate neovascularization (NV) in some situations and inhibit it in others [107, 108].

Finally, the ability of TNF-α to induce expression of proangiogenic molecules can result in different effects depending upon the makeup of the local cell population and its response to TNF-α. Therefore, the effect of TNF-α in various tissues and disease processes is difficult to predict and must be determined by experimentation.

Increased levels of TNF-α have been demonstrated in proliferative retinopathies and in animal models of retinal NV [109–112]. These increased levels of TNF-α may be collaborating with VEGF to stimulate retinal NV. TNF-α may also contribute to the process in other ways. For instance, leukocytes have been shown to play a role in the pathogenesis of ischemic retinopathies and TNF-α is a chemoattractant for leukocytes [111].

TNF-α also causes breakdown of the blood-retinal barrier [112] which may be related to its stimulation of leukostasis, and therefore TNF-α may contribute to the excessive permeability seen in ischemic retinopathies.

In early diabetic retinopathy, there is an increased release of retinal inflammatory mediators including TNF-α 85, IL-1β, ICAM-1, and angiotensin II [113] along with activation of microglial cells [114]. A soluble TNF-α receptor-Fc hybrid, such as etanercept [115], is able to normalize vascular permeability and leukostasis; this suggests that TNF-α contributes to diabetic retinopathy, perhaps by preventing endothelial-cell damage from adhering leukocytes [86].

4.2. IL-1, IL-6, and IL-8. Patients with PDR have increased vitreal levels of IL-1 and TNF-α, which induce ICAM-1 expression [116]. Aqueous humour levels of IL-6 and VEGF correlate with respective levels in the vitreous and their concentrations increase with severity of disease [117]. Early stage DR is associated with elevated levels of serum CD105 (which is thought to be involved in vascular remodelling) and vitreal VEGF which then decrease through the course of disease progression to severe PDR [118]. IL-6 (T-cell activation), IL-8 (neutrophil chemotaxis), MCP-1, and VEGF levels are also significantly higher in the vitreous of patients with PDR [119]. IL-18, which induces macrophage activation via interferon-gamma, is raised in the sera of patients with diabetes mellitus type 2 and background DR [120]. In line

with previous findings, it has been shown that intraocular production of IL-6 rather than IL-8 appears to be associated with the neovascularization activity in PDR [117], although a significant linear correlation between IL-6 and IL-8 has been demonstrated [121]. However, severity grade of PDR is not related to either IL-6 or IL-8 expression levels in vitreous fluid. This reveals that increased IL-6 levels in vitreous might be a master regulator and an important clinical marker for neovascularization activity. In addition, IL-8 expression seems to be differentially regulated compared with IL-6 response in PDR process. Thus, interleukins play an important role in mediating the inflammation and neovascularization in the development of PDR.

4.3. High Mobility Group Box-1. High-mobility group box-1 (HMGB1) protein was originally described 30 years ago as a nonhistone DNA-binding protein [122], involved in nucleosome stabilization and gene transcription [123]. HMGB1 is expressed in ganglion cells layer, inner nuclear layer, outer nuclear layer, the inner and outer segments of photoreceptors, and in the retinal pigment epithelial cells in normal retina [124, 125].

In addition to advanced glycation end products (AGEs), HMGB1 is another ligand of the receptor for AGEs [126], which can contribute to the accelerated micro- and macrovasculopathy observed in diabetes [127]. However, HMGB1 may play a key role in the protection of retinal injury after ischemia-reperfusion [128] and is also implicated as an important endogenous danger signalling molecule amplifying the activities of immunostimulatory molecules in a synergistic manner [129, 130].

HMGB1 stimulates membrane ruffling and repair of a mechanically wounded endothelial cell monolayer, causes endothelial cell sprouting, and stimulates neovascularization of chicken embryo chorioallantoic membrane via RAGE [131]. The crucial role of HMGB1 has also been demonstrated in diabetic mice for ischemia-induced angiogenesis through a VEGF-dependent mechanism [132].

HMGB1 might play a role in the upregulation of VEGF-A in retinal ganglion cells after exposure to AGEs. It has been demonstrated that blocking HMGB1 with glycyrrhizin successfully inhibits AGE-BSA-induced upregulation of VEGF-A [133].

Therefore, HMGB1 works as a cytokine or a cofactor that amplifies the effect of the AGE-RAGE axis, in an autocrine/paracrine manner, and mediates the secretion of survival factors including VEGF-A for counteracting the oxidative stress.

5. Chemokines

Chemokines are multifunctional mediators that can direct the recruitment of leukocytes to sites of inflammation, promote inflammation, enhance immune responses, and promote stem cell survival, development, and homeostasis.

They are classified by structure into four groups, designated C, CC, CXC, and CX3C depending on the number and spacing of the cysteine residues in the mature protein.

The CXC chemokines are divided into two subgroups depending on the presence or absence of the sequence glutamic acid-leucine-arginine (ELR) that immediately precedes the first cysteine amino acid in the primary structure of these cytokines. The ELR-containing CXC chemokines are angiogenic. Most non-ELR CXC chemokines such as interferon-c-inducible protein of 10 KDa (CXCL10/IP-10) potently chemoattract activated T lymphocytes and are angiostatic [134].

5.1. Monocyte Chemotactic Protein-1. Abu El-Asrar et al. [135] demonstrated that in the vitreous humor of eyes with proliferative vitreoretinal disorders, the CC MCP-1 and the CXC chemokine IP-10 are detected at high levels not correlating to serum levels, suggesting an increased local production. Furthermore myofibroblasts in PDR and proliferative vitreoretinopathy membranes express MCP-1 and stromal-cell-derived-factor- (SDF-) 1, and vascular endothelial cells in PDR membranes express MCP-1, SDF-1, and the chemokine receptor CXCR3. The same authors found that MCP-1 levels in the vitreous from cases of active PDR are significantly higher than those in inactive PDR cases.

Collectively these findings provide evidence that increased MCP-1 expression contributes to the development of neovascularization and fibrosis in proliferative vitreoretinal disorders.

Furthermore Hong et al. [136] showed that MCP-1 induces VEGF expression in endothelial cells; therefore, a positive regulatory feedback loop between VEGF and MCP-1 expression by vascular endothelial cells in mediating angiogenesis might exist.

5.2. Fractalkine. Fractalkine (FKN), the sole member of the CX3C chemokine family, is named for its fractal geometry. Silverman et al. demonstrated the presence of FKN in normal cultured microvascular endothelial and stromal cells of the iris and retina *in vitro* [137].

Vitreous sample from patients with PDR revealed higher FKN concentrations compared with the control and immunodepletion of soluble FKN from PDR vitreous samples caused 36.6% less migration of bovine retinal capillary endothelial cells [138].

Therefore, FKN appears, to be a potent angiogenic mediator *in vitro* and *in vivo* and may play an important role in ocular angiogenic disorders such as PDR.

5.3. Monokine Induced by Interferon-γ. Monokine induced by interferon-γ (Mig) is principally known as a chemoattractant of activated T cells, but also has an angiostatic activity. Wakabayashi et al. [139] have recently documented a significant elevation of vitreous Mig concentration in DR patients compared with control subjects. The authors also found a significant correlation between vitreous concentrations of Mig and VEGF. It is not clear why Mig, an angiostatic factor, is elevated in the vitreous in DR, where angiogenesis is one of the main pathologies. One possibility is that Mig is elevated as a response to the upregulation of angiogenic factors such as VEGF. A second hypothesis is that Mig in DR

might be related to chemotaxis of leukocytes rather than to angiostatic functions, because leukostasis is considered one of the pathogenic mechanisms of DR [140].

5.4. Stromal Cell-Derived Factor-1. Stromal cell-derived factor-1 (SDF-1/CXCL12) is a member of the CXC chemokine family that was originally isolated from murine bone marrow stromal cells. CXCR4, a 7-transmembrane-spanning G protein-coupled receptor, is one of the two receptors for SDF-1.

Recent studies have shown that SDF-1/CXCR4 interaction plays an important role in endothelial progenitor cells (EPCc) migration differentiation, proliferation, and survival [141–145]. SDF-1 is upregulated in ischaemic tissues, establishing an SDF-1 gradient favouring recruitment of EPCs from peripheral blood to sites of ischaemia, thereby contributing to accelerated neovascularization [141, 142].

In addition, SDF-1 promotes the chemotaxis of bone-marrow-derived CD34+ stem cells and their differentiation into EPCs in ischaemic tissue and tumours [142, 144, 145]. CXCR4 blockade profoundly inhibits VEGF- and SDF-1-induced migration of EPCs and impairs incorporation of EPCs into sites of ischaemia-induced neovascularization [143].

The finding that VEGF-mediated migration of EPCs was also influenced by CXCR4 antibodies points toward a more general involvement of CXCR4 and its downstream signalling in the homing mechanisms of EPCs.

Butler et al. [146] reported increased SDF-1 levels in vitreous from patients with PDR. In a murine model of retinal ischaemia, upregulation of SDF-1 and CXCR4 was detected in ischaemic retinas. A substantial amount of the increase in CXCR4 was caused by influx of CXCR4-expressing bone-marrow-derived cells. Pharmacological blockade of CXCR4 suppressed ischaemia- and VEGF-induced retinal neovascularization.

In a murine model of proliferative retinopathy, Blom et al. [147] demonstrated that intravitreal injection of blocking antibodies to SDF-1 prevented retinal neovascularization, even in the presence of VEGF.

Recently, it has been demonstrated that stromal CXCR4+ CD34+ cells are closely associated with the new vessels within the epiretinal membranes in eyes with PDR [148].

6. Transcriptional Factors

6.1. Hypoxia-Inducible Factor. Hypoxia-inducible-factor-(HIF-) 1 is a transcription factor that plays an essential role in the systemic homeostasis response to hypoxia. HIF-1 controls the expression of most genes involved in adapting to hypoxic conditions. HIF-1 triggers the activation of several genes that result in the production of VEGF and other angiogenic factors [149–153].

Several researchers have shown that diabetic factors result in HIF-1 production and angiogenesis. Treins et al. [154] have shown that insulin growth factor 1 stimulates accumulation of HIF-1 in human retinal pigment epithelial cells.

VEGF expression seems to be regulated through dual interdependent mechanisms. One involves HIF-1 directly and the other indirectly through NF-kappa B-mediated COX-2 expression and prostaglandin E2 production. Acute intensive insulin therapy exacerbates diabetic bloodretinal barrier breakdown through HIF-1 and VEGF [155].

This could explain why intensive control can result in transient worsening of diabetic retinopathy.

Recently, the presence of HIF-1a in the diabetic membranes has been shown [156]. HIF-1 is found more often and more intensely in diabetic preretinal membranes compared with nondiabetic idiopathic epiretinal membranes [157].

6.2. Nuclear Factor (NF)-κB. NF-κB is an ubiquitous inducible transcription factor that is a master regulator of immune responses, cellular proliferation, and apoptosis. NF-κB is activated under hypoxic conditions and in retinal endothelial cells and pericytes exposed to hyperglycaemia *in vitro* and *in vivo*.

Frede et al. reported for the first time the role of NF-κB in controlling HIF-1 gene expression in response to inflammatory stimuli [158]. Later on, a binding site has been identified for NF-κB within the HIF-1α promoter [159]. Hypoxia has been shown to result in the activation of NF-κB that subsequently can bind to the HIF-1α promoter. It is evident that both transcription factors might be crucial regulators for IL-6 and IL-8 expression in vitreous of PDR patients. In contrast to that hypothesis some authors [121] could not detect either NF-κB or HIF-1α activity in vitreous samples isolated from patients with PDR. However, this does not exclude locally increased NF-κB or HIF-1α activity, as it has been previously documented [160]. Locally increased NF-κB or HIF-1α activity may be covered for their total transcription factor levels in vitreous extracts. In addition, there might be a periodic regulation of NF-κB or HIF-1α activity in different hypoxic conditions [161, 162]. To date, the role of NF-κB or HIF-1α in the regulation of PDR process is weakly understood.

7. Growth Factors

7.1. Vascular Endothelial Growth Factor (VEGF). The VEGF family forms a part of the platelet-derived growth factor (PDGF) supergene family members which comprise four major and five minor isoforms: VEGF 121, VEGF 165, VEGF 189, and VEGF 206; VEGF 145, VEGF 148, VEGF 162, VEGF 165b (an inhibitory isoform binding to VEGFR-2), and VEGF 183. These isoforms derive from alternative exon splicing of the VEGF-A gene, located on chromosome 6p21.3 [163, 164] and are classified by their amino acids number. These cytokines bind to cell-surface receptors that belong to the family of tyrosine-kinase receptors [165].

VEGF binds to tyrosine-kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (Flk-1), and also to the neurophilins (NP)-1 and 2, which also function as receptors. VEGF signalling is modulated by angiopoietins that bind to Tie-2 receptors [166].

VEGF-A has been studied extensively and plays a critical role in both vasculogenesis and angiogenesis [164, 167, 168].

The function of VEGF-A isoforms can vary during ocular development. It is generally accepted that in adults the

formation of new blood vessels results exclusively from surrounding preexisting vessels by sprouting, a process referred to as angiogenesis whereas vasculogenesis, defined as the recruitment and *in situ* differentiation of vascular endothelial cells from circulating bone-marrow-derived endothelial precursor cells, is normally thought to occur only in the embryonic phases of vascular development.

In addition to angiogenesis and vasculogenesis, VEGF-A may participate in the maintenance of some vascular systems in the adult, but little is known of the role of VEGF-A in the maintenance of adult ocular vasculature. Different studies have shown that VEGF has a role in endothelial reparation after damage [169, 170].

VEGF is secreted by macrophages, T cells, retinal pigment epithelial (RPE) cells, astrocytes, pericytes, and smooth muscle cells in response to hypoxic and inflammatory stimuli. VEGF secretion is inducible by hypoxia-ischemia *in vitro* and *in vivo*, via hypoxia-inducible-factor- (HIF-) 1 dependent transcriptional activation [171]. A 3–12-fold increase in VEGF gene expression has been reported in hypoxia [172–174]. VEGF enhances the adhesion of leukocytes to vascular walls and increases ICAM-1 and VCAM-1 expression in the brain and retina [175–177].

In the eye, ischemic retinopathies such as PDR and retinopathy of prematurity are pathologic events which, through retinal capillary obliteration, promotes retinal ischemia.

Several reports suggest that VEGF is the critical proangiogenic cytokine [178, 179] and that a direct correlation clearly exists between intraocular VEGF levels and ischemic ocular neovascularization [180–182].

Increased production of VEGF and enhanced permeability of blood retinal barrier has been reported in the hypoxic retina and inhibition of VEGF production with melatonin reduces blood retinal barrier permeability [183].

VEGF is involved in retinopathy of prematurity, DR, and age-related macular degeneration, the leading causes of irreversible visual loss in developed countries from infants to the elderly [184].

7.2. Connective Tissue Growth Factor (CTGF). Connective tissue growth factor (CTGF) is a 38 kD cysteine rich heparin-binding protein and is involved in stimulation of proliferation, angiogenesis, migration, extracellular matrix production, cell attachment, cell survival, and apoptosis [147].

CTGF has been proposed to play an important role in tubule-interstitial fibrosis as one of the major mediators of TGF-β. It has been shown to be hypoxia-inducible in human breast cancer cells [185].

However, the precise signalling mechanisms of the hypoxia-induced expression of CTGF remain unclear. CTGF is expressed in vascular beds and acts on multiple cell types. It is important for vessel growth during early retinal development and promotes the fibrovascular reaction in murine retinal ischemia after laser injury [186]. CTGF overproduction is proposed to play a major role in pathways that lead to fibrosis [187] in the vitreous of PDR patients.

The vitreous of PDR patients has elevated levels of both CTGF and VEGF and the ratio between CTGF and VEGF levels dictates the degree of fibrosis and angiogenesis. Raised CTGF levels are associated with VEGF and fibrosis, but only VEGF itself is responsible for neovascularization (NV) in PDR. *In vitro*, CTGF induced production of fibronectin and VEGF expression had no direct effects on vascular endothelial cells. CTGF may promote formation of proliferative membranes in PDR but not its cicatrization. It may be implicated indirectly in modulating VEGF expression but has no effects on retinal NV [188]. Anti-VEGF therapy can temporarily tip the CTGF/VEGF ratio towards a profibrotic environment [189].

7.3. Stem Cell Factor (SCF). Stem cell factor (SCF), or kit ligand, is a peptide growth factor that exists as a membrane-bound protein but may be cleaved by proteases, such as matrix metalloproteinase-9 (MMP-9), to produce a soluble cytokine [190, 191].

SCF is important for the survival and differentiation of hematopoietic stem cells. The receptor for SCF, the proto-oncogene c-kit, is a tyrosine kinase that is expressed by bone-marrow-derived endothelial stem/progenitor cells [192, 193].

SCF ligand binding leads to phosphorylation and activation of the c-kit receptor and its downstream signaling proteins, which have been implicated in cell proliferation, cell adhesion and cell survival as well as chemotaxis [194–196].

Several studies have demonstrated that SCF/c-kit signaling promotes the survival, migration differentiation, and capillary tube formation of endothelial cells and plays an important role in ischemia-induced neovascularization [190, 192, 194, 196–198].

Abu El-Asrar et al. [199] demonstrated that (1) PDR membranes show immunoreactivity for SCF, c-kit, G-CSF, eNOS, and CXCR4 in vascular endothelial cells; (2) stromal cells expressed SCF, c-kit, eNOS, and CXCR4; (3) c-kit+ cells coexpressed the chemokine receptor CXCR4 and eNOS; (4) the number of blood vessels expressing CD34, c-kit, G-CSF, eNOS, and CXCR4 and the number of stromal cells expressing c-kit, SCF, eNOS, and CXCR4 in membranes from patients with active PDR were significantly higher than those in membranes from patients with inactive PDR; and (5) there were significant correlations between the number of blood vessels expressing the panendothelial marker CD34 and the number of blood vessels expressing SCF, G-CSF, eNOS, and CXCR4 and the number of stromal cells expressing SCF. These data support the notion that bone-marrow-derived cells contribute to neovascularization in PDR epiretinal membranes and that SCF/c-kit signaling may play a role in the pathogenesis of PDR.

7.4. Insuline-Like Growth Factor (IGF-1). IGF-1 is produced locally in the human eye by a variety of cells including RPE cells, retinal capillary pericytes, endothelial cells, Muller cells, and ganglion cells. In cultured human RPE cells, IGF-1 is thought to exert its effect by inducing a dose-dependent increase in IGF- 1R phosphorylation and in VEGF mRNA levels. IGF-I1 also stimulates VEGF promoter activity *in vitro*, mainly via HIF-1alpha and secondarily via NF-κB and AP-1 [200]. In a south Indian cohort, a CA 18-repeat genotype in

the promoter of IGF-1 is implicated in susceptibility to PDR and associated with clinical severity [201].

7.5. Fibroblast-Growth-Factor- (FGF-) 2. Fibroblast growth factor (FGF)-2 is quickly released during the wound-healing process, providing an early stimulus for endothelial cell proliferation in the acute phase immediately after injury. FGF-2 appears able to upregulate VEGF production and acts synergistically in stimulating angiogenesis-platelet-derived growth factor, transforming growth factor-3.

7.6. Erythropoietin (Epo). Erythropoietin, a stimulator of red blood cells, is also a promoter of vascular endothelial cell proliferation and angiogenesis [202]. Both Epo and VEGF respond to hypoxia [203] leading to ischemia-induced angiogenesis. Epo and VEGF are both raised in the vitreous of patients with PDR and act independently of each other [204].

Epo levels are higher than that of VEGF and its inhibition suppresses retinal NV both *in vivo* and *in vitro*. Suppression of Epo and VEGF leads to a greater inhibition of retinal NV than when either is inhibited alone. *In vitro* inhibition of Epo leads to attenuation of endothelial cell proliferation in PDR [205]. In murine models of oxygen-induced retinopathy, inhibition of Epo led to inhibition of retinal NV *in vivo* and inhibition of retinal endothelial cell proliferation *in vitro* [204]. Even though this evidence may tempt us to target Epo in the development of a retinal antiangiogenic strategy, we must be cognizant of its neuroprotective effects on retinal cells [206].

8. The Renin-Angiotensin (RAS) System

Human retinas have angiotensin receptor (ATR) type-1 and ATR-2. In human models of DR and hypoxia-induced retinal angiogenesis, the RAS is upregulated leading to the production of VEGF, PDGF, and CTGF leading to microvascular complications, angiogenesis, cell proliferation, and fibrosis [207].

The RAS exerts its effects by the generation of a family of bioactive angiotensin peptides among which angiotensin II (ANG II) and the ATR-1 and ATR-2 receptors are most well characterized [207]. Emerging evidence suggests that an ocular RAS is activated in DR and may contribute to progressive alterations to retinal cells such as pericytes, endothelial cells, neurons, and glia. In the kallikrein-kinin system (KKS), bradykinin (BK) and kallidin and their carboxypeptidase metabolites, des-Arg (9)-BK and des-Arg(10)-kallidin, are the effector peptides exerting their actions via BK type 1 (BK-B1) and BK type 2 (BK-B2) receptors. Both RAS and KKS damage the retinal vasculature and glia in DR via production of VEGF and CTGF [207]. The RAS is also implicated in progression of DR via Ang II. Ang II induces VEGF, which leads to the loss of tight junction proteins causing a breach in the integrity of the BRB. Angiotensin receptor blockers that block Ang II receptors reduce VEGF production by retinal endothelial cells and promote the recovery of tight junction proteins thus preventing progression of DR in its early stages [208]. Important cross-talk exists between the RAS system, advanced glycation end products (AGEs), and their receptors (RAGE). AGEs act via RAGE to cause diabetic microvascular complications leading to PDR [209]. *CCN1/Cyr61* is a member of the cysteine-rich 61/connective tissue growth factor/nephroblastoma overexpressed (CCN) family of genes. It is a downstream effector of AGE in the diabetic retina and may work synergistically with VEGF to cause ocular angiogenesis and PDR in models of oxygen induced retinopathy (OIR) in mice and streptozotocin (STZ-) induced DM in rats. Levels of both *CCN1* mRNA and protein are raised in vitreous of STZ rats and PDR patients (non-diabetics) [210]. AGEs-RAGE-induced VEGF expression is thought to lead to neovascularization in PDR. Olmesartan, an angiotensin II type-1 receptor blocker, inhibited angiogenesis by inhibiting AGE-induced NFK-b promoter activity and consequently NFKb-mediated RAGE expression [211]. AGEs also induce injury of retinal pericytes, which are protected by PEDF expression. Thus, a decrease in PEDF expression can amplify the effect of AGEs on RPE integrity leading to PDR [212].

9. Other Mediators

9.1. Periostin. Periostin is a secreted extracellular matrix (ECM) protein that is found in areas of normal fibrogenesis or pathologic fibrosis and that can directly interact with other ECM proteins such as fibronectin, tenascin-C, collagens I and V, and heparin. The high degree of structural and sequence homology of periostin with fasciclin 1 and transforming growth factor β-induced suggests that periostin plays a role in cell adhesion and migration [213].

Yoshida et al. [214] showed that the concentration of periostin in the vitreous of patients with PDR is significantly higher than that in the vitreous of patients without PDR and, differently from the concentration of VEGF or bFGF, it is significantly correlated with the presence of fibrovascular membranes (FVMs). The differences in the correlations between periostin and VEGF are probably because VEGF is upregulated in the retina at an earlier stage in response to ischemia before the development of FVMs [215, 216].

9.2. Apelin. Apelin was first identified as an endogenous ligand of the orphan G-protein-coupled receptor, APJ, from bovine stomach extracts in 1998 [217]. Apelin signaling has recently been identified as an important contributor to angiogenesis [218]. It is reported that both apelin messenger RNA and APJ messenger RNA are highly expressed in the vascular system, especially in endothelial cells [219, 220].

In vitro, apelin was found to stimulate the proliferation and migration of retinal endothelial cells and the vascular tube formation [221].

Apelin might contribute to the formation of FVMs during the development of PDR and apelin may not be directly regulated by VEGF. Consequently, apelin signalling could represent a new promising therapeutic target during pathologic neovascularization associated with PDR [222].

9.3. Adiponectin. Adiponectin (APN) is a polypeptide hormone produced exclusively in adipocytes and circulates

at very high levels in the bloodstream. In experimental studies, APN has been shown to exert anti-inflammatory and antiatherosclerotic effects and to inhibit neointimal thickening and vascular smooth muscle cell proliferation in mechanically injured arteries. Plasma APN concentrations are decreased in obesity, insulin resistance, type 2 diabetes, coronary disease, and hypertension [223]. Several studies have indicated that APN possesses anti-inflammatory properties and thus may negatively modulate the process of atherogenesis [224]. The role of APN in the development of microvascular disease (such as diabetic retinopathy and nephropathy) is largely unknown.

In patients with PDR, aqueous humor levels of APN are significantly higher than those recorded in control subjects and tend to diminish after intravitreal bevacizumab [225, 226]. These increased APN levels may represent a local reparative response to endothelial dysfunction.

Circulating APN levels well correlate with blood inflammatory marker levels, being highest in the presence of chronic inflammatory diseases. This effect is mediated by a downregulation of a TNF-α, whose levels are chronically increased in Type 2 diabetes. These remarks underline the relationships with inflammatory background and clearly indicate the role of APN as an endogenous modulator of microvascular function and inflammation.

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Review Article

Mechanism of Inflammation in Age-Related Macular Degeneration

Francesco Parmeggiani,[1] Mario R. Romano,[2,3] Ciro Costagliola,[2] Francesco Semeraro,[4] Carlo Incorvaia,[1] Sergio D'Angelo,[1] Paolo Perri,[1] Paolo De Palma,[1] Katia De Nadai,[1,5] and Adolfo Sebastiani[1]

[1] *Department of Ophthalmology, University of Ferrara, Ferrara, Italy*
[2] *Department of Health Sciences, University of Molise, Campobasso, Italy*
[3] *Department of Ophthalmology, Istituto Clinico Humanitas, Milan, Italy*
[4] *Department of Ophthalmology, University of Brescia, Brescia, Italy*
[5] *Center for Retinitis Pigmentosa of Veneto Region, ULSS 15 Alta Padovana, Camposampiero, Italy*

Correspondence should be addressed to Francesco Parmeggiani, francesco.parmeggiani@unife.it

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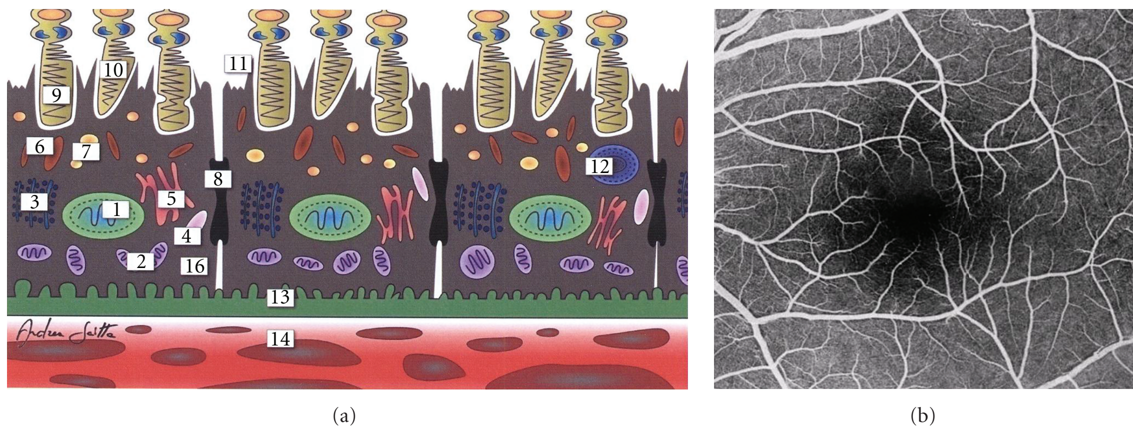
Age-related macular degeneration (AMD) is a multifactorial disease that represents the most common cause of irreversible visual impairment among people over the age of 50 in Europe, the United States, and Australia, accounting for up to 50% of all cases of central blindness. Risk factors of AMD are heterogeneous, mainly including increasing age and different genetic predispositions, together with several environmental/epigenetic factors, that is, cigarette smoking, dietary habits, and phototoxic exposure. In the aging retina, free radicals and oxidized lipoproteins are considered to be major causes of tissue stress resulting in local triggers for parainflammation, a chronic status which contributes to initiation and/or progression of many human neurodegenerative diseases such as AMD. Experimental and clinical evidences strongly indicate the pathogenetic role of immunologic processes in AMD occurrence, consisting of production of inflammatory related molecules, recruitment of macrophages, complement activation, microglial activation and accumulation within those structures that compose an essential area of the retina known as macula lutea. This paper reviews some attractive aspects of the literature about the mechanisms of inflammation in AMD, especially focusing on those findings or arguments more directly translatable to improve the clinical management of patients with AMD and to prevent the severe vision loss caused by this disease.

1. Introduction

Age-related macular degeneration (AMD) is a common disease of the central area in the ocular posterior segment, known as the *macula lutea*. This retinal area is essential for the vision of fine details and the image resolution, capturing the greatest focus of the external light stimuli. In the human macula, there are several recognizable main structures: the neuroretina (NR), composed by the inner neurosensory layer and outer photoreceptor cell layer with the underlying retinal pigment epithelium (RPE); this latter structure is separated from the choriocapillaris (CC) by the Bruch's membrane (BM), a modified basement stratum. The retina contains two types of photoreceptors, rods and cones. The rods are more numerous (about 120 million) and are more sensitive than the cones, being responsible for vision at low light levels (scotopic vision). They are not sensitive to colors and characterized by low spatial acuity. Conversely, the 6 to 7 million cones are active at higher light levels (photopic vision), are capable of color vision, and are responsible for high spatial acuity. The center of the macula, called *fovea centralis*, is an avascular zone exclusively populated by cones (Figures 1(a) and 1(b) [4]). AMD is due to multifaceted degenerative disorders involving the NR-RPE-BM-CC complex at the level of macular region [1–3].

AMD represents the main cause of legal blindness or low vision in those developed countries with the longest



(a) (b)

Figure 1: Normal human macula. (a) Schematic cross-sectional illustration of the macular outer segment and, in particular, the cells of the retinal pigment epithelium ((1) nucleus, (2) mitochondria, (3) ribosomes, (4) lysosomes, (5) Golgi apparatus, (6) melanosomes, (7) lipofuscin granules, (8) zonula occludens, (9) photoreceptor (cone), (10) outer segment of cones, (11) phagocytosis of photoreceptorial discs, (12) phagosome, (13) Bruch's membrane, and (14) choriocapillaris). (b) Fluorescein angiography of the macula with its foveal avascular zone (extracted and modified from [4]).

life expectance, especially affecting the elderly people of European descent [3]. In North America, Europe, and Australia, AMD accounts for up to 50% of all cases of central blindness [5], approximately reaching a prevalence of 3% among the general adult population [6]. In the United States, about 15% of people older than 80 years of age were estimated to have AMD on 2000, and this number is expected to rise in the next years reaching more than 2.95 million people with AMD in 2020 [7].

Numerous and heterogeneous pathological processes are likely to predispose an individual to AMD, which is considered an extremely complex, multifactorial disease. Aging represents its primary determinant, while environmental factors such as cigarette smoking [8, 9], dietary habits [10–12], and phototoxic exposure [13–15] contribute to significantly increase the risk of AMD occurrence, together with several gene polymorphisms [16–22]. In a population-based twin study including both concordant/discordant and monozygotic/dizygotic sibling pairs, Seddon and coworkers have evaluated the relative contribution of heredity and environment to AMD etiology, concluding that heritability estimates for AMD are remarkable and range from 46% to 71% [23]. More recently, the same research group has indicated that individual genotypic susceptibility interacts with behavioral and nutritional factors in the etiology of AMD by means of various epigenetic mechanisms [24], further supporting the importance of the epigenetics into AMD investigations [25, 26]. These data, along with findings of genome-wide association studies, emphasize the presence of an important rationale to practice the search for AMD-related gene variants [27, 28], despite the unavoidable efforts required to plan genetic analyses of a complex disease with late onset. In particular, remarkable correlations were documented between common or rare immunological/inflammatory gene polymorphisms and AMD, unequivocally indicating the involvement of inflammation and immune-mediated processes (complement activation) in the pathogenesis of this disease [21, 29–32]. Thus, although AMD is not considered a classic inflammatory disease, immunocompetent cells, such as macrophages and lymphocytes, are present in the chorioretinal tissues affected by AMD [33, 34]. Moreover, peculiar signs of abnormalities/dysregulation of innate immune system are observed in eyes with AMD principally at the level of the complement pathway, including complement components C3a and C5a, C5 and C5b-9 terminal complement complex, complement regulators or inhibitors, that is, complement factor H (CFH), vitronectin and clusterin, complement receptor 1 (CR1, also called CD35), membrane cofactor protein (MCP, also called CD46), and decay accelerating factor (DAF, also called CD55), but also at the level of C-reactive protein (CRP) [18, 35–41]. In particular, activation products C3a, C5a, and C5b-9 are also systemically elevated in patients suffering from AMD [42–45]. In the course of AMD, several immunopathological phenomena occur within the NR-RPE-BM-CC complex of the macular area, especially due to the pathophysiologic effects of complement system, which have a main role in the parainflammation of the aging retina [46–52]. Herein, we briefly review the literature on the involvements of inflammation in AMD, highlight the possible environmental, genetic, and/or epigenetic interactions, and discuss those therapeutic approaches potentially able to modulate inflammatory pathways and more directly translatable to the management of AMD patients.

2. Parainflammation and Age-Related Macular Degeneration

Parainflammation is defined as a condition of tissue adaptive response to noxious stress or malfunction, and it has features which are considered as intermediate between normal/basal and inflammatory/acute states. Although the physiological purposes of normal parainflammation are to preserve tissues homeostasis and to restore their functionality, when

a tissue is exposed to stress and/or malfunction for a prolonged period, it is implicated in both initiation and progression of many human age-related disorders, such as AMD [47, 53]. The risks of degenerative diseases, at least partially related to the pathophysiologic para-inflammatory response, are especially relevant in those tissues functionally dependent on nonproliferative cells and characterized by very high metabolism and other oxidative stress, such as the macular retina. In humans, the retina is a highly differentiated neuroectodermal tissue, in which an outer layer of photoreceptors, two layers of neuronal cells bodies, and two layers of synapses are present. The NR, together with RPE cells, forms the intraocular functional unit of the visual system. Like the components of central nervous system (brain and spinal cord) and several other tissues, retina also undergoes many pathophysiologic modifications with age. Because of cell and tissue damage/malfunction, mainly due to accumulative oxidative and metabolic changes in NR-RPE-BM-CC complex induced by reactive oxygen species (ROS), the vision sensitivity progressively declines during the aging process. In accordance with the "free radical theory of aging," originally expressed by Harman in 1956, age-related degeneration is basically caused by an imbalance between ROS-induced tissue damages and repair/remodelling processes [54]. This concept seems to be extremely important for human AMD; in fact, its main risk factors include increased age, smoking, augmented body mass index, phototoxicity and inflammation [8–15, 55], and all these factors augment ROS generation [14, 56–62]. Moreover, exactly the innate immune system, which plays a key role in tissue repair/remodeling processes, is also the same one that is more interconnected with AMD susceptibility and etiopathogenesis starting, respectively, from genotypic [21, 29–32] and phenotypic [18, 35–45] points of view. Particularly, the outer photoreceptor/RPE/MB complex, that is, the site of onset of the elementary AMD lesions (drusen), is considered more prone to oxidative stress because of both its proximity with the highly variable choroidal hemodynamics and its continuous exposition to photooxidation due to light stimuli [13–15, 63, 64]. In fact, unregulated blood flow may increase the fluctuations of tissue oxygen concentration, leading to elevated ROS generation by the mitochondria [63, 64]. Likewise, photooxidation in photoreceptors is associated with complement activation [65], which can increase membrane attack complex formation, an important trigger of those apoptotic processes inducing nonlethal, retinal degeneration [66–68]. ROS augmentation can also trigger angiogenic signaling that has a crucial role in the occurrence of the more severe complication of AMD, that is, choroidal neovascularization (CNV) [69–72]. In other words, several factors, linked to AMD etiopathogenesis, lead to increased ROS generation and can mediate apoptosis and angiogenesis, which are more implicated in the atrophic and neovascular AMD forms, respectively [14, 46, 63–72]. Finally, the critical position of complement must be, once again, emphasized. In fact, dysregulation of complement pathways can lead to that autologous damage which, at the macular level, is manifested by the development of drusen. Starting from this rational (even if notional) point of view, the earliest hallmarks of AMD may act as foci of chronic inflammation [49, 52, 73].

During the normal aging, in the NR, the number of neuronal and ganglion cells decreases, as also happens in the case of RPE cells which generally suffer the greatest losses in the macular and surrounding areas. Lipofuscin, the main aging-associated retinal end product, accumulates in the RPE cells with age, and its autofluorescent properties are routinely used in the clinical practice for the diagnostic imaging of various macular disorders. Another crucial age-related retinal change is the BM alteration, characterized by an increased thickness, accumulations of basal laminar deposits and/or drusen formation, and frequently accompanied by pigmentary irregularities due to RPE cell hypertrophy, hyperplasia, or atrophy. Usually, all these occurrences are more evident at the posterior pole in comparison with retinal periphery. In addition, both biochemical constitutions and biophysical properties of the BM modify with age, also influencing a further RPE cell dysfunction as well as noticeable CC disorders. Although the retina has been traditionally considered as an "immunologically privileged" tissue, at present it is known to have an endogenous immune system, actively coordinated by immunocompetent cells (microglia and dendritic cells), along with a rare population of perivascular macrophages; moreover, also RPE cells possess a variety of immunological functions. Retinal microglia and RPE cells, together with choroidal macrophages/dendritic cells, physiologically play an essential role in retinal homeostasis [47, 74–78]. In the aging retina, all these elements represent important factors in both dealing with the retinal malfunctions and restoring retinal homeostasis or rebalancing the homeostatic points. Several sight-threatening retinal diseases have a higher prevalence among the elderly persons, but the most common of these is AMD that can be diagnosed in its early (Figures 2(a) and 2(b) [4]) or intermediate (Figures 3(a) and 3(b) [4]) drusen/RPE-atrophy/pigmentary forms, as well as in its advanced forms, that is, geographic atrophy and neovascular AMD (Figures 4(a)–4(d)) [3]. Even if the clinical pattern of the above-mentioned types of AMD significantly differs, low-grade/subclinical degree of inflammation (parainflammation) is implicated in every AMD forms, reaching a high level when maculopathy is complicated by CNV development [71]. In neuroretinal structure, para-inflammatory modifications are characterized by the breakdown of blood-retinal barrier, microglial activation, and subretinal migration, whereas, in the choroid they become evident with an increased number of $CD45^+$ $CRIg^+$ macrophages, morphologic abnormalities of melanocytes, tissue's thickening, and fibrosis. At the retinal/choroidal interface, these AMD-related changes are particularly manifested by complement activation in RPE-BM cells and microglia accumulation in subretinal space [47, 48, 71]. Insightful knowledge on the mechanisms of retinal parainflammation, as well as of complement dysregulation, is fundamental to comprehensively understand the pathogenesis of AMD and to develop better curative therapeutic strategies for the different forms of this harmful disease.

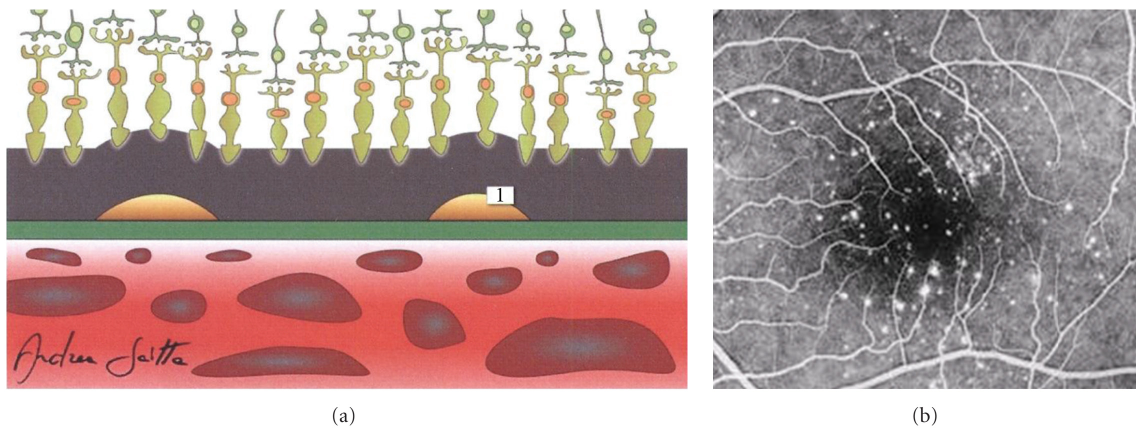


FIGURE 2: Early age-related macular degeneration. (a) Schematic cross-sectional illustration of the macula with an early stage of the disease ((1) drusen). (b) Fluorescein angiography of the macula affected by an early form of the disease (nonconfluent *hard* drusen); in this eye, the best best-correct visual acuity was 20/20 (Snellen equivalent) (extracted and modified from [4]).

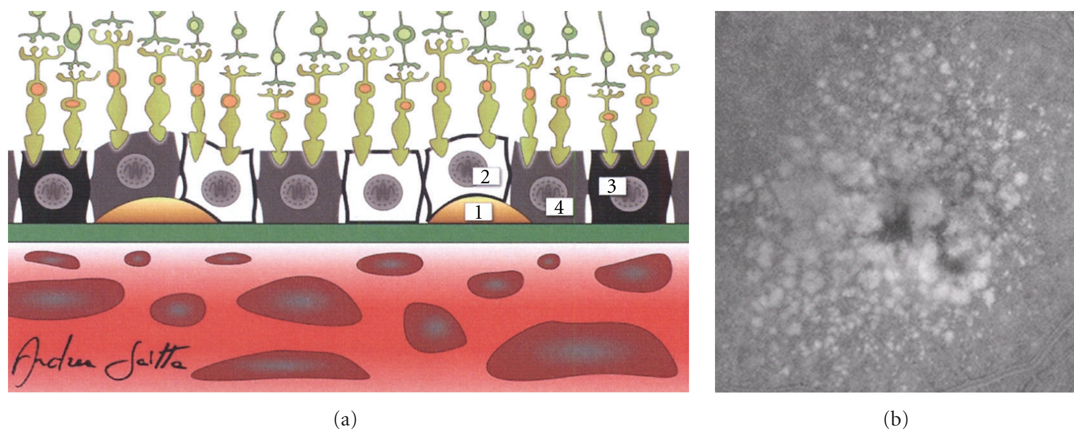


FIGURE 3: Intermediate age-related macular degeneration. (a) Schematic cross-sectional illustration of the macula with an intermediate stage of the disease ((1) drusen; (2) atrophy of a cell of the retinal pigment epithelium; (3) hypertrophy or hyperplasia of a cell of the retinal pigment epithelium; (4) a normal cell of the retinal pigment epithelium). (b) Fluorescein angiography of the macula affected by an intermediate form of the disease (confluent *soft* drusen and pigmentary irregularities); in this eye, the best best-correct visual acuity was 20/50 (Snellen equivalent) (extracted and modified from [4]).

3. Complement System and Age-Related Macular Degeneration

Complement system consists of over 40 proteins and regulators which are detectable in the blood circulation. It plays a key role in host defense against pathogens, adaptive immune responses, removal of the immune complexes and apoptotic cells [79]. In humans, three complement-mediated pathways complementarily act, and each of them is characterized by a specific trigger as follows:

(i) antibody-antigen complex for the classical pathway;

(ii) binding to host cell or pathogen surface for the alternative pathway;

(iii) polysaccharides on microbial surfaces for the lectin pathway.

Dysregulation and/or dysfunction of the complement pathways can result in various critical autologous damages, with consequent pathogenetic implications in a wide spectrum of diseases [52]. Both pathogenesis and progression of AMD represent complex events, in which complement system is directly or indirectly implicated. Pathobiologic studies have identified numerous complement proteins inside drusen (i.e., the elementary clinical lesions identifiable in the macula of AMD patients), and genetic analyses have discovered the existence of common or rare polymorphisms in several complement-related genes that significantly increase or reduce the risk for AMD late in life [49]. In fact, the phenotypic features of drusen (i.e., clinical pattern and time of onset) and the genotypic individual background for AMD seem to be mutually and closely intersected with each other, figuratively sharing, as lowest common denominator, the local dysregulation of the complement system in the NR-RPE-BM-CC complex due to acquired and/or inherited risk factors. In particular, the development of AMD-related drusen occurs between the basal surface of

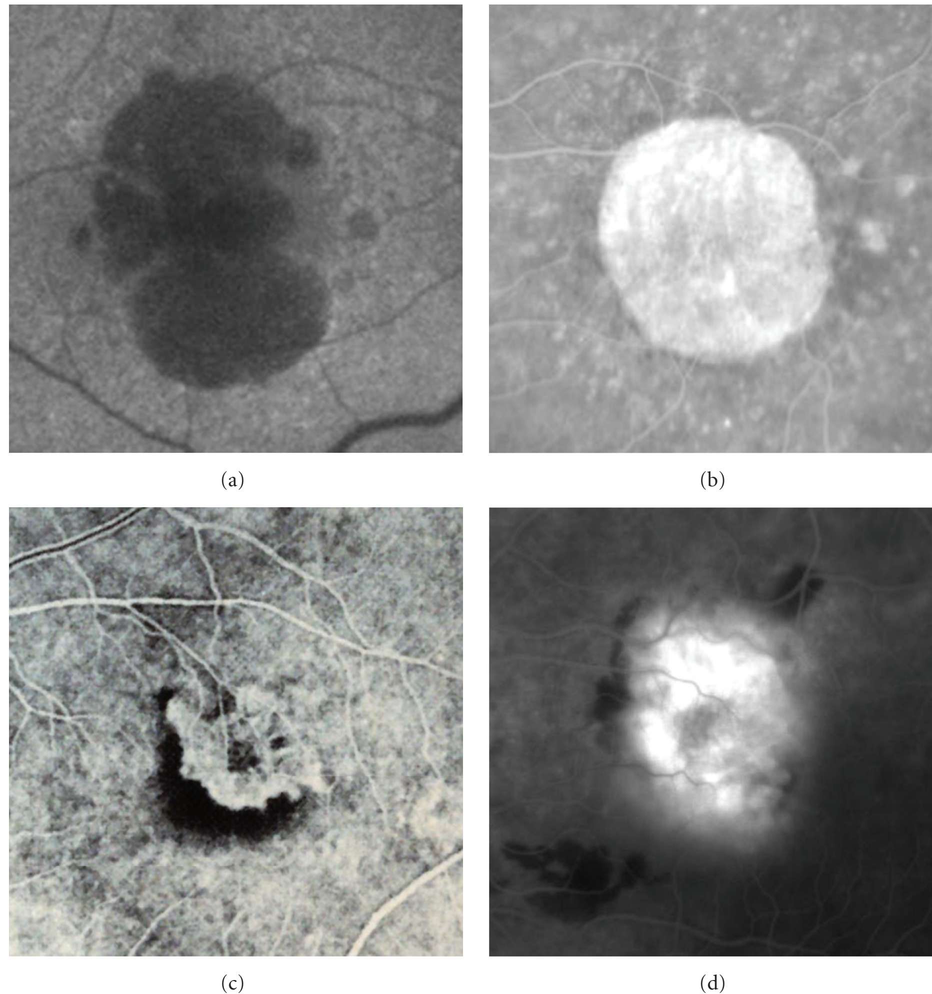


Figure 4: Advanced forms of age-related macular degeneration. (a) Autofluorescent retinography and (b) fluorescein angiography of two different cases of severe central geographic atrophy; in these eyes, the best-correct visual acuities were, respectively, 20/125 and 20/160 (Snellen equivalent). (c) Early and (d) late fluorescein angiograms of two different cases of subfoveal choroidal neovascularization; in both these eyes, the best-correct visual acuity was 20/200 (Snellen equivalent).

RPE and the BM, a single-stratified extracellular matrix in contact with CC (Figures 2(a) and 3(a) [4]) [80]. Since the mid 1990s, increasing experimental and clinical evidences clearly indicate that a lot of complement-related molecules, such as complement activators, complement components, and complement regulatory proteins, represent substantial constituents of the drusen [35, 36, 65, 66, 73, 81–93]. Starting from the beginning of the 2000s, the more and more exact identification of their compositional profile has been essential to create the basis for a new paradigm of AMD pathogenesis, in which macular and perimacular drusen should be considered as the earliest diagnosable byproducts of chronic local para-inflammatory phenomena at BM level. According to this model of AMD occurrence and progression, parainflammation of retinochoroidal tissues, accompanied by complement activation, immune-mediated processes, and bystander cell lysis, becomes the most crucial aspect of this neurodegenerative maculopathy [47–52, 73, 85, 94].

In the course of the past few years, a definitive support for the "immuno-inflammatory" model of AMD pathogenesis has been evidenced by the clinical-genetic findings of numerous studies, which revealed highly significant correlations between AMD and polymorphisms of genes encoding for several molecules directly involved in the activities of the complement alternative pathway. Of these genes/loci, the most studied ones are:

(i) complement factor H (CFH) [16–18, 95–99],

(ii) complement component 3 (C3) [100–103],

(iii) complement factor I (CFI) [104, 105],

(iv) complement factor B (CFB) [19, 106, 107],

(v) complement component 2 (C2) [19, 106, 107],

(vi) CFH-related genes (CFHR) type 1–5 [108–110].

Even if some of these relationships between these genes/loci and AMD are incompletely understood, their

comprehensive consideration indicates, once again, that complement-related polymorphic alleles are able to increase (CFH, C3, CFI, and CFHR-2-4-5) or reduce (CFB, C2, and CFHR-1-3) AMD risk, representing a central key point on which the evidences of the high heritability of AMD are based [23, 106, 111]. Then again, genetic susceptibility to AMD is a very multifaceted issue that also includes several other immunological/inflammatory aspects, either just indirectly linked or not linked to complement system such as, for example:

(i) interactions between C-reactive protein (CRP) and Y402H variant of CFH gene (rs1061170), a very common single nucleotide polymorphism (SNP) located within the chromosome 1q32 region and unequivocally identified in association with AMD among multiple study populations—providing for the first time a logical basis by which to assess the disease's risk in over 50% of affected individuals [49, 50, 52, 112];

(ii) potential synergisms between the above-mentioned SNPs in genes/loci encoding for factors or components of the alternative complement pathway and some noncomplement-related genes, located on the chromosome 10q26 region and extensively described as strongly implicated in AMD pathogenesis, that is, the rs10490924 SNP of the age-related maculopathy susceptibility 2 (ARMS2), and the rs11200638 SNP of the high-temperature requirement factor A of serine peptidase 1 (HTRA1) [21, 22, 26, 49, 111].

CRP is a biomarker of acute-phase inflammation. It plays an essential role in the innate immune response to tissue injury and/or infection. Because CRP induces complement activation via the alternative pathway, it is plausible that CRP may have a direct responsibility in AMD pathogenesis by causing macular damages via complement-mediated mechanisms, as also happens in the case of CFH [46, 50, 71, 113, 114]. In fact, although several facets of the CFH-CRP interaction are not yet well defined [115], several findings have indicated that in carriers of the polymorphic H402 variant of CFH gene a lower affinity for CRP exists in respect of the individuals with the Y402 protein [92, 116, 117]. Moreover, a more recent study has confirmed that native CRP-CFH interaction is evident at high plasma CRP concentrations (as happens during the acute-phase response, i.e., when the H402 protein inadequately binds to CRP) [118], and also a large meta-analysis has documented that serum levels of CRP >3 mg/L are related to a double AMD risk in comparison with CRP concentrations <1 mg/L [119]. Starting from these latter results, it is not surprising that homozygous CFH-Y402H polymorphic genotype, together with elevated serum/plasma CRP levels, leads to a very high risk of both AMD and its progression (with odds ratios of 19.3 and 6.8, resp.) [120], even if the CRP elevation is not related to any variant of the CRP gene and no polymorphism in this gene is directly associated with AMD [121, 122]. As well, at the levels of RPE and choroid of CFH-H402 homozygous carriers, a greater amount of CRP was detected in comparison with that found in Y402 homozygotes, but there was no significant difference in CFH protein concentrations among individuals with diverse Y402H genotypes. This lack of local CRP expression indicates that CRP is present in the posterior segment of the eye as a consequence of deposition through chronic low-grade local inflammation [91].

Based on early genome-wide linkage analyses, which have established that the 10q26 locus is closely associated with AMD [123–126], several clinical-genetic studies, specifically focused on this chromosomal region, discovered two major hereditary predisposing factors for AMD: the *ARMS2* [127, 128] and the *HTRA1* genes [129, 130]. At present, ARMS2 locus is considered a noncomplement-related gene because its potential role in the inflammatory process, if any, remains to be clarified [49]. In fact, although rs10490924-ARMS2 mRNA is detected in the human retina, both prevalent expression and cellular location of its putative protein are still under debate, having been initially observed in the mitochondrial outer membrane [131], and later in the cytosol and extracellular compartment [132, 133]. In any case, it seems extremely unlikely that deficiency of ARMS2-related protein could be a direct pathogenic mechanism responsible for AMD [134]. Also HTRA1 locus, encoding for a secreted protein belonging to the high-temperature requirement A family of serine proteases, can be still labeled as noncomplement-related gene [49]. However, because some molecules involved in the complement activities (i.e., clusterin, vitronectin, and fibromodulin) represent specific substrates for HTRA1 serine protease, an implication of *HTRA1* in complement system has been notionally indicated [135]. The initial investigations documented the correlation between the rs11200638 promoter variant of the HTRA1 gene and an increased expression of its protein [129, 130], whereas other studies have not replicated these outcomes [131, 136]. Nevertheless, more recent reports showed that HTRA1 mRNA expression is higher in cultured RPE cells homozygous for the HTRA1 allele related to AMD risk, also supporting the perception that *HTRA1* could be one of the causal genes in AMD patients [135, 137, 138].

In consideration of the heterogeneous gene-gene relationships between the major risk variants of the CFH, ARMS2, and HTRA1 loci, several Authors have emphasized the consistent possibility of an independent multiplicative joint effect in AMD, also taking into account that each of them should be contextualized within gene-environment interactions and epigenetic aspects [22–25, 49, 139, 140]. Exclusively limiting the focus on those well-recognized SNPs which confer increased or decreased risk of inflammation (i.e., CFH, CX3CR1, IL-8, and TLR3 and 4), and voluntarily ignoring the other, suspected or ascertained, AMD-related gene variants (i.e., APOE, ABCR, LIPC, TIMP3, PON1, ERCC6, ELOVL4, fibulin-5, hemicentin-1, SERPING1, VLDLR, LRP6, VEGF, and KDR), the etiopathogenetic scenario of AMD is exactly that of a complex/polygenic disease characterized by (i) multiple clinical phenotypes with non-Mendelian transmission; (ii) environmental effects; (iii) increased incidence with age; (iv) specific susceptibility genes with variant alleles (Table 1) [20,

Table 1: Main AMD-susceptibility genetic loci.

Locus	Role in immunoinflammatory pathways	Possibility of AMD-risk elevation
CFH	Yes (complement system)	High (in carriers of polymorphic allele)
ARMS2	Not clarified	High (in carriers of polymorphic allele)
HTRA1	Possible (complement system)	High (in carriers of polymorphic allele)
CFB	Yes (complement system)	Intermediate (in carriers of wild allele)
C2	Yes (complement system)	Intermediate (in carriers of wild allele)
C3	Yes (complement system)	Intermediate (in carriers of polymorphic allele)
CFI	Yes (complement system)	Low (in carriers of polymorphic allele)
TIMP3	Yes (immunity in extracellular matrix)	Low (in carriers of polymorphic allele)
LIPC	Not clarified	Low (in carriers of polymorphic allele)
ABCR	No	Low (in carriers of polymorphic allele)
APOE	No	Low (in carriers of polymorphic allele)

Legend: CFH: complement factor H; ARMS2: age-related maculopathy susceptibility 2; HTRA1: high-temperature requirement factor A of serine peptidase 1; CFB: complement factor B; C2: complement component 2; C3: complement component 3; CFI: complement factor I; TIMP3: tissue inhibitor of metalloproteinases 3; LIPC: hepatic lipase gene; ABCR: ATP-binding cassette transporter; APOE: apolipoprotein E.

22, 25, 26, 29, 46, 140–162]. The next section of this paper focuses on those immunological/inflammatory topics more directly translatable to improve the therapeutic strategies against AMD and, in particular, against its neovascular form, often responsible for the cases of most severe visual loss.

4. Agents Directed against the Immune Response and Age-Related Macular Degeneration

The responses of human immune system are necessary to defend our organism against several diseases, external antigens, invading microorganisms and/or acute tissue injuries. However, the contribution of the immune system in the occurrence of chronic age-related pathologic conditions has not been yet fully understood. In the course of the normal aging, as well as during chronic diseases, low-grade tissue stress (caused by noninfectious insults) may be related to subclinical damages resulting in the release of endogenous molecules, collectively called “alarmins”, that activate immunocompetent cells capable to support both innate and acquired immunity. In fact, they recruit and/or trigger receptor-expressing cells of the innate immune system, such as dendritic cells and macrophages, and consequently can also promote adaptive immunity in either direct or indirect manner [47]. To restore tissue homeostasis, by means of a mounting localized para-inflammatory response, immune system must be able to early identify such minimal biopathological changes in each specific district. Conversely, dysregulation or dysfunction of the immune system in chronically facing low-grade stress conditions may lead to manifest pathologies.

The retina, like the brain, is a high-metabolism tissue and is sensitive to noxious microenvironmental stimulations; however, unlike the brain, it is constantly exposed to the light, which can produce loads of photooxidized materials. Light-related stress and other oxidative damages increase in retinal tissue with the aging, as does the para-inflammatory response. In this view, although AMD is not a classic inflammatory disease, innate immunity and autoimmune components (i.e., complement factors, chemokines, cytokines, macrophages, and ocular microglia) have a reliable role in both pathogenesis and progression of AMD [47, 49, 163]. During the last two decades, directly or indirectly targeting these specific molecules/components, implicated in the immunoinflammatory pathways, has been assessed in the attempt to improve the therapeutic management of patients affected by the different clinical forms of AMD (Figures 2–4).

Since the mid 2000s, the Age-Related Eye Disease Study (AREDS, a large multicenter randomized clinical trial evaluating the long-term effects of high-dose antioxidant nutritional supplements on the incidence and progression of AMD and cataract) has documented a significantly lower incidence of advanced AMD in patients with drusen maculopathy treated with appropriate dosages of antioxidants than in a placebo group [164–169]. Also other studies and evidences indicate the opportunity to indirectly counteract para-inflammatory changes minimizing the retinal oxidative stresses in AMD patients [11, 13–15, 170, 171]. In particular, oral lutein intake results in beneficial effects on various visual function tests, and recent findings show that it is able to influence immune/inflammatory responses, not only diminishing the manifestation of various ocular inflammation models, but also suppressing NF kappa-B activation and/or inhibiting the expression of iNOS and COX-2 [48, 171].

On the other hand, during the last few years, numerous trials have been started to verify the therapeutic effects of various drugs aimed to directly downgrade the retinochoroidal immune response in AMD patients. In the next future, the outcomes of these ongoing clinical studies (156 studies found at http://clinicaltrials.gov/ searching, on June, 26 2012, with the keywords “age-related macular degeneration” and “anti-inflammatory”) together with the already reported findings [163, 172–175] will be able to provide a more exact delineation of the role of the agents directed against the immune response in therapeutic recommendations for AMD patients [165–168, 176–186]. The majority of these

interventional trials are conducted on patients affected by neovascular AMD, employing corticosteroids (i.e., dexamethasone and triamcinolone acetonide), nonsteroidal anti-inflammatory drugs (i.e., low-dose acetylsalicylic acid, bromfenac, diclofenac, and nepafenac), immunosuppressive agents (i.e., methotrexate and rapamycin), and biologics (i.e., anti-TNF-α agents such as infliximab and adalimumab, IL-2-receptor antagonists such as daclizumab, and complement inhibitors/regulators such as ARC1905, TNX-234, eculizumab, and POT-4), with the exception of rapamycin which has been also evaluated in cases of geographic atrophy secondary to AMD [163, 187].

The pathogenetic scenario that gives rise to the first RPE-BM-CC alterations in AMD is extremely complex. It includes a variety of predisposing genetic backgrounds, which can take effect on an heterogeneous plethora of para-inflammatory causative factors: cigarette smoking, phototoxic oxidative exposure, dietary habits, alterations of iron and lipid homeostasis, buildup of advanced glycation endproducts, microbial infection, lipofuscin and beta-amyloid toxicity, excessive immune-complex generation, choroidal hemodynamic insufficiency and ischemia, phagocytic overload, and/or RPE autophagy [49]. However, regardless of what are the conditions that can initially trigger the macular degenerative pattern in each individual AMD patient, it is indisputable that the decisive downstream consequences are the deposition and/or sequestration of both cellular and acellular debris at sub-RPE level. In the course of the normal human aging, mid- or long-term malfunctions in the tissue processing of these debris can be sufficient to locally generate abnormal para-inflammatory signal with a consequent aberrant activation of the complement system. This macular status would most likely result in persistent complement attack, further sub-RPE deposits, continuous formation of drusen, bystander injury to neighboring cells and, finally, irreversible photoreceptor degeneration and/or deconstruction (especially in those lots of adult or elder individuals who are more genetically susceptible to AMD for the presence of polymorphisms influencing the immune-inflammatory pathways and, in particular, the alternative complement-modulating activity) [25, 47, 49]. Starting from this rationale, our current knowledge regarding the role of both inflammation and complement systems in AMD should be refined to the point where it can be more easily translated in an innovative enhancement of AMD treatments, by means of either comparative randomized clinical trials or interventional pilot studies or biogenetic therapeutic researches.

5. Final Remarks

In the recent years, a substantial amount of evidences and/or arguments document the crucial responsibility of immune-inflammatory processes in the pathogenesis of AMD [3, 47–52, 71, 188], clearly indicating the importance not only of specific complement-modulation agents, but also of nonspecific anti-inflammatory drugs, as adjunctive therapies for both non neovascular AMD (conventionally treated with AREDS formula and lutein) [163, 165–171] and, most of all, neovascular AMD (routinely treated with intravitreal administration of drugs acting against vascular endothelial growth factor (anti-VEGF) and/or with photodynamic therapy with verteporfin (PDT-V)) [163, 172–186, 189–194]. The fact that para-inflammatory dysregulation is already present in the early stage of AMD may notionally support the preventive employment of agents directed against the immune-inflammatory response in combination with high-dose nutritional supplements (particularly in those patients with a disabling form of maculopathy in one eye, younger than 65 years, and/or carrier of significant genetic susceptibility to AMD) [30, 164–171]. On the other hand, the existence of variable mid-term responsiveness of CNV to either anti-VEGF or PDT-V regimen (often resulting in elevated risks of legal blindness, high societal costs and expensive economic burden) practically recommends, above all in patients with advanced AMD in one eye, the adjunctive utilization of drugs directed against the immune-inflammatory response in combination with anti-VEGF injections and/or PDT-V [114, 171, 176–179, 183–185, 195–217].

Returning to focus on the above-mentioned translational concepts about the opportunity of pharmacologic modulation toward the immune-inflammatory pathways in AMD, a comprehensive approach is warranted to verify the chances of a prompt application of this curative modality in the clinical setting. In theory, to modulate the complement attack and minimize the local parainflammation in AMD patients who carry one or more complement-related gene polymorphisms predisposing to the disease, the most specific approach would augment the retinochoroidal bioavailability of the native/protective form of those complement factors or components responsible for the genetic susceptibility to AMD [218–220]. Adhering to this work hypothesis, a variety of delivery systems (i.e., gene transfer, cell-based therapies, organ (liver) transplantation, systemic or intraocular injections) can be envisioned to slow or arrest AMD by reasserting control over the complement system and, in particular, over its alternative pathway. If this biogenetic "augmentation" concept will be applicable also in the clinical AMD patterns, new complement-modulation therapeutics could be added to those several drugs directed against the immune-inflammatory response and already being tested on humans [49, 163]. However, at the moment, pending the concrete applicative possibilities of these biogenetic and/or pharmacologic complement-targeted treatments, open-label clinical trials are recommend, especially in patients with neovascular AMD, to better evaluate the therapeutic anti-CNV rationale in combining intravitreal corticosteroids either with the conventional anti-VEGF regimens or with anti-VEGF plus PDT-V customized protocols. In this view of good postmarketing study practice, as additional anti-CNV treatment, a promising anti-inflammatory strategy is that which involves the use of drug delivery systems (i.e., nonbiodegradable insert or biodegradable implant), able to provide a sustained release of intravitreal corticosteroids (fluocinolone acetonide or dexamethasone) for several months [221, 222]. In fact, taking into account both that immunoinflammatory phenomena are very active during

the occurrence of an AMD-related CNV [71, 223], and that corticosteroids act upstream in immunoinflammatory cascades with consequent inhibition of the alternative-amplification of complement pathway [224, 225], and that a prolonged pharmacologic action represents an important parameter for the final efficacy of any therapy against neovascular AMD [176–180], the above-described intraocular devices, already approved for the treatment of peculiar forms of macular edema and of noninfectious posterior uveitis [221, 222], could represent a rational adjunctive therapeutic approach for patients with neovascular AMD undergoing repeated intravitreal injections of anti-VEGF drug.

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Review Article

Vitreous Analysis in the Management of Uveitis

Erika M. Damato,[1] Martina Angi,[2] Mario R. Romano,[3] Francesco Semeraro,[4] and Ciro Costagliola[5]

[1] *Regional Ocular Inflammatory Service, Bristol Eye Hospital, Bristol BS1 2LX, UK*
[2] *Department of Molecular and Clinical Cancer Medicine, University of Liverpool, Liverpool L69 3BX, UK*
[3] *Department of Ophthalmology, Istituto Clinico Humanitas, Milan, Italy*
[4] *Department of Ophthalmology, Spedali Civili di Brscia, University of Brescia, Brescia, Italy*
[5] *Department of Health Sciences, University of Molise, Campobasso, Italy*

Correspondence should be addressed to Erika M. Damato, erikadamato@hotmail.com

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A correct diagnosis of uveitis is often challenging, given the wide range of possible underlying conditions and the lack of typical phenotypes. Management decisions may be difficult in view of the risk of visual loss with either inappropriate or delayed therapy. Analysis of the vitreous may therefore be used to provide the clinician with valuable information. In this paper, we describe the main clinical situations in which vitreous sampling is indicated and provide some guidance to clinicians for tailoring their requests. These situations include suspected intraocular infection and suspected intraocular malignancy. We describe the principal tests carried out on vitreous samples, including cultures, polymerase chain reaction-based testing, and cytokine analysis. Limitations of the tests used are likely to become less as more advanced testing methods are introduced. The importance of selecting the appropriate investigations to support a clinical suspicion is emphasised, as is the interpretation of test results within a clinical context.

1. Introduction

The term "uveitis" encompasses a wide spectrum of conditions resulting in intraocular inflammation. Standardised uveitis nomenclature (SUN) defines uveitis depending on the predominant site of inflammation within the eye [1]. At the most severe end of the spectrum, uncontrolled or inadequately treated posterior uveitis may result in irreversible visual loss.

Uveitis may be associated with an underlying systemic disease or may exclusively involve the eye [2]. There are a vast number of causes and conditions related to the development of uveitis; however, these may be broadly divided into infectious, autoinflammatory, and neoplastic causes. Extensive investigations are often carried out to establish one, as the clinical phenotype may not be specific for a diagnosis. Common investigations include angiography, blood tests, urinalysis, chest X-rays, and CT scans. In certain situations, incorrect treatment may be catastrophic for vision and could potentially threaten the patient's life [3]. Hence, a rapid and accurate diagnosis based on intraocular sampling may be essential, mainly to exclude infection or malignancy before the introduction of powerful immunosuppressive or steroid therapy. The vitreous gel is amenable to sampling, either by vitreous tap, where a small amount of gel is aspirated with a needle or by a formal vitrectomy, where most or all of the vitreous gel is removed surgically [4].

In this paper, we describe the clinical situations in which vitreous sampling may become necessary, providing a guide to clinicians for tailoring their requests for laboratory analyses. We also review the salient immunological findings in the setting of experimental autoimmune uveitis and clinical studies, which may become relevant in future clinical practice.

1.1. Sampling Intraocular Fluids. Analysis of a small sample of aqueous humour may be adequate in order to confirm

a clinically suspected intraocular infection, in particular in the context of suspected viral retinitis [5].

Anterior chamber paracentesis has the advantage of being quick, relatively straightforward to perform and can be carried out in the outpatient setting [6]. Main limitations are that: (1) only about 0.2 mL of fluid are obtained, which may only be sufficient for one molecular test and (2) if there is relatively mild inflammation at the anterior part of the eye, then a false negative result may occur [5].

In order to obtain a larger sample (0.5 mL–1 mL) of intraocular fluid, vitreous sampling is necessary. This can be obtained by either a vitreous cutter or by using a 23 G needle. Formal pars plana vitrectomy requires an operation and needs to be carried out by a skilled ophthalmic surgeon. This allows up to 2 mL of undiluted vitreous to be sampled and sent for analysis.

1.2. What Are the Indications for Sampling the Vitreous? The three main indications for sampling the vitreous that will be described are

(1) suspected intraocular infection;

(2) suspected intraocular lymphoma;

(3) atypical response to therapy during the treatment of presumed autoimmune intraocular inflammation.

2. Suspected Intraocular Infections

The two most common vision threatening intraocular infections are viral retinitis and infectious bacterial endophthalmitis. In both situations, irreversible visual loss can occur rapidly. Immediate therapy is warranted with appropriate antimicrobial agents, and often the clinical phenotype and clinical history will strongly direct the clinician toward a diagnosis. Vitreous sampling helps to confirm the clinical suspicion and to identify a causative agent, which has therapeutic implications.

Tests carried out on the vitreous include cytological examination, culture of suspected organisms, and molecular analyses [7]. In clinical ophthalmology, the introduction of molecular diagnostics, mainly based on the polymerase chain reaction (PCR), has changed the management of patients, as it enables a rapid and tailored therapy [8]. PCR is more sensitive than culture for the detection of many organisms, and by utilizing the two together increased specificity can be assured [9]. PCR may; however, be affected by contaminants or by sample degradation, resulting in false positive results. Also there is a limit of detection below which the pathogen cannot be detected reliably, thus resulting in a false negative result. PCR techniques have been developed since their introduction, evolving from qualitative (presence/absence of pathogen) to quantitative or real-time PCR. Quantitative PCR is particularly useful in the clinical setting because it measures the pathogen (especially viral) load, hence allows monitoring of response to treatment over consecutive samples [10]. Multiplex PCR has also been introduced, which runs several primers at once, thus allowing several organisms to be detected and quantified simultaneously.

2.1. Viral Retinitis. The classic clinical phenotype of viral retinitis is a rapidly progressive, confluent retinitis associated with a dense vitritis, anterior chamber activity, and raised intraocular pressure. The most common causative agents are the herpetic viruses including varicella zoster (VZV) and herpes simplex (HSV) [11]. The clinical picture may be pathognomonic, in which case empirical therapy with intravitreal and systemic antiviral agents is commenced independent of a PCR-based test. In some circumstances, however, the diagnostic certainty is less [12]. Other viruses including cytomegalovirus (CMV) and possibly Epstein-Barr virus (EBV) can also be involved [13]. In immunocompromised patients, the clinical phenotype may be consistent or concurrent with other infections, which must be excluded, including syphilis and toxoplasmosis [14]. In addition, the clinical picture in such patients is less extensive that what may be expected. Therefore, when a vitreous sample is sent from a patient with suspected infectious retinitis, PCR testing for HSV, VZV, CMV, EBV, and toxoplasmosis is often requested.

Importantly, the presence of viral antigen, detected by qualitative PCR may not always be clinically relevant, for example, in viruses which can remain latent in host cells may be detected by PCR without actually being the cause of the retinitis. This is especially the case for testing EBV and CMV.

The sensitivity and specificity of PCR testing in the context of viral retinitis has been investigated both on aqueous and vitreous humour specimens. For instance, Harper et al. reported that out of 113 patients, using the final clinical diagnosis as the gold standard, a true positive result was obtained by PCR in 76 patients, whilst a true negative result was obtained in 38. There were one false positive result and 18 false negative results [15]. The result may have been influenced by the use of anterior chamber paracentesis. Other authors have reported that vitreous sampling enables a much higher sensitivity for demonstrating viral PCR as compared with anterior chamber tap [16].

The likelihood of dual pathology is higher in immunocompromised patients, as these patients are more predisposed to developing retinitis in the first place. In such patients, vitreous sampling is preferred over aqueous sampling, as volumes for testing are greater. As mentioned previously, more than one positive PCR result may, however, simply reflect the "detection" of a "latent" infection, for example, in the case of EBV. In one study by Cochrane et al., more than one infectious agent was demonstrated in 12 out of 77 patients [16].

2.2. Bacterial and Fungal Endophthalmitis. Intraocular infection can occur secondary to bacteria, either introduced into the eye during surgery or from another source in the body travelling to the eye from the systemic circulation [17]. Intraocular bacterial endophthalmitis can be devastating for vision. Classically, postoperative infection presents as a painful red eye with a hypopyon and significant vitritis in the week following intraocular surgery. In this situation, a vitreous biopsy is taken and broad-spectrum antibiotics such as vancomycin and ceftazidime are introduced into the vitreous cavity while waiting for the laboratory results.

In the laboratory, part of the vitreous specimen is put into culture, while the remainder is examined following immunohistochemical staining [18]. The most common responsible organisms identified in this setting include *Staphylococcus aureus*, *Staphylococcus epidermidis*, and *Streptococcus* species.

Sensitivity can be significantly improved by the use of PCR, where correlation with culture results is high. A recent paper evaluated the efficacy of quantitative real-time PCR in the diagnosis of postoperative bacterial endophthalmitis among 64 patients who underwent cataract surgery. PCR allowed the detection of bacterial DNA in 66% of patients, compared to 34% with traditional culture. Only one patient had a positive result by culture (*Nocardia* species) but negative result by PCR [19].

Bispo et al. have analysed aqueous and vitreous taken from 14 eyes with suspected bacterial endophthalmitis. Gram staining and culture were followed by PCR testing of ocular fluids looking for bacterial infection. Testing was carried out for 31 clinically prevalent bacteria, including both gram positive and gram negative organisms. It was possible to perform gram staining in all samples; however, culture was only successful in just under half of patients. The use of PCR enabled a positive result to be obtained in 95% of patients. In this study the rate of false positive PCR results was low, occurring in 3.4% of patients [20].

Endogenous endophthalmitis refers to infection occurring within the eye as a consequence of systemic infection. In this situation, the patient is often clinically unwell or septic. The spectrum of causative organisms is different, and fungal infections are more relevant [21]. Patients also require systemic investigations, such as blood cultures, cardiac echocardiogram, or urine cultures. Systemic antimicrobial treatment also plays a major role. In a paper by Schiedler et al. [21], fungal infections such as *Candida* and *Aspergillus* were most commonly demonstrated on culture. Targeting the investigation to the clinical context increases the diagnostic yield, as a significant proportion of patients also had demonstrable fungaemia on blood culture.

2.3. Chronic Postoperative Bacterial Endophthalmitis. Following intraocular surgery, such as phacoemulsification and intraocular lens insertion, some patients may be affected by chronic low-grade inflammation. It has been shown that it can be due to a low-grade infection caused by fastidious organisms such as *Propionibacterium acnes*, *Actinomyces israelii*, or *Corynebacterium spp.* [22]. Demonstration of such organisms by culture and microscopy is difficult, and sensitivities are low. This is attributable to the fact that these organisms may be present in very low numbers and grow very slowly in culture. A positive diagnosis of such fastidious organisms is greatly enhanced by the use of PCR. In a study investigating the delayed onset endophthalmitis following cataract surgery, vitreous testing allowed a causative organism to be identified in 92% of eyes, compared with 6% of eye using culture. In this setting, testing is guided by clinical suspicion, as the clinical phenotype may closely mimic idiopathic intraocular inflammation. Identification of an infectious cause will enable decisions regarding future therapy, such as surgical intervention and antimicrobial medication. The optimal management is controversial and strategies include systemic antibiotics, intracapsular antibiotics, and surgical removal of the intraocular lens, lens capsule, and vitreous.

As well as the fastidious bacteria mentioned, mycobacterial species or fungi may be the cause of chronic post operative or delayed onset endophthalmitis. These organisms are also demonstrable by PCR, however, require a degree of clinical suspicion for these diagnoses to be considered.

2.4. Toxoplasmosis. Toxoplasmosis is parasitic protozoan infection that can infect the retina either in utero or as a primary infection resulting in a characteristic chorioretinal scar. At times, the parasite may become reactivated, resulting in intraocular inflammation and evidence of activity or fluffy white areas around the scar. There is often vitritis and perivascular change associated with reactivation. The clinical phenotype is often typical, and usually treatment with the appropriate antimicrobials is commenced based on fundoscopic findings.

In some patients, however, especially in patients who are immunocompromised, intraocular toxoplasmosis can result in a clinical phenotype very similar to acute retinal necrosis. In such cases, accurate diagnosis is imperative as incorrect therapy with antiviral therapy will not be effective, and retinitis may rapidly progress. Patients with ocular toxoplasmosis usually have antibodies (IgG) circulating in peripheral blood, and a negative serology may often be used to exclude the diagnosis. The most accurate diagnostic testing, however, is by using intraocular fluid [23].

Ocular fluids can be tested for local antibody production or for the presence of microbial DNA using PCR. Local antibody production can be detected using immunoblotting techniques, and a Goldmann-Witmer coefficient can be calculated to compare intraocular antibody production with serum antibody levels. A ratio of greater than 1.0 is abnormal and ratios of 2-3 are significant.

In immunocompromised patients, however, antibody production is impaired, and molecular diagnostic plays an important role. In a study of 15 patients in whom a clinical diagnosis was unclear, PCR for toxoplasmosis enabled a diagnosis in 7. The remaining patients were diagnosed as having alternative conditions following further testing. In this paper, a volume of 0.4 mL of vitreous was used and qualitative PCR was utilised [24].

The use of both tests together increases the sensitivity of diagnosing toxoplasmosis as both may be affected by the immune status of the individual and by the stage of the disease. Toxoplasma DNA may not be detected until 2-3 weeks after the initiation of infection, therefore early testing may fail to demonstrate the organism, leading to a false negative result. Antibody testing is more likely to be positive in the early stages of infection and may also be affected by the use of steroids, commonly used in association with antibiotics, to treat the inflammatory component of the reactivation. This is supported by more than one study

investigating the combined use of PCR and Goldmann-Witmer coefficient, noting, however, that this was carried out using aqueous humour samples [25, 26].

2.5. Intraocular Tuberculosis (TB). Tuberculosis is implicated in intraocular inflammation either causing direct infection of intraocular tissues, where TB can be demonstrated within the eye, or resulting in immune-mediated inflammation affecting intraocular tissues. In the latter case, the presumption is that the presence of TB outside the eye results in intraocular inflammation due to an immune-mediated attack on intraocular tissues, presumably due to mimicry between TB antigen and retinal antigens. There has been significant interest in the use of interferon release assays such as QuantiFERON-Gold testing of blood in patients with presumed idiopathic uveitis or retinal vasculitis.

Testing ocular fluids using PCR to detect TB is not routinely employed in UK uveitis clinics; however, this can be carried out. A larger volume of vitreous is required, compared to the amount required in testing for herpetic viruses.

In countries where tuberculosis is more common than that in the UK, patients with uveitis demonstrating consistent clinical features, such as choroidal granulomas and retinal vasculitis, are often empirically treated with antituberculous therapy with good results. There is evidence to suggest that "idiopathic" retinal vasculitis, where there is clinical evidence of inflammation around blood vessels in the retina, or patients with presumed "Eales disease," may actually have TB demonstrable inside the eye as demonstrated by PCR testing of vitreous fluid. This would suggest that vitreous sampling in such cases would be advocated.

In support of this, in a recent study by Singh et al. 57% of patients with a diagnosis of Eales disease had a demonstrable intraocular TB demonstrated by PCR testing of vitreous samples [27]. It is unclear whether similar results would be obtained if the same study have to be carried out in a population with a lower TB prevalence such as the UK.

2.6. Other Intraocular Infections. Several other organisms may invade and infect the eye including fungi such as *Candida* and rarer bacterial infections, such as Whipples disease, Lyme disease, or *Bartonella* [28].

3. Suspected Intraocular Lymphoma

Intraocular lymphoma is an important masquerade of intermediate uveitis. In most cases, intraocular lymphoma involves the vitreous and the choroid and is a non-Hodgkins CD20+ B cell lymphoma, which is part of the spectrum of central nervous system (CNS) lymphoma. Approximately, 25% of patients with primary CNS lymphoma of this type develop intraocular involvement. Conversely, patients presenting with intraocular lymphoma have a high risk of developing CNS pathology, with over 50% developing disease [29].

Establishing a diagnosis of intraocular lymphoma is challenging and the gold standard requires demonstration of malignant cells or tissue. Often, a patient will have been treated with corticosteroids to address "uveitis." This affects the yield and the phenotype of the cells in the vitreous. Ideally therefore, steroids should be rapidly tapered prior to vitreous sampling in order to increase the yield of lymphoma cells within the eye. A negative vitreous biopsy in the face of ongoing clinical suspicion is an indication for repeating a vitreous biopsy. Repeatedly negative sampling may necessitate a chorioretinal biopsy to be undertaken. Lymphoma cells are fragile and rapidly disintegrate, meaning that [30] obtaining an adequate vitreous sample requires special considerations and procedures [31]. Ideally, at least 2 mL of undiluted vitreous should be sampled, and the pathologist analysing the sample should be made aware to expect the sample and to analyse it, ideally within one hour of the procedure. If this is not possible, the specimen should be placed in a mild cytofixative, such as hepes-glutamic acid buffer mediated organic solvent protection effect (HOPE) or CytoLyt [32].

Features of lymphoma cells include atypical lymphoid cells with scant basophilic cytoplasm and a high nuclear : cytoplasmic ration and prominent nucleoli [33]. Haematoxylin and eosin staining can be used, however, Giemsa may be better at demonstrating the presence of lymphoma cells. As well as lymphoma cells, the vitreous may also contain inflammatory cells, fibrin, and cellular debris. Accurate diagnosis requires the skill of an experienced ocular pathologist [34].

Immunohistochemistry is used to stain for specific surface immune cell markers, including CD22, CD20, and CD19, thus further characterising the lymphoma cells. Germinal centre markers can also be identified, including CD10 [35].

Flow cytometry can also be employed in order to analyse the cells allowing characterisation of surface markers and surface antibodies. This technique also enables monoclonality to be demonstrated. There are several caveats to the use of flow cytometry; however, useful adjunctive information can be obtained by using the technique [7].

Analysis of the cytokines presented in the vitreous can be used as an adjunctive test in the diagnosis of lymphoma, and cytokine levels can be measured using enzyme immunoassay. Inflammatory cytokines, such as IL-6 or TNF-alpha, are found to be present in the eyes of patients with idiopathic inflammation [36]. This has also been demonstrated experimentally. Patients with lymphoma are found to have low levels of proinflammatory IL-6, but higher levels of the anti-inflammatory cytokine IL-10 are produced by B cells. The IL-10 : IL-6 ratio has been studied as a marker to support the presence of intraocular lymphoma, and it has been proposed that a ration greater that 1.0 is highly suggestive of intraocular lymphoma.

It has also been proposed that the IL-10 level alone can also be used as a surrogate marker of lymphoma and can be obtained from an anterior chamber paracentesis with reportedly good sensitivity and specificity. In this study, the authors reported an aqueous level of 50 pg/mL to have a sensitivity and specificity of 0.89 and 0.93, respectively. Vitreous levels of 400 pg/mL yielded a specificity of 0.99 and a sensitivity of 0.8 [37].

Finally, monoclonality of B-cell populations is a feature of lymphoma and can be detected using molecular analysis. PCR is used to show rearrangements of the IgH gene, especially affecting the IgH variable region. Monoclonality of the more rare T-cell lymphomas can be demonstrated through the identification of TCR gene rearrangements [38].

In summary therefore, although cytology is the "gold standard" for diagnosing lymphoma, it is seen that the availability of these adjunctive techniques to test the vitreous can enhance the diagnosis especially when the laboratory technician is faced with a poor cellular yield from a vitreous sample.

4. Vitreous Analysis in Patients with Autoinflammatory Uveitis

The majority of uveitis encountered in western uveitis clinics is diagnosed as being autoimmune or autoinflammatory. In approximately half of patients, intraocular inflammation occurs as part of a systemic disease, and intraocular findings may adhere to a characteristic phenotype [2].

Analysis of the vitreous in autoimmune or autoinflammatory uveitis has been undertaken mainly in a research setting. Animal models enable testing to be undertaken, whilst clinical studies offer an insight into the nature of the inflammatory environment.

Experimental models of uveitis support the proposal that inflammation occurs due to immune-mediated attack on retinal antigen [39]. Experimental autoimmune uveitis (EAU) is an immune-mediated response against soluble retinal antigens, found mainly around photoreceptor segments. T-cell-mediated attack against intraocular antigen is believed to be central in the mechanism of autoimmune uveitis.

Following stimulation of T cells by antigen (which may be presented by antigen presenting cells in the eye), T cells differentiate into 3 main subtypes, which are characterised by the types of cytokines that they release. These subtypes include Th1, Th2, and Th17. In the context of EAU, T cells are polarised toward a Th1 response, whilst resolution of disease is associated with polarisation toward Th2 and regulatory T-cell phenotype.

Ooi et al. reviewed the relevance of cytokines in both experimental autoimmune uveitis and also in patients affected with uveitis [36]. Proinflammatory cytokines are found to be present in patients and animal models of uveitis at high levels. These include Il1, IL2, IL6 IFNy, and TNFa.

TNF-alpha is a significant cytokine in autoimmune uveitis [40] and is the focus of targetted biologic therapy in the treatment of noninfectious uveitis [41]. It is released from monocytes, macrophages natural killer cells, and T cells and stimulated increased cellular infiltration by activating macrophages, increased in leukocytic infiltration and upregulating adhesion molecules [42]. Analysis of vitreous samples from animals models demonstrates high levels of TNF-a within the eye during inflammation.

Studies examining the findings in the vitreous of patients with a prediagnosed condition have been undertaken. These have demonstrated different cytokine environments within the eyes of these patients, sometimes supporting the underlying diagnosis and enabling further understanding of the inflammatory process. Testing vitreous for cytokine levels is certainly not routine in clinical practice, and as seen from papers such as this, high levels of different inflammatory cytokines may occur with a range of inflammatory or infectious aetologies.

In a paper by Nagata et al., the authors aim to report cytokines that are upregulated in the vitreous fluid of patients with ocular sarcoidosis to see whether a characteristic pattern can be observed [43]. They found that when levels of 27 different cytokines were measured, the vitreous levels of 17 cytokines were elevated in the patients with sarcoidosis compared with patients with idiopathic epiretinal membrane. As well as some cytokines being elevated, there were some that were lower in the patient group with sarcoidosis. The authors also correlated levels of inflammatory cytokines with the degree of cystoid macular oedema observed.

5. Conclusion

Analysis of the vitreous is shown to be a valuable adjunct to the management of patients with intraocular inflammation [44]. Limitations of the tests are likely to become less as more advanced testing methods are introduced. The importance of selecting the appropriate tests to support a clinical suspicion is emphasised, as is the interpretation of test results within a clinical context.

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Research Article

High-Mobility Group Box-1 and Endothelial Cell Angiogenic Markers in the Vitreous from Patients with Proliferative Diabetic Retinopathy

Ahmed M. Abu El-Asrar,[1,2] Mohd Imtiaz Nawaz,[1] Dustan Kangave,[1] Marwan Abouammoh,[1] and Ghulam Mohammad[1]

[1] *Department of Ophthalmology, College of Medicine, King Saud University, Riyadh 11411, Saudi Arabia*
[2] *Department of Ophthalmology, King Abdulaziz University Hospital, Old Airport Road, P.O. Box 245, Riyadh 11411, Saudi Arabia*

Correspondence should be addressed to Ahmed M. Abu El-Asrar, abuelasrar@yahoo.com

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The aim of this study was to measure the levels of high-mobility group box-1 (HMGB1) in the vitreous fluid from patients with proliferative diabetic retinopathy (PDR) and to correlate its levels with clinical disease activity and the levels of vascular endothelial growth factor (VEGF), the angiogenic cytokine granulocyte-colony-stimulating factor (G-CSF), the endothelial cell angiogenic markers soluble vascular endothelial-cadherin (sVE-cadherin), and soluble endoglin (sEng). Vitreous samples from 36 PDR and 21 nondiabetic patients were studied by enzyme-linked immunosorbent assay. HMGB1, VEGF, sVE-cadherin, and sEng levels were significantly higher in PDR patients than in nondiabetics ($P = 0.008$; <0.001; <0.001; 0.003, resp.). G-CSF was detected in only 3 PDR samples. In the whole study group, there was significant positive correlation between the levels of HMGB1, and sVE-cadherin ($r = 0.378$, $P = 0.007$). In PDR patients, there was significant negative correlation between the levels of sVE-cadherin and sEng ($r = -0.517$, $P = 0.0005$). Exploratory regression analysis identified significant associations between active PDR and high levels of VEGF (odds ratio = 76.4; 95% confidence interval = 6.32–923) and high levels of sEng (odds ratio = 6.01; 95% confidence interval = 1.25–29.0). Our findings suggest that HMGB1, VEGF, sVE-cadherin and sEng regulate the angiogenesis in PDR.

1. Introduction

Ischemia-induced angiogenesis and expansion of extracellular matrix in association with the outgrowth of fibrovascular membranes at the vitreoretinal interface is the pathological hallmark in proliferative diabetic retinopathy (PDR). Vascular endothelial growth factor (VEGF), an endothelial cell mitogen that also enhances vascular permeability, is thought to be the major angiogenesis factor in PDR [1]. In addition, strong evidence indicates that chronic low-grade inflammation is implicated in the pathogenesis of diabetic retinopathy [2, 3]. Sustained proinflammatory responses in diabetic retinopathy are often associated with angiogenesis [2–5]. The causal relationship between inflammation and angiogenesis is now widely accepted [6]. An emerging issue in diabetic retinopathy research is the focus on the mechanistic link between chronic, low-grade inflammation and angiogenesis.

High-mobility group box-1 protein (HMGB1) was initially discovered as a nuclear chromatin-binding protein that stabilizes nucleosome formation and facilitates transcription. Necrotic cell death can result in passive leakage of HMGB1 from the cell as the protein is then no longer bound to DNA. In addition, HMGB1 can be actively secreted by different cell types, including activated monocytes and macrophages, mature dendritic cells, natural killer cells, and endothelial cells. Extracellular HMGB1 functions as a proinflammatory cytokine [6–10] and exhibits angiogenic effects [10–14]. HMGB1 signals through the receptor for advanced glycation end products (RAGE) leading to activation of the transcription factor nuclear factor kappa B (NF-κB) and induces the expression of various leukocyte adhesion

molecules and proinflammatory cytokines, chemokines, and angiogenic factors [6–9]. These findings suggest that HMGB1 might provide the mechanistic link between chronic low-grade inflammation and angiogenesis. In a previous report, we demonstrated that HMGB1 and RAGE were expressed by vascular endothelial cells and stromal cells in PDR fibrovascular epiretinal membranes and that there were significant correlations between the level of vascularization in PDR epiretinal membranes and the expression of HMGB1 and RAGE [15]. In addition, we demonstrated increased levels of HMGB1 in the vitreous samples from patients with PDR and that HMGB1 expression was upregulated in the retinas of diabetic mice. Moreover, there were significant correlations between the vitreous levels of HMGB1 and the levels of the inflammatory biomarkers monocyte chemoattractant protein-1 (MPC-1) and soluble intercellular adhesion molecule-1 (sICAM-1) [16].

Over the years, great effort has been made to find specific markers for the angiogenic endothelial cells that can be exploited by vascular targeting agents. Among these markers, the endothelial cell activation markers vascular-endothelial-(VE-) cadherin and endoglin (Eng) stand out as reliable biomarkers of angiogenesis activity. VE-cadherin is a cell adhesion molecule localized at the endothelial junction. VE-cadherin plays a key role in angiogenesis, signaling, endothelial cell survival, and endothelial cell barrier function. The regulation of its biological activity may be the central mechanism in normal or pathological angiogenesis [17, 18]. This molecule can be shed from the cell surface and elevated serum levels of soluble VE-chaderin (sVE-cadherin) seem to be a reliable marker of endothelial angiogenic activity and/or injury [19–25].

Endoglin (Eng) (also known as CD105), a type I transmembrane glycoprotein highly expressed on proliferating vascular endothelial cells, has been identified as an accessory receptor for transforming growth factor-β (TGF-β) and is essential for angiogenesis. Eng is expressed at low to non-detectable levels in resting endothelial cells within normal tissues, but its expression strongly increases in vascular endothelial cells in sites of active angiogenesis during embryogenesis, in inflamed tissues, in healing wounds, and in tumor vessels. Therefore, Eng detection is used as a marker to analyze angiogenesis and microvascular density in tumors and has been found to be an independent prognostic indicator. Expression of Eng can be induced by hypoxia and is also upregulated in ischemic tissues [26, 27]. Furthermore, a soluble form of Eng (sEng) has been observed in the serum of patients with different types of solid malignancies [28] and of pregnant women suffering from preeclampsia [29]. Circulating levels of sEng were found to be a reliable biomarker that correlates with disease severity and has prognostic significance [28, 29]. This soluble form, which results from partial shedding of the membrane-bound form of Eng by the matrix metalloproteinase-14 (MT1-MMP) [30], has been proposed to act as a scavenger or trap for circulating TGF-β family ligands such as bone morphogenetic proteins 9 and 10, thus impairing binding to their physiological receptors indicating an important role of sEng in the regulation of angiogenesis [31].

The aim of this study was to measure the levels of HMGB1 in the vitreous fluid from patients with PDR and to correlate its levels with clinical disease activity and vitreous levels of VEGF, the angiogenic cytokine granulocyte-colony stimulating factor (G-CSF) [32–34] and the endothelial cell angiogenic markers sVE-cadherin and sEng.

2. Materials and Methods

2.1. Vitreous Samples. Undiluted vitreous fluid samples (0.3–0.6 mL) were obtained from 36 patients with PDR and 21 patients with rhegmatogenous retinal detachment (RD) without proliferative vitreoretinopathy during pars plana vitrectomy. The indications for vitrectomy in patients with PDR were traction retinal detachment and/or nonclearing vitreous hemorrhage. In patients with PDR, the severity of retinal neovascular activity was graded clinically at the time of vitrectomy using previously published criteria [35]. Neovascularization was considered active if there were visible perfused new vessels on the retina or optic disc present within tractional epiretinal membranes. Neovascularization was considered inactive (involuted) if only nonvascularized, white fibrotic epiretinal membranes were present. Active PDR was present in 19 patients and inactive PDR was present in 17 patients. Vitreous samples were collected undiluted by manual suction into a syringe through the aspiration line of vitrectomy, before opening the infusion line. The samples were centrifuged (500 rpm for 10 min, 4°C) and the supernatants were aliquoted and frozen at −80°C until assay. The study was conducted according to the tenets of the Declaration of Helsinki, and informed consent was obtained from all patients. The study was approved by the Research Centre, College of Medicine, King Saud University.

2.2. Enzyme-Linked Immunosorbent Assay Kits. Enzyme-linked immunosorbent assay (ELISA) kits for human VE-cadherin (Human VE-cadherin, Cat No: DCADV0), human VEGF (Human vascular endothelial growth factor, Cat No: SVE00), human Eng (Human Endoglin/CD105, Cat No. DNDG00) and human G-CSF (Human granulocyte-colony stimulating factor, Cat No: DCS50), were purchased from R&D Systems, Minneapolis, MN, USA. The ELISA kit for HMGB1 (human high-mobility group box-1, Cat No: ST51011) was purchased from IBL International GMBH, Hamburg, Germany.

The minimum detection limit of each ELISA kit for VE-Cadherin, VEGF, Eng, G-CSF, and HMGB1 is 113, 9, 7, 20, and 100 picograms/mL (pg/mL), respectively. The ELISA plate readings were done using FLUOstar Omega-Miroplate reader from BMG Labtech, Offenburg, Germany.

2.3. Measurement of VE-Cadherin, VEGF, Eng, G-CSF, and HMGB1. The quantification of human VE-cadherin, VEGF, Eng, G-CSF, and HMGB1 in the vitreous fluid was determined using ELISA kits according to the manufacturer's instruction. For each ELISA kit, the undiluted standard served as the highest concentration and calibrator diluents served as the blank. Depending upon the detection range for

each ELISA kit, vitreous samples were either directly used or diluted with calibrator diluents supplied with ELISA kit.

For the measurement of VE-caherin and VEGF, 100 μL of 5-fold and 2-fold diluted vitreous (sample diluents, supplied with the kit) was used in the respective ELISA assay for their analysis. For measurement of Eng and G-CSF, 100 μL of undiluted vitreous was used and added to the wells of respective ELISA plates. For the quantification of HMGB1 within the high sensitivity range, 50 μL of diluents buffer (Dilbuf, IBL International) was added to each well of microtiter plate followed by the addition of 50 μL of standard, positive control, and vitreous fluid.

As instructed in the kit manual, samples were incubated into the each well of ELISA plates. The antibody against VE-cadherin, VEGF, Eng, G-CSF, and HMGB1,conjugated to horseradish peroxidase was added to each well of the ELISA plate. After incubation, substrate mix solution was added for colour development. The reaction was stopped by the addition of 2N sulfuric acid and optical density was read at 450 nm in microplate reader. Each assay was performed in duplicate. Using the 4-parameter fit logistic (4-PL) curve equation, the actual concentration for each sample was calculated. For the vitreous fluid that has been diluted, the concentration for each sample was calculated after multiplying with the dilution factors to get the actual reading for each sample.

2.4. Statistical Methods. Because of the large variances that we had in our data, we used the non-parametric Mann-Whitney test to compare means from two independent groups, and the nonparametric Kruskal-Wallis test was used for conducting Analysis of Variance (ANOVA) to compare means from more than two independent groups. Correlation between continuous variables was investigated by computation of the Pearson correlation coefficient. A P value less than 0.05 indicated statistical significance. Post-ANOVA pairwise comparisons of means were conducted using the Kruskal-Wallis test. For three groups, the critical Z-value for determining statistical significance was $Z = 2.39$. Exploratory logistic regression analysis involving forcing entry, into a logistic model, the variables of interest, was conducted to discover whether active PDR was associated with high or low levels for the variables that were investigated. The mean level of each variable was used as the cut-off value for high versus low levels. SPSS version 15 and programs LR and 3S from Bio-Medical Data Processing Version 2007 (BMDP 2007) Statistical Software (Cork Technology Pack, Model Farm Road, Cord, Ireland) were used for the statistical analyses.

3. Results

3.1. Levels of Angiogenesis Biomarkers in Vitreous Samples. HMGB1, sVE-cadherin, and sEng were detected in all vitreous samples from patients with PDR and nondiabetic patients. VEGF was detected in 36 (90%) vitreous samples from patients with PDR and in 10 (45%) vitreous samples from nondiabetic patients. G-CSF was detected in only 3 (7.5%) vitreous samples from patients with PDR and in 6 (27%) vitreous samples from nondiabetic patients.

The mean levels of HMGB1, VEGF, sVE-cadherin, and sEng in vitreous samples from PDR patients were significantly higher than those in nondiabetic patients ($P = 0.008$; $P < 0.001$; $P < 0.001$; $P = 0.003$, resp.; Mann-Whitney test) (Table 1).

3.2. Relationship between Angiogenesis Biomarkers and Activity of PDR. Comparison of mean levels of angiogenesis biomarkers among active PDR patients, inactive PDR patients, and nondiabetic patients was conducted using the Kruskal-Wallis test and the results are shown in Table 2. Mean levels differed significantly between the 3 groups from HMGB1 ($P = 0.028$), VEGF ($P < 0.001$), sVE-cadherin ($P < 0.001$), and sEng ($P = 0.006$). Post-ANOVA pairwise comparisons of means indicated that mean HMGB1 level was significantly higher in patients with active PDR than in nondiabetic patients ($Z = 2.53$). For VEGF, the mean levels were significantly higher in patients with active PDR than that in inactive PDR patients and nondiabetic patients ($Z = 3.88$; $Z = 5.46$, resp.). For sVE-cadherin, the mean levels were significantly higher in patients with active PDR and patients with inactive PDR than those in nondiabetic patients ($Z = 4.72$; $Z = 4.42$, resp.). For sEng, the mean level in patients with inactive PDR was significantly higher than that in nondiabetic patients ($Z = 3.16$).

3.3. Correlations. In the whole study group, there was a significant positive correlation between vitreous fluid levels of HMGB1 and sVE-cadherin ($r = 0.378$, $P = 0.007$). In PDR patients, there was a significant negative correlation between vitreous fluid levels of sVE-cadherin and sEng ($r = -0.517$, $P = 0.005$).

3.4. Logistic Regression Analysis. We conducted exploratory logistic regression analysis to investigate further the association between the angiogenesis biomarkers and active PDR. Active PDR was significantly associated with high levels of VEGF (odds ratio = 76.4; 95% confidence interval = 6.322–923) and high levels of sEng (odds ratio = 6.01; 95% confidence interval = 1.25–29.0).

4. Discussion

In the present study, the levels of HMGB1, VEGF, sVE-cadherin, and sEng were significantly higher in the vitreous fluid from PDR patients compared with nondiabetic patients. In contrast, G-CSF was detected in only few samples consistent with a previous study [36]. There was a significant positive correlation between the vitreous levels of HMGB1 and sVE-cadherin in the whole patient group and a significant negative correlation between sVE-cadherin and sEng in patients with PDR. Among the angiogenic factors that we investigated, VEGF and sEng had a stronger influence on the activity of PDR than the other factors.

In the present study, HMGB1 levels were significantly elevated in the vitreous fluid from patients with PDR. Furthermore, the levels were higher in patients with active PDR compared with patients with quiescent PDR. In a previous study, we demonstrated that HMGB1 expression

Table 1: Comparisons of mean angiogenesis biomarker levels in proliferative diabetic retinopathy (PDR) and rhegmatogenous retinal detachment (RD) patients.

Disease group	HMGB1 (ng/mL)	VEGF (ng/mL)	sVE-cadherin (ng/mL)	sEng (ng/mL)
PDR ($n = 36$)	5.69 ± 8.5	0.85 ± 1.2	77.3 ± 63.5	3.64 ± 1.8
RD ($n = 14$)	1.70 ± 2.10	0.04 ± 0.1	10.7 ± 9.6	2.22 ± 0.7
P value (Mann-Whitney test)	0.008*	<0.001*	<0.001*	<0.003*

*Statistically significant at 5% level of significance.
HMGB1: high-mobility group box-1; VEGF: vascular endothelial growth factor; sVE-cadherin: soluble vascular endothelial-cadherin; sEng: soluble endoglin.

Table 2: Comparisons of mean angiogenesis biomarker levels in proliferative diabetic retinopathy (PDR) patients with or without active neovascularization.

Disease group	HMGB1 (ng/mL)	VEGF (ng/mL)	sVE-cadherin (ng/mL)	sEng (ng/mL)
Active PDR ($n = 19$)	7.28 ± 11.1	1.67 ± 1.4	75.8 ± 53.4	3.28 ± 1.9
Inactive PDR ($n = 17$)	4.02 ± 4.1	0.18 ± 0.4	78.8 ± 74.9	4.04 ± 1.6
RD ($n = 21$)	1.70 ± 2.1	0.04 ± 0.1	10.7 ± 9.6	2.22 ± 0.7
P value (ANOVA)	0.028*	<0.001*	<0.001*	0.006*

*Statistically significant at 5% level of significance.
HMGB1: high-mobility group box-1; VEGF: vascular endothelial growth factor; sVE-cadherin: soluble vascular endothelial-cadherin; sEng: soluble endoglin; RD: rhegmatogenous retinal detachment.

was upregulated in the retinas of diabetic mice [16]. Similarly, increased vascular [37] and renal [38] HMGB1 expression was recently demonstrated in diabetic animals. In addition, hyperglycemia-induced reactive oxygen species production increased the expression of HMGB1 and RAGE in endothelial cells [39]. In patients with type 1 diabetes, serum HMGB1 levels were positively associated with markers of low-grade inflammation and endothelial dysfunction. In addition, higher serum HMGB1 levels were associated with greater prevalence and severity of albuminuria [40]. Activation of HMGB1/RAGE signaling axis is important in promoting proinflammatory pathways considered to play an important role in diabetes-induced retinal vascular inflammation. In endothelial cells, HMGB1 induces the expression of RAGE and adhesion molecules, such as ICAM-1, vascular cells adhesion molecule-1, and E-selectin, to release tumor necrosis factor-α (TNF-α), G-CSF, interleukin-8, and MCP-1 and to increase neutrophil adhesion. This proinflammatory phenotype was mediated by the activation of NF-κB and was RAGE dependent as it was inhibited by antibodies directed toward RAGE [7–10]. In our laboratory, we recently demonstrated that intravitreal administration of HMGB1 to normal rats induced significant upregulation of ICAM-1, HMGB1, and RAGE and NF-κB activation in the retina (Mohammad et al., unpublished data). In turn endothelial cells secrete HMGB1 in response to TNF-α treatment [41], suggesting a role for HMGB1 in positive feedback loop promoting inflammation. Recently, HMGB1 has been recognized as an angiogenic cytokine [10–14]. HMGB1 treatment of endothelial cells induced a proangiogenic gene expression program evidenced by the induction of VEGF and its receptors, platelet-derived growth factor receptors, integrins and matrix metalloproteinases [10]. In addition, HMGB1 induced endothelial cell migration, and sprouting [10]. HMGB1 was also identified as a specific marker of tumor endothelium [14] and as a tumor angiogenesis marker [10]. Moreover, anti-HMGB1 antibodies inhibited tumor angiogenesis [10]. Another interesting role of HMGB1 in neovascularization is its ability to attract endothelial progenitor cells to sites of tissue injury and tumors to improve neovascularization in a RAGE-dependent manner [13].

Several studies demonstrated that sVE-cadherin serum levels may reflect the intensity of angiogenesis. sVE-cadherin serum level was increased in untreated multiple myeloma patients and decreased after chemotherapy in patients in remission [19]. Similarly, circulating sVE-cadherin levels were increased in pregnant women (a physiological condition associated with increased angiogenesis) and cancer patients and were particularly increased in patients affected by hematological malignancies and decreased to normal values in patients achieving complete remission [20]. Reverse transcriptase-polymerase chain reaction was used to profile gene expression of proteins closely associated with angiogenesis. Results showed 10-fold increase in VE-cadherin during angiogenesis [25]. These findings are in agreement with another study that demonstrated that VE-cadherin was a selective marker for assessing microvessel density in breast cancer [42]. Serum sVE-cadherin levels were also increased in other pathologic states associated with endothelial dysfunction such as Behçet's disease [23], rheumatoid arthritis [21], coronary atherosclerosis [22], and ovarian hyperstimulation syndrome [24]. *In vitro* studies demonstrated that treatment of endothelial cells with TNF-α [21], VEGF [43], matrix metalloproteinase-9 [44], and the diabetic metabolite advanced glycation end products [44] resulted in shedding of the VE-cadherin extracellular domain and loss of cell-cell contact which may lead to increased vascular permeability. The present study is the first report documenting increased levels of sVE-cadherin in the vitreous fluid from patients with PDR. In addition, our analysis showed a significant positive correlation between

the vitreous levels of HMGB1 and sVE-cadherin. It is well established that endothelial dysfunction is a key feature of diabetic retinopathy [44]. On the basis of our findings, we propose that elevated levels of sVE-cadherin in the vitreous fluid from patients with PDR could be a reflection of endothelial cell activation or injury associated with angiogenesis, inflammation, and breakdown of the inner blood-retinal barrier.

The current study is the first to demonstrate that sEng is significantly upregulated in the vitreous fluid from patients with PDR. Our results are consistent with a previous report showing that plasma sEng concentration could serve as an indicator of diabetes-associated vascular pathologies such as retinopathy, hypertension, endothelial dysfunction, and cardiovascular risk [45]. Similarly, another study demonstrated that sEng could be a marker to predict cardiovascular events in patients with chronic coronary artery disease [46]. In addition, Li et al. [28] showed that plasma sEng is a valuable surrogate angiogenic marker for identifying breast cancer patients who are at high risk of developing metastasis. In a previous study, we demonstrated that Eng was expressed by vascular endothelial cells in PDR fibrovascular epiretinal membranes [47]. Therefore, it is possible that the increase in sEng in the vitreous fluid from patients with PDR resulted from Eng proteolytic shedding of the membrane-bound form associated with angiogenesis. A previous study showed elevated levels of matrix metalloproteinase-14 in the retinas of diabetic animals [48]. Matrix metalloproteinase-14 was shown in a previous report to induce shedding of the membrane-bound form of Eng [30]. Among the studied biomarkers of angiogenesis, exploratory logistic regression analysis revealed that higher levels of VEGF and sEng were associated with active PDR. These findings suggest that sEng may also represent a surrogate marker of angiogenic activity in PDR.

Endothelial dysfunction is a major characteristic of patients with diabetic retinopathy [44]. Several studies demonstrated that sEng plays an important role in endothelial cell function and in regulating angiogenesis. Forced expression of sEng increased vascular permeability. *In vitro* studies on endothelial cell lines showed that sEng interferes with TGF-β signaling and endothelial nitric oxide activation and thereby causes endothelial dysfunction. sEng also seems to be a regulator of vascular tone, as administration of sEng to mice induces an increase in arterial pressure by increasing vascular resistance [49]. Recently, Walshe et al. [50] demonstrated that sEng increased vascular and neural cell apoptosis in the retina, which was associated with decreased retinal function and breakdown of the blood-retinal barrier. In addition, *in vitro* and *in vivo* studies demonstrated that sEng is capable of inhibiting angiogenesis [30, 31, 49]. Our analysis demonstrated a significant negative correlation between sEng levels and the levels of sVE-cadherin in the vitreous from patients with PDR. These findings suggest a lower angiogenic activity in patients with higher levels of sEng and that the upregulation of sEng in the vitreous fluid from patients with PDR may be a protective antiangiogenesis eye response to suppress progression of PDR.

In conclusion, these data suggest that, along with HMGB1 and VEGF, sVE-cadherin and sEng might play a role in the pathophysiology of PDR. In addition, sVE-cadherin and sEng might be valuable angiogenic markers for PDR.

Conflict of Interests

The authors declare that they have no conflict of interest.

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Clinical Study

Osteopontin and Other Regulators of Angiogenesis and Fibrogenesis in the Vitreous from Patients with Proliferative Vitreoretinal Disorders

Ahmed M. Abu El-Asrar,[1,2] Mohd Imtiaz Nawaz,[1] Dustan Kangave,[1] Mohammed Mairaj Siddiquei,[1] and Karel Geboes[3]

[1] *Department of Ophthalmology, College of Medicine, King Saud University, Riyadh, Saudi Arabia*
[2] *Department of Ophthalmology, King Abdulaziz University Hospital, Old Airport Road, P.O. Box 245, Riyadh 11411, Saudi Arabia*
[3] *Laboratory of Histochemistry and Cytochemistry, University of Leuven, Leuven, Belgium*

Correspondence should be addressed to Ahmed M. Abu El-Asrar, abuasrar@ksu.edu.sa

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The aim of this study was to determine the levels of the angiogenic and fibrogenic factors osteopontin (OPN), high-mobility group box-1 (HMGB1), and connective tissue growth factor (CTGF) and the antiangiogenic and antifibrogenic pigment epithelium-derived factor (PEDF) in the vitreous fluid from patients with proliferative diabetic retinopathy (PDR), proliferative vitreoretinopathy (PVR), and rhegmatogenous retinal detachment with no PVR (RD). Vitreous samples from 48 PDR, 17 PVR and 30 RD patients were studied by enzyme-linked immunosorbent assay. OPN, HMGB1, CTGF, and PEDF levels were significantly higher in PDR patients than in RD patients ($P < 0.001$; 0.002; <0.001; <0.001, resp.). CTGF and PEDF levels were significantly higher in PVR patients than in RD patients ($P < 0.001; 0.004$, resp.). Exploratory logistic regression analysis identified significant associations between PDR and high levels of HMGB1, CTGF and PEDF, between PDR with active neovascularization and high levels of CTGF and PEDF, and between PDR with traction retinal detachment and high levels of HMGB1. In patients with PDR, there were significant correlations between the levels of PEDF and the levels of OPN ($r = 0.544, P = 0.001$), HMGB1 ($r = 0.719, P < 0.001$), and CTGF ($r = 0.715, P < 0.001$). In patients with PVR, there were significant correlations between the levels of OPN and the levels of HMGB1 ($r = 0.484, P = 0.049$) and PEDF ($r = 0.559, P = 0.02$). Our findings suggest that OPN, HMGB1, and CTGF contribute to the pathogenesis of proliferative vitreoretinal disorders and that increased levels of PEDF may be a response to counterbalance the activity of angiogenic and fibrogenic factors in PDR and PVR.

1. Introduction

Ischemia-induced pathologic growth of new blood vessels and expansion of extracellular matrix (ECM) in association with the outgrowth of fibrovascular epiretinal membranes at the vitreoretinal interface is the pathological hallmark in proliferative diabetic retinopathy (PDR) and often leads to catastrophic loss of vision due to vitreous hemorrhage and/or traction retinal detachment. Proliferative vitreoretinopathy (PVR) is a process of fibrocellular proliferation on either sides of the retina that may complicate rhegmatogenous retinal detachment. The formation and gradual contraction of epiretinal membranes causes a marked distortion of the retinal architecture and results in complex retinal detachments that are difficult to repair.

Angiogenesis, the growth of new vascular networks from preexisting ones, is under tight regulation by a dynamic balance between angiogenic stimulators and inhibitors [1]. The biological process of fibrosis, typically associated with an abnormal accumulation of ECM, occurs in response to

various stimuli in many biological systems. The key cellular mediator of fibrosis is the myofibroblast, a cell type differentiated from quiescent fibroblasts. These are contractile cells, characterized by the expression of α-smooth muscle actin (α-SMA), and their presence is a marker of progressive disease. They have the capacity to produce several ECM components including collagen resulting in fibrosis [2]. Previous studies have shown that α-SMA-expressing myofibroblasts are the principal cellular component of PDR and PVR epiretinal membranes [3–6]. Inflammation, angiogenesis, and fibrosis are processes involved in the pathogenesis of proliferative vitreoretinal disorders, and the interplay between these events is under intense investigation [3–8]. A number of proinflammatory, proangiogenic, profibrogenic, and immunomodulating factors may be linked to the development and progression of proliferative vitreoretinal disorders, such as osteopontin (OPN), high-mobility group box-1 (HMGB1), connective tissue growth factor (CTGF), and pigment epithelium-derived factor (PEDF).

Osteopontin is a phosphorylated acidic arginine-glycine-aspartate- (RGD-)containing glycoprotein that exists both as an immobilized ECM component and as a soluble, multifunctional, proinflammatory cytokine that plays important roles in promoting inflammation [9, 10], tissue remodeling, fibrosis [9, 11–14], and angiogenesis [15–18]. Many of these effects are mediated by the binding of OPN to CD44 receptors and the surface integrin receptor $\alpha_v\beta_3$ [15, 16, 19]. HMGB1 is a nonhistone DNA-binding nuclear protein that is highly conserved during evolution. Necrotic cell death can result in passive leakage of HMGB1 from the cell as the protein is then no longer bound to DNA. In addition, HMGB1 can be actively secreted by different cell types, including activated monocytes and macrophages, mature dendritic cells, natural killer cells, and endothelial cells. Extracellular HMGB1 functions as a proinflammatory cytokine [20–23] and exhibits angiogenic [24–27] and fibrogenic [28–31] effects. CTGF is a cysteine-rich secretory protein that functions as a downstream mediator of transforming growth factor-β action on connective tissue cells [32]. It acts as a fibroblast chemoattractant and mitogen and also stimulates the production of ECM components in various fibroblast cultures [32–34]. In addition, *in vitro* and *in vivo* studies demonstrated that CTGF exhibits angiogenic activities [35, 36].

PEDF is a 50 KDa secreted glycoprotein that belongs to the noninhibitory serpin family group. PEDF has been described as a natural inhibitor of both physiological and pathological angiogeneses with antioxidant, and anti-inflammatory effects. It has been demonstrated to function as a potent and broadly acting neurotrophic and neuroprotective factor that induces cell differentiation and protects neurons in the brain, eye, and spinal cord against a wide range of neurodegenerative insults [37, 38]. In addition, PEDF was recently shown to have antifibrogenic activity [39].

To address mechanisms involved in the pathogenesis of proliferative vitreoretinal disorders and to identify molecular targets for treatment and/or preventive intervention, we measured the levels of OPN, HMGB1, CTGF, and PEDF in the vitreous fluid from patients with PDR, PVR, and rhegmatogenous retinal detachment with no PVR (RD). In addition, we correlated their levels with PDR clinical disease activity.

2. Materials and Methods

2.1. Vitreous Samples. Undiluted vitreous fluid samples (0.3–0.6 mL) were obtained from 48 patients with PDR, 17 patients with PVR, and 30 patients with RD during pars plana vitrectomy. The indications for vitrectomy in patients with PDR were traction retinal detachment and/or nonclearing vitreous hemorrhage. In patients with PDR, the severity of retinal neovascular activity was graded clinically at the time of vitrectomy using previously published criteria [40]. Neovascularization was considered active if there were visible perfused new vessels on the retina or optic disc present within tractional epiretinal membranes. Neovascularization was considered inactive (involuted) if only nonvascularized, white fibrotic epiretinal membranes were present. Active PDR was present in 28 patients, and inactive PDR was present in 20 patients. Traction retinal detachment was present in 21 patients. Vitreous samples were collected undiluted by manual suction into a syringe through the aspiration line of vitrectomy, before opening the infusion line. The samples were centrifuged (500 rpm for 10 min, 4°C), and the supernatants were aliquoted and frozen at −80°C until assay. The study was conducted according to the tenets of the Declaration of Helsinki, and informed consent was obtained from all patients. The study was approved by the Research Centre, College of Medicine, King Saud University.

2.2. Enzyme-Linked Immunosorbent Assay Kits. Enzyme-linked immunosorbent assay (ELISA) kit for human OPN (Human Osteopontin, DuoSet, Cat no. DY1433) was purchased from R&D Systems, Minneapolis, MN. An ELISA kit for HMGB1 (human high-mobility group box-1, Cat no. ST51011) was purchased from IBL International GMBH (Hamburg, Germany). ELISA kits for human CTGF (human connective tissue growth factor, Cat No: E0010h) and human PEDF (human pigment epithelium-derived factor, Cat no. CSB-E08818h) were purchased from USCN life science & Tech Co., Ltd. and Cusabio Biotech Co., Ltd. Wuhan, China, respectively. The minimum detection limit of each ELISA kit for OPN, HMGB-1, CTGF, and PEDF are 2.5, 200, 15.6, and 40 picograms/mL (pg/mL), respectively. The ELISA plate readings were done using FLUOstar Omega-Miroplate reader from BMG Labtech, Offenburg, Germany.

2.3. Measurement of Human OPN, HMGB-1, CTGF, and PEDF. The quantification of human OPN, HMGB-1, CTGF and PEDF in the vitreous fluid was determined using ELISA kits according to the manufacturer's instruction. For each ELISA kit, the undiluted standard serves as the highest standard and calibrator diluents serve as the blank. Depending upon the detection range for each ELISA kit and the expression level of the particular molecule, vitreous samples were either directly used or diluted with calibrator diluents supplied with ELISA kit.

For the measurement of OPN, 100 μL of 1000-fold diluted vitreous samples were added into each of the ELISA plate for the analysis. For the quantification of HMGB1, 60 μL of diluent buffer was added to each well of microtiter plate followed by the addition of 40 μL of standard, positive control, and vitreous fluid. For the measurement of CTGF, and PEDF, 100 μL of 3-fold and 6-fold diluted vitreous were used, respectively, for ELISA assay.

As instructed in the kit manual, vitreous samples were incubated into each well of ELISA plates. Antibodies against OPN, HMGB1, CTGF and PEDF conjugated to horseradish peroxidase were added to each well of the ELISA plate. After incubation, the substrate solution was added for colour development. The reaction was stopped by the addition of 2N sulfuric acid, and optical density was read at 450 nm in microplate reader. Each assay was performed in duplicate. Using the 4-parameter fit logistic (4-PL) curve equation, the actual concentration for each sample was calculated. The concentration for each sample was calculated after multiplying with the dilution factors to get the actual reading for each sample.

2.4. Statistical Analysis. Because of the large variances that we had in our data, we used the nonparametric Mann-Whitney test to compare means from two independent groups, and the nonparametric Kruskal-Wallis test was used for conducting analysis of variance (ANOVA) to compare means from more than two independent groups. Correlation between continuous variables was investigated by computation of the Pearson correlation coefficient. A P value less than 0.05 indicated statistical significance. Post-ANOVA pairwise comparisons of means were conducted using the Kruskal-Wallis test. For three groups, the critical Z-value for determining statistical significance was $Z = 2.39$. Exploratory logistic regression analysis, involving forcing entry into a logistic model the variables of interest, was conducted to identify the angiogenic and fibrogenic factors that had a significant association with the studied diseases. The mean level of each variable was used as the cut-off value for high versus low levels. SPSS version 15 and programs LR and 3S from Bio-Medical Data Processing Version 2007 (BMDP 2007) Statistical Software (Cork Technology Pack, Model Farm Road, Cord, Ireland) were used for the statistical analyses.

3. Results

3.1. Levels of Angiogenesis and Fibrogenesis Regulatory Factors in Vitreous Samples. OPN, HMGB1, and PEDF were detected in all vitreous samples from patients with RD, PVR, and PDR. CTGF was detected in all vitreous samples from patients with PDR and PVR and in 11 (36.6%) samples from RD patients.

Mean levels of OPN, HMGB1, CTGF, and PEDF in vitreous samples from PDR patients were significantly higher than those in RD patients ($P < 0.001$; $P = 0.002$; $P < 0.001$; $P < 0.001$, resp.; Mann-Whitney test). Mean levels of CTGF and PEDF in vitreous samples from PVR patients were significantly higher than those in RD patients ($P < 0.001$; $P = 0.004$, resp.; Mann-Whitney test). Mean levels of OPN and HMGB1 from PVR patients were higher than those in RD patients, but the differences between the means were not statistically significant ($P = 0.425$; $P = 0.571$, resp.; Mann-Whitney test) (Table 1).

3.2. Relationship between Angiogenesis and Fibrogenesis Regulatory Factors and Activity of PDR. Comparison of mean levels of angiogenesis and fibrogenesis regulatory factors among active PDR patients, inactive PDR patients, and RD patients was conducted using the Kruskal-Wallis test, and the results are shown in Table 2. Mean levels differed significantly between the 3 groups for OPN ($P < 0.001$), HMGB1 ($P = 0.002$), CTGF ($P < 0.001$), and PEDF ($P < 0.001$). Post-ANOVA pairwise comparisons of means indicated that the mean OPN level was significantly higher in patients with active PDR than in RD patients ($Z = 4.11$). For HMGB1, the mean levels were significantly higher in patients with active PDR and patients with inactive PDR than in RD patients ($Z = 2.92$; $Z = 2.97$, resp.). For CTGF, the mean levels were significantly higher in patients with active PDR and patients with inactive PDR than those in RD patients ($Z = 6.24$; $Z = 4.2$, resp.). For PEDF, the mean levels were significantly higher in patients with active PDR and patients with inactive PDR than in RD patients ($Z = 6.89$; $Z = 3.59$, resp.). In addition, mean PEDF level was significantly higher in patients with active PDR than in patients with inactive PDR ($Z = 2.57$).

3.3. Relationship between Angiogenesis and Fibrogenesis Regulatory Factors and Traction Retinal Detachment. When patients with PDR were divided into those with or without traction retinal detachment, the mean levels of angiogenesis and fibrogenesis regulatory factors differed significantly between PDR patients with traction retinal detachment, PDR patients without traction retinal detachment, and RD patients for OPN ($P = 0.002$), HMGB1 ($P = 0.003$), CTGF ($P < 0.001$), and PEDF ($P < 0.001$) (Table 3). Post-ANOVA pairwise comparisons of means indicated that, for OPN, the mean levels in PDR patients with or without traction retinal detachment were significantly higher than those in RD patients ($Z = 5.18$; $Z = 5.64$, resp.). For HMGB1, the mean levels in PDR patients with or without traction retinal detachment were significantly higher than those for RD patients ($Z = 2.53$; $Z = 3.26$, resp.). For CTGF, the mean levels in PDR patients with or without traction retinal detachment were significantly higher than those in RD patients ($Z = 4.72$; $Z = 5.87$, resp.). For PEDF, the mean levels in PDR patients with or without traction retinal detachment were significantly higher than those in RD patients ($Z = 5.17$; $Z = 5.62$, resp.).

3.4. Exploratory Logistic Regression Analysis. PDR was significantly associated with high levels of HMGB1 (odds ratio = 7.39; 95% confidence interval = 2.11–25.9), CTGF (odds ratio = 11.4; 95% confidence interval = 2.87–45.3), and PEDF (odds ratio = 7.70; 95% confidence interval = 1.77–33.5). Active PDR was significantly associated with high levels of

Table 1: Comparisons of mean angiogenesis and fibrogenesis regulatory factor levels in proliferative diabetic retinopathy (PDR), proliferative vitreoretinopathy (PVR), and rhegmatogenous retinal detachment (RD) patients.

Disease group	OPN (ng/mL)	HMGB1 (ng/mL)	CTGF (ng/mL)	PEDF (ng/mL)
PDR ($n = 48$)	837.36 ± 1012.3	4.47 ± 10.1	1.91 ± 2.2	4.06 ± 7.9
RD ($n = 30$)	209.33 ± 192.5	0.98 ± 0.9	0.22 ± 0.3	0.32 ± 0.2
P value (Mann-Whitney test)	<0.001*	0.002*	<0.001*	<0.001*
PVR ($n = 17$)	737.95 ± 996.5	2.79 ± 5.4	0.65 ± 0.5	0.98 ± 0.9
RD ($n = 30$)	209.33 ± 192.5	0.98 ± 0.9	0.22 ± 0.3	0.32 ± 0.2
P value (Mann-Whitney test)	0.425	0.571	<0.001*	<0.004*

*Statistically significant at 5% level of significance.
OPN: osteopontin; HMGB1: high-mobility group box-1; CTGF: connective tissue growth factor; PEDF: pigment epithelium-derived factor.

Table 2: Comparisons of mean angiogenesis and fibrogenesis regulatory factor levels in proliferative diabetic retinopathy (PDR) patients with or without active neovascularization.

Disease group	OPN (ng/mL)	HMGB1 (ng/mL)	CTGF (ng/mL)	PEDF (ng/mL)
Active PDR ($n = 28$)	882.54 ± 1024.4	5.48 ± 11.7	2.15 ± 2.4	4.98 ± 9.2
Inactive PDR ($n = 20$)	579.66 ± 816.0	2.74 ± 2.0	1.05 ± 1.1	1.69 ± 2.0
RD ($n = 30$)	209.33 ± 192.5	0.98 ± 0.9	0.22 ± 0.3	0.32 ± 0.2
ANOVA P value	<0.001*	0.002	<0.001*	<0.001*

*Statistically significant at 5% level of significance.
OPN: osteopontin; HMGB1: high-mobility group box-1; CTGF: connective tissue growth factor; PEDF: pigment epithelium-derived factor.
RD: rhegmatogenous retinal detachment.

CTGF (odds ratio = 8.29; 95% confidence interval = 2.44–28.1) and PEDF (odds ratio = 7.66; 95% confidence interval = 2.00–29.4). PDR with traction retinal detachment was significantly associated with high levels of HMGB1 (odds ratio = 5.07; 95% confidence interval = 1.36–18.9).

None of the studied angiogenesis and fibrogenesis regulatory factors was significantly associated with PVR.

3.5. Correlations. In patients with PDR, there were significant correlations between the vitreous fluid levels of PEDF and the levels of OPN ($r = 0.544$, $P = 0.0011$), HGMB1 ($r = 0.719$, $P < 0.001$), and CTGF ($r = 0.715$, $P < 0.001$). In addition, there were significant correlations between the vitreous fluid levels of CTGF and the levels of OPN ($r = 0.490$, $P = 0.002$) and HMGB1 ($r = 0.369$, $P = 0.027$) (Table 4).

In patients with PVR, there were significant correlations between the vitreous fluid levels of OPN and the levels of HGMB1 ($r = 0.484$, $P = 0.049$) and PEDF ($r = 0.559$, $P = 0.020$) (Table 4).

4. Discussion

In this study, we examined the levels of the angiogenic and fibrogenic factors OPN, HMGB1, and CTGF and the antiangiogenic and antifibrogenic PEDF in the vitreous fluid from patients with PDR, PVR, and RD and their relationship with PDR clinical disease activity. We found upregulation of OPN, HMGB1, CTGF, and PEDF in the vitreous from PDR patients with active neovascularization compared with patients with quiescent PDR, PVR, and RD. Exploratory logistic regression analysis identified a significant association between PDR and high levels of HMGB1, CTGF, and PEDF, between active PDR and high levels of CTGF and PEDF, and between PDR with traction retinal detachment and high levels of HMGB1. Furthermore, there were significant correlations between the levels of PEDF and the levels of OPN, HMGB1, and CTGF in patients with PDR and between the levels of OPN and the levels of HMGB1 and PEDF in patients with PVR.

In the present study, we report that OPN was significantly upregulated in the vitreous fluid from patients with PDR and that OPN levels were nonsignificantly elevated in the vitreous fluid from patients with PVR. In a previous study, Kase et al. [41] demonstrated increased levels of OPN in the vitreous fluid from patients with diabetic retinopathy; however, they studied only 11 cases. Our subgroup analysis showed that OPN levels in vitreous samples from active PDR cases were higher than those in quiescent cases. These results are in agreement with a previous report in which we demonstrated that OPN was expressed by vascular endothelial cells and stromal cells in PDR fibrovascular epiretinal membranes and by α-SMA-expressing myofibroblasts in PVR epiretinal membranes and that there was a significant correlation between the level of vascularization in PDR epiretinal membranes and the expression of OPN [42]. Taken together, these findings suggest a role for OPN in the progression of PDR. *In vitro* and *in vivo* studies demonstrated that OPN is an important angiogenic factor [15–18]. In addition, OPN is required for the activation, migration, proliferation, and differentiation of fibroblasts into α-SMA-expressing myofibroblasts [11–13] and is upregulated in several fibrotic diseases [9, 11, 12, 14]. Our results are consistent with previous reports showing that the proinflammatory cytokine OPN plays a role in the development of diabetic vascular complications [9, 43–45].

The proinflammatory cytokine HMGB1 [20–23] exhibits angiogenic [24–27] and fibrogenic [28–31] effects. Another

Table 3: Comparisons of mean angiogenesis and fibrogenesis regulatory factor levels in proliferative diabetic retinopathy (PDR) patients with or without traction retinal detachment (TRD).

Disease group	OPN (ng/mL)	HMGB1 (ng/mL)	CTGF (ng/mL)	PEDF (ng/mL)
PDR with TRD ($n = 21$)	584.15 ± 910.9	4.08 ± 6.9	1.38 ± 1.9	3.18 ± 5.5
PDR without TRD ($n = 27$)	868.2 ± 962.6	4.57 ± 10.7	1.94 ± 2.1	3.94 ± 8.4
RD ($n = 30$)	209.33 ± 192.5	0.98 ± 0.9	0.22 ± 0.3	0.32 ± 0.2
ANOVA P value	0.002*	0.003*	<0.001*	<0.001*

*Statistically significant at 5% level of significance.
OPN: osteopontin; HMGB1: high-mobility group box-1; CTGF: connective tissue growth factor; PEDF: pigment epithelium-derived factor; RD: rhegmatogenous retinal detachment.

Table 4: Pearson correlation coefficients between variables in proliferative diabetic retinopathy (PDR) and proliferative vitreoretinopathy (PVR) patients.

Disease	Variable	PEDF	OPN	HMG1
	OPN			
		$r = 0.544$		
		$P = 0.001$*		
	HMGB1			
PDR		$r = 0.719$	0.255	
		$P = <0.001$*	0.145	
	CTGF			
		$r = 0.715$	0.490	0.369
		$P = <0.001$*	0.002*	0.027*
	OPN			
		$r = 0.559$		
		$P = 0.020$*		
	HMGB1			
PVR		$r = 0.374$	0.484	
		$P = 0.140$	0.049*	
	CTGF			
		$r = 0.015$	0.293	−0.033
		$P = 0.953$	0.253	0.899

*Statistically significant at 5% level of significance.
OPN: osteopontin; HMGB1: high-mobility group box-1; CTGF: connective tissue growth factor; PEDF: pigment epithelium-derived factor.

interesting role of HMGB1 in neovascularization is its ability to attract endothelial progenitor cells to sites of tissue injury and tumors to improve neovascularization [26]. Several studies showed overexpression of HMGB1 in other fibrotic disorders [28, 29, 31]. *In vitro* studies demonstrated that HMGB1 stimulated the proliferation and migration of fibroblasts [28, 30]. In addition, exposure of epithelial cells to HMGB1 resulted in the transition from an epithelial to myofibroblast-like phenotype, with a significant increase in the mesenchymal markers α-SMA and vimentin [31]. Recently, Arimura et al. [46] demonstrated that HMGB1 stimulated the migration of human retinal pigment epithelial cells. In the present study, we report that HMGB1 was significantly upregulated in the vitreous fluid from patients with PDR, particularly in patients with active neovascularization in agreement with our previous report [47]. Furthermore, exploratory logistic regression analysis demonstrated significant associations between high levels of HMGB1 and all PDR and PDR with traction retinal detachment. These findings suggest a role for HMGB1 in the progression of PDR. In addition, in this study, we demonstrated that PVR eyes had a 3-fold increase in the vitreous level of HMGB1 when compared with those with RD. These results are in agreement with a previous report in which we demonstrated that HMGB1 was expressed by α-SMA-positive myofibroblasts in PVR epiretinal membranes [42]. In addition to its role in mediating inflammation, angiogenesis, and fibrogenesis, several studies demonstrated that extracellular HMGB1 can aggravate tissue damage in neuronal tissue after ischemia [48–50].

Recently, various studies suggested an important role for the proangiogenic [35, 36] and profibrotic [32–34, 51] CTGF in the development of chronic diabetes-related end-organ complications, including diabetic nephropathy [52]. In the present study, CTGF levels in the vitreous fluid from patients with PDR and PVR were significantly higher than those in the vitreous fluid from patients with RD. Our observations are consistent with previous reports showing increased CTGF levels in the vitreous fluid from patients with PDR [53–55] and PVR [54]. However, levels of CTGF in the vitreous fluid from patients with PDR were 3-fold higher than those in patients with PVR and levels of CTGF were particularly high in PDR patients with active neovascularization. In addition, exploratory logistic regression analysis demonstrated significant associations between high levels of CTGF and all PDR and PDR with active neovascularization. Our results are not in agreement with a previous report, in which Kuiper et al. [55] showed that CTGF levels in the vitreous fluid from patients with PDR were significantly associated with the degree of fibrosis. Our results are in agreement with a previous report in which we demonstrated increased expression of CTGF in the retinas from subjects with diabetes and that CTGF was expressed by vascular endothelial cells and α-SMA-expressing myofibroblasts in PDR epiretinal membranes and by myofibroblasts in PVR epiretinal membranes. In addition, there was a significant correlation between the level of vascularization in PDR epiretinal membranes and the expression of CTGF [6].

PEDF has been shown to be the most potent endogenous inhibitor of angiogenesis. The activity of PEDF is selective in that it targets only new vessel growth and spares the preexisting vasculature [37, 38, 56]. The results of different studies on PEDF expression in the vitreous from patients

with PDR are conflicting. Several studies found significant decrease in vitreous PEDF levels in patients with PDR [57–59]. Other studies found that PEDF levels in patients with PDR were not different from those in the controls [60, 61]. However, Duh and associates [62] demonstrated significant increase of PEDF levels in the vitreous from patients with active PDR. We do not have an explanation for the differences; however, differences maybe method related.

In the present study, the levels of PEDF in the vitreous from patients with PDR and PVR were significantly greater than those in patients with RD. In addition, PEDF levels in the vitreous from patients with PDR were higher than those in patients with PVR. Our subgroup analysis showed that PEDF levels were significantly higher in the vitreous from patients with active PDR compared with patients with quiescent PDR. Furthermore, exploratory logistic regression analysis demonstrated significant associations between high levels of PEDF and all PDR and active PDR. Similarly, studies of other angiogenic eye diseases demonstrated increased levels of PEDF in the aqueous humor in patients with choroidal neovascularization [63] and macular edema secondary to branch retinal vein occlusion [64].

In the present study, we demonstrated that the vitreous fluids from patients with PDR and PVR express different regulators of angiogenesis and fibrogenesis. Recently, Lenga et al. [13] showed that OPN is required for the presence of HMGB1 in the focal adhesions of fibroblasts and for CTGF expression by fibroblasts in response to the proinflammatory cytokine transforming growth factor-β1. These findings suggest that HMGB1, and CTGF serve to mediate the immune response attributed to OPN and that the interaction between OPN, HMGB1, and CTGF modulates fibroblast functions. The significant positive correlations between PEDF levels and the levels of OPN, HMGB1, and CTGF in the vitreous from patients with PDR in the present study echoed the findings of Matsuoka et al. [65] that both PEDF and the angiogenic VEGF have been strongly expressed temporally and spatially in the retina of diabetic rats. Similarly, there was a significant positive correlation between the expression of VEGF and PEDF in patients with choroidal neovascularization [63]. Our findings suggest that increased levels of PEDF in the vitreous of patients with PVR and PDR, particularly active PDR, may be a response to counteract the activity of the angiogenic and fibrogenic factors. In addition, our data suggest that a positive regulatory feedback loop may exist in PDR, such that increased OPN, HMGB1, and CTGF induced synthesis of PEDF.

In conclusion, our data suggest that the upregulation of OPN, HMGB1 and CTGF contribute to the pathogenesis of proliferative vitreoretinal disorders and that increased levels of PEDF may counteract the activity of angiogenic and fibrogenic factors during the progression of PDR and PVR. The OPN/HMGB1/CTGF pathway maybe a novel therapeutic target to inhibit progression of PDR and PVR.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

Vitreous Diagnosis in Neoplastic Diseases

Mónica Asencio-Duran,[1] José Luis Vallejo-Garcia,[2] Natalia Pastora-Salvador,[1] Agustín Fonseca-Sandomingo,[1] and Mario R. Romano[2]

[1] *Hospital La Paz, Madrid, Spain*
[2] *Istituto Clinico Humanitas, Milano, Italy*

Correspondence should be addressed to Mónica Asencio-Duran, masedur@hotmail.com

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Vitreous body is an intraocular structure, origin of diverse pathologies, but is also the place where cells and inflammatory mediators are released coming from several pathologic processes. These inflammatory reactions can happen in any other ocular location like choroid, retina, optic nerve, or ciliary body and vitreous humor constitutes a stagnant reservoir for these resulting substances and debris. Through the recent techniques of vitreous collecting, handling, and analysis, increasingly more sophisticated and with fewer complications, cellularity and molecules in the vitreous of challenging pathologies for the ophthalmologist can now be studied. The most usefulness for vitreous diagnosis would be the masquerade syndromes, and the best exponent in this group is the primary vitreoretinal lymphoma (PVRL), in which cytology and an IL-10/IL-6 ratio more than 1 is fundamental for the diagnosis.

1. Introduction

Vitreous body is the clear gel that fills the vitreous chamber or posterior chamber (PC) of the eyeball, the space between the lens and the eyewall, whose inner layer is the neurosensorial tissue that receives and transmits the image to the central nervous system called the retina. Its functions are to give volume to the eye, to support the retina attached, and to maintain its transparency to allow light beams to reach onto the retina.

Unlike the fluid in the anterior segment of the eye (aqueous humour), which is continuously replaced, vitreous humor is stagnant, and its composition remains quite constant throughout life. The vitreous gel is avascular, composed mainly of water (98-99%), and 0.9% of inorganic salts (sodium, potassium, and chloride). The remaining 0.1% is divided between protein, polysaccharide components, and ascorbic acid. Most of the protein is forming fibrils composed of a small collagen type V/XI core wrapped in a thick layer of collagen type II (75% of the fibril by mass) [1, 2]. It also contains very few cells, mostly phagocytes, whose function is to remove undesired cellular debris from the visual field, as well as hyalocytes of the surface of vitreous, which act as macrophages [3, 4].

The vitreous is feebly antigenic and is characterized by the absence of gamma-globulins and immunocompetent cells [5]. Because it only exhibits phagocytosis, this represents an incomplete and primitive immunological system, reacting like an embryonic tissue. The immune privilege, also, a physiologic mechanism characteristic of the internal compartments of the eye, is designed to provide protection against pathogens, protecting the delicate visual axis from the sight-destroying effect of immunogenic inflammation [6]. At the same time, there is a sustained suppressive microenvironment in the PC of the eye that inhibits the local expression of preexisting systemic immunity and participates in modifying the primary immune responses to ocular antigen [7, 8]. In the vitreous cavity it would develop a deviant form of immunity, similar to that in the anterior chamber [9], in which antigen-specific suppressor T cells are generated and delayed hypersensitivity reactivity is selectively impaired. Both the vitreous and the retina supply immunosuppressive molecules to the PC, but it has been suggested that retinal cells contribute more significantly to the immune suppressive microenvironment than vitreous cells: TGF β is produced by retinal astrocytes and retinal pigment epithelium; and Muller's cells (glia) of the retina suppress T cell proliferation

by a direct contact mechanism. In addition, the retinal vascular endothelium, Bruch's membrane, and the pigment epithelium together form the so-called ocular-blood barrier [7].

2. Ocular Diseases with Clinical Repercussion in Vitreous Body

Vitreous has a major role in the origin and the triggering of several ocular pathologies. Posterior vitreous liquefaction developed through years by means of dissolution of collagen fibers yields to several primary degenerative pathologies in vitreoretinal junction. Other diseases are easily diagnosable in the fundus eye and only affect the vitreous in late stages, like retinal vasculopathies as diabetic retinopathy and other vitreoretinal proliferations. Also, certain eye diseases have their beginning in other more hidden structures of the eye but may secrete molecules or even cells to the vitreous chamber, causing symptoms and helping in the diagnosis, since the vitreous is more accessible to study than other posterior pole structures.

The latter is the case of uveitis, a wide term that actually comprises a large group of diverse diseases affecting the retina, optic nerve, and also the vitreous compartment. These diseases may affect, in addition, several territories of the eye simultaneously and can have in major or minor degree manifestation in the vitreous humor, specially those concerning the posterior chamber. Fifty percent of noninfectious or "autoimmune" cases are limited to the eye (organ specific), whereas the remainder forms part of more generalized diseases, so that the pathophysiology of uveitis depends on the specific etiology, but in all types there is a breach in the ocular-blood barrier that normally prevents cells and large proteins from entering the eye. These cells then recognize antigens (autoantigen or foreign antigen) presented on the cell surface of antigen-presenting cells (APCs, like dendritic cells or macrophages), and activation and clonal expansion will take place, which results in increased production of IL-2, interferon-γ, and TNF-α [10]. Only certain responses are capable of overcoming the condition of immune privilege of the eye and unleashing the inflammatory cell accumulation and the tisular damage.

In most cases, the clinical appearance is sufficient for diagnosis, but since the majority of these patients have an unknown etiology for the intraocular inflammation, and this can be in addition related to a primarily nondiagnosed systemic disease, could be infectious, inflammatory, or even tumoral, the correct diagnosis can prove difficult. Thus, the intraocular inflammation is associated with the increased expression and action of several cytokines and growth factors, which can be determined in the vitreous and can help in the diagnosis. Several molecules have been identified along the last decades in the vitreous humor, which may be the key in the physiopathology of certain ocular diseases. In most cases this determination has investigational purposes, in others is helpful for the prognosis of the patients, and in a minority of cases its finding constitutes an indispensable diagnostic tool.

3. Diagnostic Techniques in Atypical Presentations of Uveitis

Many patients with uveitis have such characteristic ocular signs and symptoms, associated systemic disorders, and laboratory abnormalities that a satisfactory clinical diagnosis can be established without the need for invasive intraocular studies. Most other patients have mild, self-limited, and/or readily controllable disease that does not warrant aggressive invasive testing. In contrast, some patients have atypical ophthalmic and/or systemic features or do not respond to conventional anti-inflammatory therapies. Several techniques have been employed for diagnostic purposes in these cases: aqueous aspiration, vitreous aspiration, diagnostic vitrectomy, fine-needle aspiration biopsy, controlled aspiration of subretinal fluid, incisional chorio-retinal biopsy, and diagnostic enucleation.

3.1. Aqueous Aspiration. The indications of anterior chamber aspiration may be varied, but most common situations are patients with anterior chamber inflammation with suspicion of masquerade syndromes, a hypopion suspicious of infection, endophthalmitis, lens-induced uveitis and for cytopathologic examination [11]. A fine needle (30, 27, or 25 gauge) is introduced with the bevel up through clear cornea over the iris stroma, with optimal visualization by means of slit lamp or the surgical microscope, taking care to avoid the lens. A 0.1-0.2 mL of aqueous humor is withdrawn into a 3 mL syringe in a sterile technique, and then balanced salt solution may be used to reform the chamber [12]. The cytospin technique or others can be used to increase the sensitivity of the cytology specimen [13]. In the aqueous humor many other techniques can be performed for the diagnosis of infectious posterior uveitis, as polymerase chain reaction (PCR) and pathogen-specific antibody production for herpes simplex virus (HSV), varicella zoster virus (VZV), cytomegalovirus, Epstein-Barr virus (EBV), human immunodeficiency virus (HIV), Propionibacterium acnes, and Toxoplasma gondii [14]. Further studies have demonstrated for PCR of aqueous humor to yield a diagnosis in one-third of patients with posterior infectious uveitis with a sensitivity of 82% and specificity of 100%, equal or better than vitreous biopsy [15]. The advantage of anterior chamber aspiration is that it can be performed in an outpatient setting, but the disadvantage is that it retrieves a limited sample volume of 100 to 200 μL per procedure [16], which limits the number of molecular examinations that can be performed on the sample.

3.2. Vitreous Aspiration Tap. The indications for this technique are basically in posterior uveitis unresponsiveness to treatment when malignancy needs to be ruled out; when intraocular infection is considered the primary cause of inflammation in the absence or insignificant amount of vitreous cells that would preclude diagnosis through pars plana vitrectomy (PPV) [17, 18]. The major indication for vitreous aspiration would be when intraocular lymphoma is a significant diagnostic possibility. The technique of vitreous aspiration is similar to that of anterior chamber paracentesis

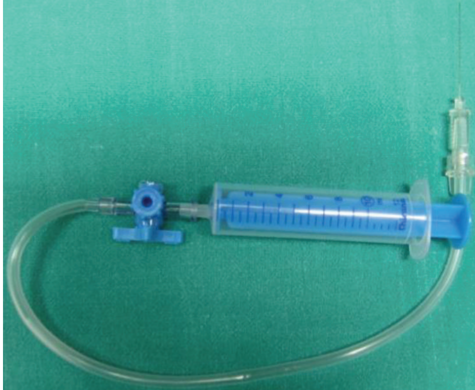

Figure 1: Set for manual Fine-needle aspiration biopsy/cytology, composed of 10 mL syringe, extension tube with a three-way stopcock, and long 27-gauge needle.

and is currently performed through the pars plana under local anesthesia with a large caliber needle, such as 21-gauge (G) hollow needle or fine, like 23 or 25-G mounted on a 1-mL syringe as an aspirating device, permitting to aspirate a volume of 100 to 250 μL of vitreous humor while the needle is directed posteriorly towards the optic nerve head. With vitreous biopsy, specimens obtained using a needle and syringe were positive in 54% of infected eyes compared with 75% of the specimens collected with vitrectomy procedures [18], although posteriorly, in the Endophthalmitis Vitrectomy Study, no significant difference was shown between needle tap versus mechanized vitreous biopsy with respect to microbiologic yield [19]. The main complications associated with the technique are retinal tears and endophthalmitis; however the risk is low, but both more common than after vitrectomy [20]. Other advantages of vitreous biopsies over vitrectomy are quickness, can be done in the outpatient setting without inpatient admission, can be repeated, and are less traumatic to the eye [20].

Another variation of the technique is the fine-needle aspiration biopsy (FNAB), in which a fine-needle gauge is directed to the localized suspected areas of intraocular tumor or lesion. Aspiration is performed automatic or manually using a 25-G to 30-G needle connected to an aspirating syringe (Figure 1). In case of intraocular tumor, the aspirated block obtained is likely to have a higher concentration of neoplastic cells than any of the adjacent intraocular fluids, decreasing the possibility of inconclusive cytological diagnoses, which often occur in vitrectomy specimens. For aqueous or vitreous humor the technique turns into an effective method for cytology that generally is able to obtain a sufficient amount of cells (100 to 500 μL of ocular fluid) to perform routine analysis like microbiologic, cytomorphological evaluation of Papanicolaou or haematoxylin- and eosin-stained cells, immunocytochemical analysis, and other applications [21]. The fine-needle aspiration technique is less invasive and has fewer complications than others [22], being in certain centers the preferred method for diagnostic purposes.

3.3. Diagnostic Vitrectomy. This technique may be the better option in selected cases, such as when vitreous removal is considered to be not only diagnostic but also therapeutic (e.g., endophthalmitis, intraocular bleeding with suspected malignant origin, and for the treatment of complications of chronic uveitis), and when the eye is inflamed and thereby patients may experience substantial discomfort during the vitreous biopsy tap [23]. Some authors recommend that vitrectomy-assisted biopsy should be considered only in cases in which FNAB fails [22], or multiple tests are needed and therefore requiring several punctures. A diagnostic standard three-port pars plana vitrectomy (VPP) provides a large amount of vitreous, retina, or choroid (though diluted), but always requires an operation theater under sterile conditions and direct visualization of the vitrectomy instruments. In order to obtain an undiluted vitreous sample, the infusion cannula of the system must be closed and the vitreous specimen is collected through undiluted lines using the vitreous cutter connected directly to a 3 mL syringe until the eye is noted to soften visibly [24]. At least 1.5 mL of undiluted vitreous can be reliably obtained with this technique. With perfluorocarbon-perfused vitrectomy, in which aspirated vitreous is compensated with perfluorocarbon liquid entry during vitreous aspiration, other authors were able to obtain an average of 2.4 mL of undiluted vitreous [25].

There is controversy whether using classic 20-G ports VPP needing suture, or the newest microincisional systems with 23-, 25-, or 27-G systems, but with anyone of these, the overall diagnostic yield of VPP varies considerably in different published studies from 14.3 to 61.5% [26–31], and the success for the procedure was greater when an intraocular infection was suspected compared with an intraocular malignancy [31], and greater for detecting primary vitreoretinal lymphoma than for detecting metastatic disease.

3.4. Chorioretinal Biopsies. Biopsies have been performed to investigate uncertain uveitis, choroiditis, and retinal and choroidal masses [32]. The Indications for biopsy included major diagnostic uncertainty, suspected cancer metastasis to the choroid without other evidence of systemic malignancy, and patient insistence on biopsy confirmation of the diagnosis prior to treatment. The procedure may be performed transsclerally or by an ab interno approach. Fine-needle aspiration biopsy is another method of obtaining retinal and choroidal tissue [33]. The limited performance of intraocular biopsy is explained by the risks for dissemination of malignant cells, eye complications (mainly hemorrhage, retinal detachment, and infection), and fears of misdiagnosis, although the literature gives little support to these [34, 35]. However, on the other hand, several authors have claimed that identifying patients with aggressive disease and a high risk for dissemination in malignant processes should be a priority and histopathological diagnosis should be mandatory [36, 37].

Various techniques have been developed to minimize the risks aforementioned. In the classic, transscleral approach, a scleral flap is created. A sharp blade then incises the choroid, and the biopsy tissue is grasped with forceps. A retinal specimen may also be obtained with the choroidal specimen

if a chorioretinal sample is the subject of study. Several modifications have been later described to facilitate the biopsy procedure and also reduce the risk of complications [38]; VPP is now often performed before creating the scleral flap, and another modification is the use of cyanoacrylate glue to provide increased stability to the tissue. In the transvitreal or ab interno approach, a retinochoroidectomy down to the sclera is performed after vitrectomy. The risk for complications is high, mostly due to hemorrhage and retinal detachment. FNAB for choroidal lesions provides the least invasive method of harvesting tissue [39]. Anterior lesions (iris and/or ciliary body) may be approached via limbal entry. The pars plana approach provides access for posterior lesions. The tip of the needle may be bent, facilitating entry into shallow choroidal lesions. Also, the risk of posterior scleral perforation is decreased.

4. Vitreal Biomarkers of Uveitis

Studies have shown increased levels of IL-6 (T-cell cytokine) in the vitreous fluid of patients with active intermediate or posterior uveitis, although it did not correlate with a specific uveitis type [40], suggesting that IL-6 is an inflammatory mediator common in various uveitis etiologies. IL-12, produced by monocytes, macrophages, B cells, and connective tissue-type mast cells has, also been found increased in aqueous humor and vitreous fluid of patients with low-grade intraocular inflammation and in uveitis in clinical remission for as long as 2 years [41]. Intraocular inflammation that fails to respond to immunosuppressive treatment raises suspicion for another different process. Since diagnostic analysis of vitreous fluid in patients with uveitis is limited, the best challenge for the study would be the masquerade syndromes.

5. Uveitis Masquerade Syndromes

Uveitis masquerade syndrome (UMS) is a group of disorders that mimic intraocular inflammation, but cells seen may be of noninflammatory origin (e.g., pigment, blood or malignant cells) or are inflammatory but secondary to another disorder [42, 43]. Theodore in 1967 was the first author who described a conjunctival carcinoma manifesting as a chronic conjunctivitis and named it *masquerade syndrome* [44]. The frequency of UMS among the patients with uveitis in a tertiary ophthalmologic center was 5% [45]. The causes of UMS may be variate, such as malignant, including hematologic malignancies, retinoblastoma, melanoma, and lung cancer metastasis; or nonmalignant, like ocular toxoplasmosis, diabetic retinopathy, hypertension, retinal detachment or degeneration, intraocular trauma, and radiation retinopathy [42, 43, 45–69] (Table 1). They are often misdiagnosed as a chronic idiopathic uveitis, but they can present in any location of the eye manifesting as panuveitis, pars planitis, vitreitis, papillitis, anterior segment cells, hypopyon or vitreal, and/or chorioretinal infiltrates (Figure 2).

Although they constitute rare presentations of uncommon diseases in the eye, the ophthalmologist must be aware because many of the UMS etiologies are malignancies with deleterious effects for the patient, for what early diagnosis and prompt treatment are mandatory. The study of vitreous body can be of great help specially in these cases, since the achievement of a small sample in doubtful cases can provide us the diagnosis.

Table 1: Ophthalmic diseases masquerading as chronic idiopathic uveitis.

Malignant diseases
Intraocular lymphoma
Non-Hodgkin's lymphoma of the central nervous system (NHL-CNS)
Systemic Non-Hodgkin's lymphoma metastatic to eye
Hodgkin's lymphoma
Other lymphomas
Lymphoid hyperplasia of uvea
Leukemia
Carcinoma metastatic to the eye
Uveal melanoma
Childhood malignancies
Retinoblastoma
Coats' disease
Leukemia
Medulloepithelioma
Juvenile xanthogranuloma
Paraneoplastic syndromes
Cancer-associated retinopathy
Melanoma-associated retinopathy
Bilateral diffuse uveal melanocytic proliferation
Nonmalignant diseases
Multiple sclerosis
Intraocular foreign body
Vascular disorders (hypertension, diabetic retinopathy, radiation retinopathy, retinal vasculitis, branch/central vein occlusion, ocular ischemic syndrome)
Retinal detachment
Vitreous and retinal degenerations (myopic, tapetoretinal)
Pigment dispersion syndrome
Intraocular infections (bacterial, fungal, viral, parasitic, propionibacterium acnes)
Postvaccination and drug-related reactions

5.1. Intraocular Lymphoma

5.1.1. Classification. Although both Hodgkin's lymphoma and non-Hodgkin's lymphoma (NHL) can present as intraocular inflammation, in the case of Hodgkin's lymphoma ocular involvement generally is rare and often occurs late in the course of the disease, whereas NHL affects more commonly the eye. NHL can be divided in two clinically different entities: systemic NHL with metastases to the eye, and NHL of the central nervous system (NHL-CNS).

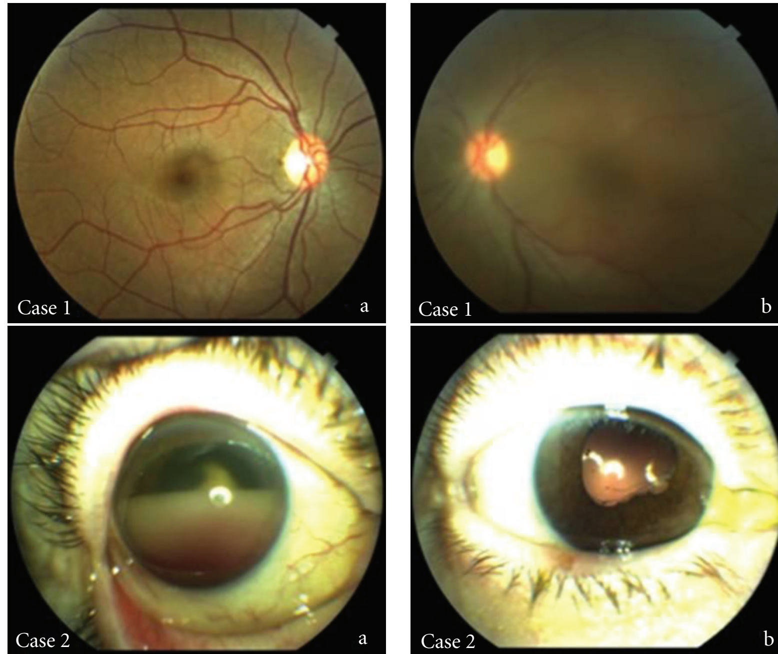

Figure 2: Case 1: (a) healthy right eye of the same patient, (b) left eye vitreitis in a healthy patient that was finally diagnosed of primary vitreoretinal lymphoma by vitrectomy. Case 2: (a) hemorrhagic hypopion in a patient with primary thoracic B-cell Hodgkin's lymphoma in clinical remission. After unsuccessful anti-inflammatory and antibiotic treatment an anterior chamber paracentesis was performed confirming the diagnosis of metastatic lymphoma, (b) external aspect of the eye after treatment with intravitreal methotrexate.

Recently, Coupland and co-workers proposed an anatomical classification according to the localization of the disease in the eye; retinal lymphomas are high-grade B-cell malignancies associated with a poor prognosis, whereas primary uveal lymphomas are typically low-grade B-cell tumours derived from the postgerminal centre (memory) B cell [70].

The variant with major ophthalmic repercussion is the primary vitreoretinal lymphoma (PVRL), a subtype of primary central (CNS) lymphoma, typically classified as a diffuse large B-cell lymphoma and most frequently develops in elderly populations. Over 15% of primary CNS lymphoma patients develop intraocular lymphoma, usually occurring in the retina and/or vitreous, and conversely, 65%–90% of PVRL patients develop CNS lymphoma [71]. Consequently, PVRL is often fatal because of ultimate CNS association, that can appear from 1 month to 10 years after [72, 73].

5.1.2. Clinical Features. Both retinal and uveal lymphoma can manifest as any form of uveitis, but PVRL typical clinical findings include vitreous cellular infiltration (lymphoma and inflammatory cells) and subretinal tumor infiltration. Choroid is the predominant location for primary uveal lymphomas and most often manifests as recurrent episodes of blurred vision and metamorphopsia secondary to exudative retinal detachment affecting the fovea. A classic finding is the presence of solitary or multiple yellow, creamy choroidal infiltrates with clear vitreous, that can evolve to diffuse thickening of the uveal tract and in some cases, to episcleral extension appearing as a nonmobile orange to yellow or "salmon" patch.

5.1.3. Sample Collection and Handling. The clinical suspicion is very important given the potential lethality if an uncorrect diagnosis is made and a proper systemic treatment is applied. Currently, PVRL is most often diagnosed using citology (the gold standard) or vitrectomy to identify lymphoma cells in the vitreous or retina [74]. In order to prevent degeneration of lymphoma cells, vitreous specimens are placed into a tube containing culture medium like RPMI (Roswell Park Memorial Institute) [75], whereas others prefer immediate placement in normal saline, taking care not fixing with alcohol with the aim to not alter the identification of PVRL cells in the vitreous sample.

5.1.4. Sample Analysis

Citology. As lymphoma cells are fragile, the general consensus recommends sending the samples immediately to an experienced cytopathologist to distinguish the malignant cells (usually B lymphocytes) from the reactive lymphocytes (T cells). The malignant B cells of PVRL exhibit characteristic features with Papanicolaou, Giemsa, or Diff-Quick stains [26, 76]: large round or oval nuclei, frequently segmented and often containing prominent nucleoli, surrounded by scant basophilic cytoplasm. Samples often are negative because of poor biopsy samples, with a reported effectivity of only 48.3% of lymphoma cases for PPV, although other authors have found for FNAB a diagnostic effectivity in 87.5% of the suspicious intraocular lymphoma cases [17]. Other authors advocate for the fixation of the samples with Cytolit or HOPE solution (Herpes-glutamic acid buffer

mediated organic solvent protection effect) in order to facilitate the transportation from the theater to the laboratory [77].

Molecular Analysis. Flow cytometric immunophenotyping (FCI) can be done in diluted samples, allows for the analysis of several different cell surface markers simultaneously, and offers a quantitative method of determining the percentage of a particular cellular phenotype, increasing the efficiency of a biopsy specimen [26]. Dilute vitreous is centrifuged and resuspended in cell culture medium, and cells are counted and stained with antibodies to detect markers that identify leukocytes, T lymphocytes, B lymphocytes (including CD19, CD20, CD22, κ, and λ light chain markers), monocyte/macrophages, and lymphocyte activation. The test relies on the finding that the majority of PVRL have restricted expression of κ or λ chains, with the most sensitive marker being a $\kappa:\lambda$ ratio ≥ 3 or ≤ 0.6 (80%), whereas CD22 and CD20 markers are not very sensitive for lymphoma (50 and 33%, resp.), although they are quite specific (94 and 89%, resp.) [31]. For patients with possible T-cell lymphoma, cell surface markers more commonly searched are CD3, CD8, CD4, CD7, CD2, CD25, and CD52 [78].

Other molecular analysis techniques like microdissection and PCR can be used. Microdissection allows for the selection of only few or poorly preserved malignant or atypical lymphoid cells that would have been nondiagnostic for PVRL by routine cytological techniques. PCR can determine monoclonality by immunoglobulin heavy chain (IGH) rearrangement and t(14; 18) translocation of the bcl-2 gene that promote cell survival and predict a more aggressive tumor course in B-cell lymphoma [75, 79, 80]. PCR has been found to be 64% sensitive for PVRL [81], and is being used to study the genotypic classification of PVRL with the goal of identifying prognostic factors; patients with a translocation in the bcl-2 gene are significantly younger than patients who lacked the translocation, suggesting that younger patients with the translocation may need to be treated aggressively [82]. Some authors have advocated an inhibition of B-lymphocyte chemoattractants (BCA-1, CXCL13, and SCYB13) and their ligands CXCR4 and CXCR5 could be a future strategy for the treatment of this disease with limited side-effects profile [83].

For Margolis, vitrectomy together with cytology and flow cytometry detected all cases of PVRL [84]. If the quality of the cytology finally is poor, then a second vitrectomy may be necessary, but because cell numbers are likely to be low in a vitrectomized eye, a retinochoroidal biopsy may be performed at the time of vitrectomy surgery [85].

Vitreous Biomarkers of Intraocular Lymphoma. Possibly, PVRL is the best example of ocular disease in which intravitreal cytokines are more useful for the diagnosis. Increased concentration of IL-10, a growth and differentiation factor for activated B lymphocytes, has been found increased in vitreous fluids of PVRL patients [75], in contrast with the increased concentration of IL-6 characteristic of uveitis, for which many authors have indicated that an IL-10/IL-6 ratio greater than 1.0 is useful for the diagnosis of PVRL. Cytokine analysis can be useful adjunctive tests in corroborating suspicion of PVRL and determining whether there is a significant response to treatment [75, 80–87], but cannot be used only to make the diagnosis, as some studies have reported false positive or false negative results [88]. The IL-10/IL-6 ratio greater than 1.0 in suspected cases of PVRL was associated with a sensitivity and specificity of 74.3 and 75.0%, respectively, [86] and Cassoux and co-workers found in 51 vitrectomies performed in patients with proven PVRL that an IL-10 cut-off value of 400 pg/mL was associated with 80% sensitivity and 99% specificity [89].

The diagnosis of intraocular lymphoma from vitreous specimens depends on proper handling of the specimens, methods of aspiration, concentration, fixation, and staining [90, 91]. Addition of culture medium with fetal calf serum can improve the survival and viability of the malignant cell [91]. Prior treatment of patients with steroids reduces the number of viable lymphoma cells, which are known to be cytolytic, so that discontinuing systemic and topical corticosteroids is strongly recommended before biopsy to increase the profitability of these cells [91].

5.2. Other Lymphoproliferative Malignancies. Leukemia has increased the variability of ocular presentations associated, due to the improvement in the survival after the new era of effective antileukemic therapy. Leukemia may involve almost every ocular tissue, with the retina being the most frequent affected structure (up to 69% of all patients show fundus changes at some point in the course disease). Hemorrhages, infiltrates, and aggregates of leukemic cells are found at all levels [48, 92], and generally the internal limiting membrane acts as a barrier; however, cells occasionally invade the vitreous possibly emerging from the optic nerve head and these cases can be diagnosed by examination of the specimens obtained from the vitreous [93, 94]. Nevertheless, primary presentation of these diseases are rarely ophthalmological and more frequently occur in patients with advanced systemic disease. As relapsing uveitis or hyphemas can be related to leukemia, are in these cases when cytological sample must be obtained, thus allowing us to ascertain the inflammatory origin or reactivation of the disease.

5.3. Uveal Melanoma. Uveal melanoma (UM) is the most frequent intraocular tumor in the adulthood. Funduscopy combined with ultrasonography actually gives an accurate diagnosis in almost 95% of the patients, but there are, however, some cases difficult to diagnose due to atypical ocular manifestations or accompanying intraocular changes, such as extensive retinal detachment, vitreous hemorrhage, or others. In these cases histopathological examination with preservation of the eyeball is the ideal method.

Cytological tests using modified Shorr's or others stains have been capable of diagnosing cells with intracytoplasmic melanin pigment granules from samples obtained in eyes harbouring choroidal or metastatic cutaneous melanomas [95, 96]. A recent study shows that 5-S-cysteinyldopa (5-S-CD), a metabolite generated during pheomelanin synthesis, may reflect a direct secretion from the tumor into the

vitreous or an alteration of dynamics of intraocular fluids, because its concentration is increased in vitreous fluid from UM patients. But the diagnosis from vitreous samples in UM probably would not become extensible in the future due to the unknown exact role of this biomarker [97], the possibility of extraocular dissemination of UM implicit in the surgical intervention [98], and to the efficiency of other simpler diagnostic methods.

5.4. Intraocular Metastasis. Intraocular metastases often appear in the choroid as solitary or multiple mass in a patient with history of systemic malignancy, although in 34% of the cases had no known primary site [99]. Together with the possibility of bilateral involvement and atypical clinical presentations, the diagnosis sometimes is difficult. Vitrectomy has helped to diagnose metastatic cutaneous melanoma in difficult cases like nonpigmented vitreous clumps [100] and thickened posterior vitreous membranes [101], and moreover can be therapeutic in these cases. Carcinomas also can metastasize directly in the vitreous or indirectly by means of vitreal seeds from an underlying choroidal, retinal, or optic nerve infiltration, and both vitrectomy or fine-needle aspiration cytology can help in the diagnosis [102, 103].

6. Conclusion

Vitreous body constitutes a little-known intraocular structure, but we are increasingly supporting our diagnostic searches in it thanks to the recent advantages in collection, handling, and analysis of vitreous samples. Inflammation is not always the cause of apparent inflammatory diseases and sometimes the origin is degenerative, traumatic, vascular, infectious, or even neoplastic. Vitreous cells in hands of experienced cytologists can be sufficient, but an accurate diagnosis needs the employment of sophisticated molecular analysis such as flow cytometric immunophenotyping (FCI), microdissection and polymerase chain reaction (PCR), or cytokine analysis like IL-10/IL-6 ratio. The major diagnostic use of vitreous sampling would be the masquerade syndromes, in which a devastating neoplastic disease can be behind a few vague and slightly specific ocular signs, and possibly the biggest representative of this group is the primary vitreoretinal lymphoma and some metastatic intraocular lesions. Even though, little information exists nowadays on the number and the specific role of different molecules acting in the pathophysiology of diseases that represent a challenge for our daily practice. Further investigation is needed to increase our knowledge on the molecular pathogenic mechanisms underliying neoplastic diseases, with which we could interact to create new targeted and powerful therapeutic pathways or at least alternatives to the current ones.

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Review Article

Mechanisms of Inflammation in Proliferative Vitreoretinopathy: From Bench to Bedside

Stavros N. Moysidis, Aristomenis Thanos, and Demetrios G. Vavvas

Retina Service, Massachusetts Eye and Ear Infirmary, Department of Ophthalmology, Harvard Medical School, 243 Charles Street, Boston, MA 02114, USA

Correspondence should be addressed to Demetrios G. Vavvas, demetrios_vavvas@meei.harvard.edu

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Proliferative vitreoretinopathy (PVR) is a vision-threatening disease and a common complication of surgery to correct rhegmatogenous retinal detachment (RRD). Several models of the pathogenesis of this disease have been described with some of these models focusing on the role of inflammatory cells and other models focusing on the role of growth factors and cytokines in the vitreous which come into contact with intraretinal and retinal pigment epithelial cells. New experiments have shed light on the pathogenesis of PVR and offer promising avenues for clinical intervention before PVR develops. One such target is the indirect pathway of activation of platelet-derived growth factor receptor alpha (PDGRα), which plays an important role in PVR. Clinical trials assessing the efficacy of 5-fluorouracil (5-FU) and low-molecular-weight heparin (LMWH), daunorubicin, and 13-cis-retinoic acid, among other therapies, have yielded mixed results. Here we review inflammatory and other mechanisms involved in the pathogenesis of PVR, we highlight important clinical trials, and we discuss how findings at the bench have the potential to be translated to the bedside.

1. Introduction

Proliferative vitreoretinopathy (PVR) is a vision-threatening disease that can occur secondary to retinal detachment (RD). RD allows macrophages, retinal pigment epithelial (RPE) cells, glial cells, and fibroblasts to migrate to the vitreous, where they proliferate, survive, form extracellular matrix proteins and assemble into a membrane [1]. This membrane can attach to the retina and subsequently contract, which can cause a new retinal detachment or failure of a surgically corrected detachment [2]. PVR occurs most commonly as a complication of surgery to correct rhegmatogenous retinal detachment (RRD) and is the most common reason for the failure of this operation [3, 4]. In one study of 119 patients with RRD and no previous vitreoretinal surgery, there was a 52.9% prevalence of PVR and 26.9% prevalence of severe PVR with mean retinal detachment duration of 58.4 ± 129.1 days [5]. Visual outcomes and the anatomical success of surgery are worse for RD that is complicated by PVR and may require twice as many resources to care for as those cases of RD without PVR [6]. Here we review inflammatory and other mechanisms involved in the pathogenesis of PVR, we highlight important clinical trials, and we discuss how findings at the bench have the potential to be translated to the bedside.

2. The Macrophage Hypothesis for Development of PVR

Some of the hypotheses regarding the pathogenesis of PVR have focused on the role of macrophages [7–9]. In one experiment, rabbits were injected intravitreally with cells obtained from their peritoneal cavity, consisting of 85% macrophages, 10% lymphocytes, a few neutrophils, and less than 1% erythrocytes [7]. One week after injection, intravitreal strands had developed containing macrophages and fibroblasts, with massive epiretinal membranes developing between 4 to 9 weeks after injection in 17 of 24 eyes, posterior vitreous separation in 16 of 24 eyes, and retinal detachment in 15 of 24 eyes. The researchers suggested that macrophage-derived enzymes produced changes in the structure of the vitreous by

proteolysis of matrix proteins and also that the development of fibrotic membranes was due to the synthesis of fibroblast growth factor by the macrophages, but not due to cellular transdifferentiation of macrophages into fibroblast-like cells [7]. Immunohistochemical analysis of surgical specimens of patients with post-traumatic PVR indicated the presence of macrophages and transferrin in periretinal membranes [8]. It was suggested that the secretion of PDGF by macrophages was central to the pathophysiology of PVR in these specimens, since PDGF increases the density of the cell surface receptor for transferrin [8, 10]. This hypothesis is also supported by the development of PVR-models in rabbits and rats in which injected macrophages acquire fibroblastic characteristics and contribute to the formation of fibrocellular membranes [9, 11]. Macrophages (CD68-positive) were intravitreally injected into rats' eyes and by day 7, the majority of the rats (29/32) had white proliferative membranes attached to their retina [11]. This was followed by the development of neoformative membranes by day 14, but the rats did not develop complete retinal detachment; 20 control rats that received PBS injection did not have any proliferation or membrane formation. Furthermore, by day 28 a dense fibrous connective tissue had formed that on histology had a multilayer of fibroblast-like cells which on immunohistochemical analysis stained positive for vimentin (marker for mesenchymal cells), but not cytokeratin (marker of epithelial cells) or CD68 (marker of macrophages), suggesting the primary cells of the PVR membranes were fibroblasts [11]. Injected macrophages retained a round shape and CD68 on day 3, but on day 28 had developed a spindle shape with staining of vimentin and absence of CD68; the macrophages had acquired a fibroblast-like phenotype and contributed to the fibrocellular membranes directly [11]. It is likely that the role of macrophages in the pathogenesis of PVR is multifactorial and involves a combination of macrophage-secreted factors including enzymes and growth factors (e.g., PDGF) and also transdifferentiation of macrophages into fibroblast-like cells.

3. Injection of Cells into the Vitreous as a Model of PVR

In early models of PVR, a piece of dermal tissue was delivered to the vitreous of rabbit eyes through a small cauterized hole in the pars plana; growth of this tissue ensued, with the development of vitreous strands between the tissue and the retina, and ultimately retinal detachment in the majority of cases [12]. This was followed by experiments in which fibroblasts were intravitreally injected into rabbits [13–15]. Autotransplanted, cultured skin fibroblasts injected intravitreally resulted in vitreous strands, preretinal pucker, and traction detachment in 32 of 51 eyes [13]. In another rabbit model, gas compression was used to simulate vitrectomy and followed a week later by injection of autologous tissue-cultured fibroblasts; by post-op day 28, 10 of 10 eyes injected with 50,000 fibroblasts had developed transvitreal strands and severe retinal detachment [14]. Meanwhile, in eyes injected with 25,000 fibroblasts, 7 of 11 showed transvitreal strands, and 10 of 11 developed retinal detachment [14]. In the epiretinal membranes of patients undergoing vitreoretinal surgery for retinal detachment complicated by PVR, all 16 samples contained myofibroblasts expressing the contractile protein α-smooth muscle actin [16]. *In vitro*, the addition of bovine vitreous to cultures of RPE cells and fibroblasts stimulated the proliferation of these two cell types [17]. Furthermore, pathologic vitreous from patients with PVR stimulated contraction of cultured fibroblasts *in vitro* [18]. In rats, intravitreal injection of rat RPE (RPE-J) cells and platelet-rich plasma resulted in proliferative membranes and retinal detachment by post-injection day 28 [19]. Immunohistochemical analysis of membranes at days 14 and 28 revealed RPE cells expressing cytokeratin-18, glial cells expressing GFAP, fibroblasts expressing vimentin, and ED-1 positive macrophages [19]. This evidence, along with the macrophage model of PVR, suggests that it may be the introduction of cells into the vitreous that triggers processes leading to PVR, rather than the particular cell injected.

4. The Growth Factor and Cytokine Hypothesis for Development of PVR

In the proposed growth factor and cytokine model for the development of PVR, a break in the retina, such as that occurring in RRD, creates an opening for vitreous to come into contact with intraretinal cells and retinal pigment epithelial (RPE) cells. Vitreal growth factors and cytokines, now with access to these cells, promote an environment of cell migration, proliferation, survival, and formation of extracellular matrix proteins (Figure 1) [20]. As these structures form, they may physically attach to the retina, contract, and cause retinal tears. Support for this hypothesis stems from the presence of many growth factors and cytokines in the pathological vitreous or epiretinal membrane, including platelet-derived growth factor (PDGF) isoforms [21, 22], hepatocyte growth factor (HGF) [22, 23], vascular endothelial growth factor (VEGF) [24], epidermal growth factor (EGF) [25], pigment epithelium-derived factor (PEDF) [26], transforming growth factor β (TGFβ) [27, 28], tumor necrosis factor α (TNFα) [29, 30], TNFβ [29], granulocyte colony-stimulating factor (G-CSF) [29], fibroblast growth factors (FGF) [29, 31], basic fibroblast growth factor (bFGF) [32], insulin [25], insulin-like growth factor-1 (IGF-1) [33], connective tissue growth factor (CTGF) [22, 23], glutamine synthetase [32], interleukin 1 (IL-1) [34], IL-6 [29, 31], IL-8 [29, 35], IL-10 [29], interferon γ (IFNγ) [28, 29], monocyte chemotactic protein [35, 36], macrophage-colony stimulating factor [35], granulocyte colony-stimulating factor (G-CSF) [29], chemokine ligand 2 (CCL2) [29], CCL3 [29], CCL4 [29], CCL5 [29], and protein [31].

5. Tumor Necrosis Factor Alpha as a Promoter of PVR

Tumor necrosis factor (TNF)α is a cytokine that promotes inflammation, in part, by activating endothelial cells to display leukocyte adhesion molecules such as E-selectin, intercellular adhesion molecule-1, and vascular cell adhesion

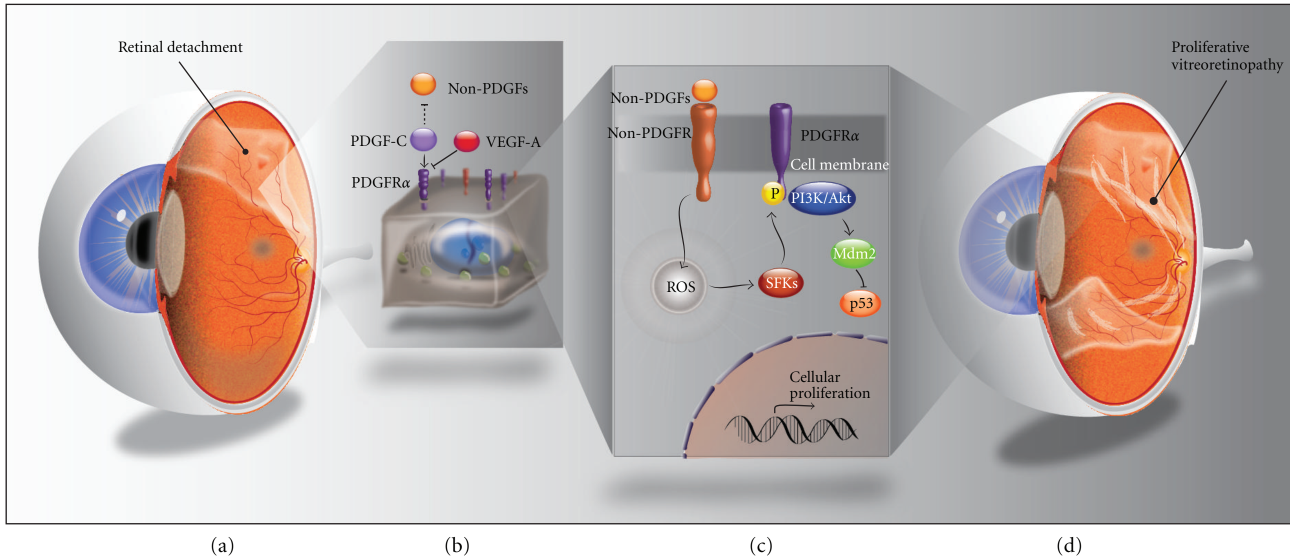

Figure 1: Indirect activation of PDGFRα by non-PDGFs triggers the events leading to proliferative vitreoretinopathy (PVR). A retinal tear or detachment (a) creates an opening via which vitreal growth factors and cytokines interact with intraretinal cells and retinal pigment epithelial (RPE) cells. Vitreal VEGF-A competitively inhibits the binding of platelet-derived growth factors (PDGFs), including the predominant isoform isolated in the vitreous of patients with PVR, PDGF-C, to the receptor PDGFR-α (b). In doing so, VEGF-A prevents direct activation of PDGFRα by PDGFs. Direct activation of PDGFRα promotes rapid clearance of this receptor from the cell surface and subsequent intracellular degradation; this rapid receptor cycling interferes (b) with the ability of non-PDGFs to activate the PDGFRα through an indirect pathway as follows. Non-PDGFs, including basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), insulin, and hepatocyte growth factor (HGF), activate their receptors, which results in an elevation of the level of intracellular reactive oxygen species (ROS), which leads to activation of Src family kinases (SFKs) that promote phosphorylation and activation of PDGFRα (c). This pathway of indirect activation results in persistent PDGFRα signaling and induces prolonged activation of phosphatidylinositol 3-kinase (PI3K)/Akt, which phosphorylates murine double minute (Mdm2), which then suppresses p53 levels (c). This promotes an environment of cell survival, proliferation, organization into a membrane, and subsequent membrane contraction, the processes intrinsic to PVR (d). Therefore, VEGF-A inhibits physiological, direct activation of PDGFRα by PDGFs and favors pathological, persistent, indirect activation of the receptor by non-PDGFs, triggering the events leading to PVR.

molecule-1 [37, 38]. TNFα was found in 22 of 26 epiretinal membranes of patients with proliferative vitreoretinopathy, with positive TNFα staining both intracellularly and in the extracellular matrix [39]. TNFα is associated with the production and secretion of the receptors sTNF-RI and sTNF-RII, which are found on the majority of nucleated cells; after activation by TNFα, these receptors are cleaved by metalloproteinases [40] and found in the soluble form in serum [41]. sTNF-RI and sTNF-RII are thought to neutralize the inflammatory effects of TNFα *in vitro* and *in vivo* and can be used clinically as markers of disease activity [42, 43]. The levels of sTNF-RI and sTNF-RII were significantly higher ($P < 0.0003$) in the vitreous of patients with PVR (244–4290 and 128–4429 pg/mL, resp.) compared to cadaveric controls (101–836 and 96–551 pg/mL, resp.) [44]. Groups in the aforementioned study were not matched for age; another study suggests that sTNF-RI and sTNF-RII are significantly increased in the serum of healthy older people (mean 71 years) and centenarians compared to younger, healthy controls (mean 27.9 years) [45]. Genetic analysis of blood samples from 138 patients with post-rhegmatogenous retinal detachment PVR demonstrated a significant association ($P = 0.0283$) with the nonsynonymous, single nucleotide polymorphism (SNP) rs2229094(T → C) compared to controls [46]. This is a SNP in the lymphotoxin alpha gene at the tumor necrosis factor locus (6p21.3), which encodes a cysteine to arginine change—from a neutral, hydrophobic amino acid to a hydrophilic, positively charged amino acid—and may have an effect on protein topology or its interactions [46]. Future studies on protein function may further elucidate the role of this SNP at the TNFα locus in PVR.

6. PDGFRs Are Involved in the Pathogenesis of PVR

PDGF is an important link in the cell-cell interactions of retinal cells and functions as a trophic factor during the development of the retina [47, 48]. PDGFR has been identified on the cell membranes of RPE cells, retinal glial cells, and fibroblasts, some of the cell types involved in PVR [49, 50]. PDGF and activated PDGFR have been noted in the epiretinal membranes, RPE, and glial cells of patients with PVR, with high levels of PDGF in the vitreous closely associated with PVR (8/9 patients with PVR had detectable levels of vitreal PDGF compared to 1/16 patients with a different retinal disease requiring surgery/vitrectomy) [51, 52]. Only

the PDGF-C isoform was isolated, which is produced mainly by the protease plasmin [53]. This finding was corroborated by a high level of PDGF-C in the vitreous of rabbit models of PVR induced by fibroblast injection [52, 54]. Additionally, in experimental models, cells that lacked the PDGFR gene had a low potential for PVR and reexpressing the wild type PDGFR in these cells greatly increased the potential for PVR [21, 50]. Inhibition of PDGFRs decreased cellular PVR potential [55, 56]. Of the three different PDGFRs: cells expressing PDGFRα induce PVR much more effectively than cells expressing PDGFRβ in rabbits, and cells expressing the heterodimer PDGFR$\alpha\beta$ had intermediate potency in inducing PVR [21]. This is supported clinically by analysis of human specimens demonstrating that a greater percentage of PDGFRα is activated [51]. In addition, PDGF-C, the predominant PDGF isoform isolated in the vitreous of patients with PVR, activates PDGFRα and PDGFR$\alpha\beta$ but not PDGFRβ [52, 57].

7. Indirect Activation of PDGFR by Non-PDGFs Triggers the Events Leading to Experimental PVR

Non-PDGFs can also activate PDGFRα; for example, bFGF, EGF, insulin, and HGF induce tyrosine phosphorylation of PDGFRα [25]. Non-PDGFs activated both full-length PDGFRα and mutant receptors that lacked the extracellular domain to a comparable extent, through the following pathway: non-PDGFs activate their receptors, resulting in an increase of intracellular reactive oxygen species (ROS), then activation of Src family kinases (SFK), which leads to phosphorylation of PDGFRα (Figure 1) [58]. New evidence suggests that this indirect pathway involving non-PDGFs as agonists of PDGFRα is the primary pathway for activation of this receptor and an important part of the pathogenesis of PVR. VEGF-A prevents binding of PDGF to PDGFRα, inhibiting the direct pathway of PDGFRα activation and downstream extracellular signal-related kinase (Erk) activation [59]. Neutralizing VEGF-A by adding anti-VEGF-A antibodies to the vitreous of rabbits with PVR resulted in a significant increase in the activation of PDGFRα; VEGF-A influences the mechanism of PDGFRα activation, inhibiting the direct pathway and creating an environment favoring non-PDGFs to indirectly activate PDGFRα [59]. While direct activation of PDGFRα results in rapid clearance of the receptor from the surface and subsequent degradation, indirect activation by non-PDGFs promotes persistent receptor signaling and induces prolonged activation of phosphatidylinositol 3-kinase (PI3K)/Akt, which activates murine double minute (Mdm2) to suppress p53 levels, driving processes intrinsic to PVR-survival, proliferation, and contraction (Figure 1) [59, 60].

8. Therapeutic Targeting of the PDGF/PDGFR Pathway

Attempts to prevent retinal detachment and PVR with antibodies directed against PDGFs have yielded mixed results. In photoreceptors of transgenic mice overexpressing PDGF-B, the universal ligand for all three PDGF receptors, intravitreal injection of an aptamer against PDGF-B was protective against retinal detachment [61]. In a rabbit model, antibodies against vitreal PDGFs inhibited them effectively but did not prevent PVR compared to controls (Table 1), suggesting that the PDGFRs in this model were activated by non-PDGFs [25]. Attempts were then made to inhibit the indirect pathway of PDGFR activation, a pathogenesis that involves an increase in ROS. In a comparison of cells null for all PDGFRs and cells containing a truncated PDGFRα that could only undergo indirect activation, both bFGF (which increases ROS) and then separately rabbit vitreous, caused the cells with truncated PDGFRα to robustly contract but did not cause contraction in control cells null for the receptor. The experiment was then repeated in the presence of N-acetyl-cysteine (NAC), an antioxidant that inhibits ROS formation. At concentrations of 2.5 mmol/L NAC and above (NAC-induced toxicity began to occur at 20 mmol/L), contraction of the PDGFRα-cell lines was prevented, as was the proliferative advantage of PDGFRα-containing cells over control cells [62]. These findings were then applied *in vivo* to PVR-model rabbits, where a vitreal concentration of 10 mmol/L of NAC was found to significantly reduce the PVR response compared to injection of buffer, with suppression persisting 3 weeks post-NAC injection; while the development of membranes occurred in most of the treated rabbits, they did not progress to retinal detachment, and analysis of PVR membranes revealed that control rabbits had 2.6 times the PDGFRα activation compared to treated rabbits (Table 1) [62]. NAC also prevented contraction of primary RPE cells isolated from a human PVR membrane which was subjected to the donor vitreous of five patients with PVR; NAC may be used to suppress receptor activation and retinal detachment but not to target pathological cells' viability [62].

9. Neutralizing a Subset of Non-PDGFs and Cytokines to Prevent PVR

Approaches with a cocktail of neutralizing reagents to target multiple growth factors and cytokines have also been studied. One *in vitro* study assessed for the minimum possible neutralizing set of antibodies that could be delivered to prevent cellular contraction in the presence of pathologic PVR vitreous. The minimum neutralizing set blocking PVR-related signaling was found to be a cocktail of antibodies that neutralized PDGFs, TGFα, EGF, HGF, FGF-2, TGFβ, IL-8, and IGF-1 [63]. The rationale for neutralizing PDGFs, despite evidence suggesting that the direct pathway of PDGFRα activation plays only a minor role in PVR, was to preempt against the possibility that inhibiting the indirect pathway would then potentiate and increase the bioactivity of the direct pathway. These findings were then applied *in vivo* to rabbit PVR-models by treating twelve rabbits with the minimum neutralizing cocktail and another twelve with nonimmune IgG. Of the control rabbits, 8 (67%) developed stage 3 PVR or higher with retinal detachments and the other 4 (33%) developed stage 2 PVR. In contrast, none

Table 1: Outcomes of therapeutic agents used in animals to prevent proliferative vitreoretinopathy (PVR).

Agent(s)	Dose and target	*In vivo* model	Treatment groups	Outcomes	Ref.
ARC126, ARC127, ARC128	20 μg, aptamers against PDGF-B	Rho/PDGFB transgenic mice treated on P7, sacrificed P12	Intravitreal inj. on P7: PBS OD; ARC126, ARC-127, ARC128 OS	PBS: mean area of GSA+ cells 1.82 mm^2, 11/19 totRD, 4/19 pRD, 79% RD ARC126: mean area of GSA+ cells 0.79 mm^2 ($P = 0.0003^*$), 0 totRD, 1/6 pRD, 17% RD ARC127: mean area of GSA+ cells 0.55 mm^2 ($P = 0.0001^*$), 0/7 totRD and pRD, 0% RD ARC128: mean area of GSA+ cells 2.00 mm^2 ($P > 0.05$), 3/6 totRD, 2/6 pRD, 83% RD	[61]
(a) Mouse mAb IgG1 HH1-57; (b) Trap PDGFRα-Fc5	(a) 200 μg, inhibits PDGF-C (b) 386 μg, inhibits all PDGF isoforms	Rabbits with OD PVR-induced by injection of PRP and fibroblasts	Intravitreal inj. on day 0: control ($n = 8$) versus (a) mAb IgG1 HH1-57 ($n = 9$) and (b) Trap ($n = 13$)	Control: 1*Stage1*, 2*S2*, 4*S3*, 1*S4* (day 3); 1*Stage2*, 2*S3*, 5*S4* (d5); 1*Stage2*, 1*S3*, 6*S4* (d7) (a) HH1-57: 2*S1*, 2*S2*, 4*S3*, 1*S4* (d3, $P = 0.229$); 2*S2*, 4*S3*, 3*S4* (d5, $P = 0.108$); 2*S2*,3*S3*, 3*S4*, 1*S5* (d7, $P = 0.406$) (b) Trap: 1*S0*, 6*S1*, 4*S2*, 1*S3*, 1*S4* (d3, $P = 0.041^*$); 3*S1*, 3*S2*, 4*S3*, 3*S4* (d5, $P = 0.063$); 4*S1*, 2*S2*, 3*S3*, 4*S4* (d7, $P = 0.058$)	[25]
N-acetyl-cysteine (NAC)	10 mmol/L, reduces ROS levels	Rabbits with OD PVR-induced by injection of PRP and fibroblasts	Intravitreal inj. on days 0, 2, 4, and 7: control ($n = 10$) versus NAC ($n = 10$)	Control: 2*Stage0*, 8*S1* (d1); 1*S0*, 7*S1*, 2*S2* (d3); 8*S1*, 1*S2*, 1*S4* (d5); 5*S1*, 2*S2*, 2*S3*, 1*S4* (d7); *S1*, 3*S2*, 2*S3*, 1*S4*, 1*S5* (d14); 4*S1*, 2*S2*, 1*S3*, 1*S4*, 2*S5* (d21); 3*S1*, 3*S2*, 1*S4*, 3*S5* (d28); 2.6x ↑ phosphorylation of PDGFR in membranes of control compared to NAC NAC: 8*S0*,2*S1* (d1, $P = 0.0232^*$); 9*S0*,1*S1* (d3, $P = 0.0011^*$); 9*S0*,1*S1* (d5 $P = 0.0007^*$); 7*S0*,3*S1* (d7, $P = 0.0015^*$); 4*S0*,6*S1* (d14, $P < 0.0011^*$); 3*S0*,5*S1*,2*S2* (d21, $P < 0.032^*$); 3*S0*,4*S1*,3*S2* (d28, $P < 0.0185^*$); no progression to RD	[62]
Minimum neutralizing antibody set (MNAS)	Antibody set against PDGFs, TGFα, EGF, HGF, FGF-2, TGFβ, IL-8, and IGF-1	Rabbits with OD PVR-induced by injection of PRP and fibroblasts	Intravitreal inj. on day 0: control ($n = 12$) versus MNAS ($n = 12$)	Control: 4/12 (33%) stage 2 PVR, 8/12 (67%) stage 3 PVR or higher with RD MNAS: 3/12 (25%) no pathology, 5/12 (42%) epiretinal membrane, 4/12 (33%) stage 2 PVR, no RD	[63]

PDGF: platelet-derived growth factor. ARC128: nonfunctional version of ARC127. P7: postnatal day 7. P12: postnatal day 12. inj.: injection. PBS: phosphate-buffered saline. OD: right eye. OS: left eye. GSA+ cells: ectopic cells in the inner retina staining positive for Griffonia simplicifolia lectin. totRD: total, pRD: partial, RD: retinal detachment. *statistically significant difference compared to control. mAb: monoclonal antibody. PDGFR: platelet-derived growth factor receptor. PRP: platelet-rich plasma. #*S0*–#*S5*: number of cases observed at a given Fastenberg Stage of PVR 0–5. d: day. ROS: reactive oxygen species. TGFα: transforming growth factor α. EGF: epidermal growth factor. HGF: hepatocyte growth factor. FGF-2: fibroblast growth factor 2. TGFβ: transforming growth factor β. IL-8: interleukin 8. IGF-1: insulin-like growth factor-1. Ref: Reference number.

of the treated rabbits developed retinal detachment, with 3 (25%) having no pathology, 5 (42%) developing an epiretinal membrane, and 4 (33%) developing stage 2 PVR (Table 1) [63]. Furthermore, treated eyes did not develop vitreal or anterior chamber white cells, and the histology of one of these treated rabbits revealed no retinal damage compared to histology of the noninjected eye of the same rabbit [63].

10. 5-Fluorouracil (5-FU) and Low-Molecular-Weight Heparin (LMWH): Clinical Trials

5-Fluorouracil (5-FU) is an antimetabolite that inhibits DNA synthesis and fibroblast proliferation [64]. Low-molecular-weight heparin (LMWH) is an anticoagulant that binds fibronectin, bFGF, PDGF, and other growth factors [65]. Animal studies have found some efficacy of 5-FU for the treatment of vitreoretinal scarring [66, 67], but results in human clinical trials have been mixed (Table 2) [68–70]. In one prospective, randomized, double-masked, placebo-controlled trial, 174 high-risk patients were randomized to receive 5-FU and LMWH ($n = 87$) versus placebo ($n = 87$) after primary vitrectomy for rhegmatogenous retinal detachment [68]. The study reports a significantly ($P = 0.02$) lower incidence of postoperative PVR in the 5-FU and LMWH therapy group (11/87, 12.6%) compared to placebo (23/87, 26.4%). In the treatment group, 19.5% (17/87) of patients required more than one operation with 52.9% (9/17) due to PVR compared to 25.3% (22/87) of patients in the placebo group requiring reoperation with 72.7% (16/22) due to PVR. There was no significant difference in visual acuity (VA) outcomes in the two groups although patients with postoperative PVR had worse VA, nor were there significant differences in the complication rates of the two groups [68]. In another randomized, controlled trial of 5-FU and LMWH in patients with established anterior or posterior grade C PVR, patients were randomized to receive a perioperative infusion with or without 200 μg/mL of 5-FU and 5 IU/mL LMWH during vitreoretinal surgery and silicone oil exchange [69]. The trial looked at the primary outcome of posterior retinal reattachment after removal of silicone oil without any reoperations at 6 months and found no significant difference ($\chi^2 = 2.9$, $P = 0.59$) between the treatment group (56%, $n = 73$) and the placebo group (51%, $n = 84$) [69]. These trials were followed by a large, randomized, controlled trial of 5-FU and LMWH versus placebo in 615 patients presenting with unselected primary rhegmatogenous retinal detachment [70]. The main outcome measure was retinal reattachment after primary vitrectomy without any reoperations at 6 months with secondary outcome measures including occurrence and grade of PVR and best-corrected visual acuity. Retinal reattachment after primary vitrectomy was 82.3% in the combined 5-FU and LMWH group ($n = 327$) and 86.8% in the placebo group ($n = 288$; $P = 0.12$), with no statistically significant difference in development of PVR (7% in treatment group compared to 4.9% in placebo group; $P = 0.072$), nor was there a significant difference in the median final visual acuity of the two groups [70]. Evidence for adjuvant therapy with 5-FU and LMWH for the prevention of PVR is mixed; additional trials targeting prevention in patients with high risk for PVR may provide greater insight [71].

11. Daunorubicin in the Treatment of PVR: *In Vitro*, *In Vivo*, and Clinical Trials

Daunorubicin or daunomycin is an anthracycline that inhibits cell proliferation and migration [72, 73]. Early use of intraocular daunorubicin *in vitro* and *in vivo* in rabbits determined that the concentration that caused a 50% inhibition of colony-forming units was 700 nM; the half-life of daunomycin was determined to be 131 minutes in the vitreous, conveying that critical concentrations of the drug can be maintained for more than 4 hours after injection, with safe elimination across the retina [73]. Daunorubicin used in humans to reduce the failure rate of surgery for traumatic proliferative vitreoretinopathy due to postoperative cellular proliferation reported anatomic success in 14 out of 15 patients; daunorubicin was delivered at 7.5 μg/mL over a ten-minute period after vitrectomy and before silicone oil or gas injection, with no reported toxicity to the optic nerve, retina, lens, or cornea [74]. In a controlled clinical trial, 286 patients with advanced preoperative PVR were randomized into standardized surgery with adjunctive daunorubicin or surgery alone (Table 2) [75]. Outcomes included retinal attachment with no additional vitreoretinal surgery to 6 months postop, number of and time to additional surgery within 1 year of the first operation, and best-corrected visual acuity at 1 year postop. The trial demonstrated no significant difference ($P = 0.07$) in retinal attachment at 6 months post-op between the two groups with the daunorubicin group having 62.7% (89/142) attachment and 54.1% (73/135) in the control group. In secondary outcomes, there was a statistically significant difference in the need for another vitreoretinal operation within 1 year of the first surgery ($P = 0.005$), with the daunorubicin group requiring fewer such operations 34.5% (50/145) compared to the control group 46.1% (65/141); there was no difference in best-corrected visual acuity [75]. There are a limited number of trials studying the efficacy of daunorubicin in prevention of PVR, but it appears to be ineffective when used as a single agent.

12. Corticosteroids, 13-Cis-Retinoic Acid, Cyclin-Dependent Kinases, and Novel Compounds

Experiments in rabbits found that a single intravitreal injection of 1 mg of triamcinolone acetonide effectively inhibited fibroblast growth in a fibroblast autotransplantation model, reducing retinal detachment from 83.7% (36/43) to 34.1% (15/44) as well as the rate of retinal neovascularization from 72.1% (31/43) in controls to 18.2% (8/44) in treated rabbits [76]. In a prospective clinical trial, however, a much weaker response was seen; patients treated with systemic steroids had a 63.3% incidence of retinal fibrosis compared to 75.4% of patients given placebo following retinal detachment

Table 2: Outcomes of randomized, controlled, clinical trials in humans with and without proliferative vitreoretinopathy (PVR).

Agent(s)	Dose and target	Patients	Treatment groups	Outcomes	Ref.
5-FU and LMWH	200 μg/mL 5-FU and 5 IU/mL LMWH, 5-FU inhibits DNA synthesis and fibroblast proliferation; LMWH binds fibronectin, bFGF, PDGF, and other growth factors	174 patients at High risk for PVR	Intravitreal inf.: placebo versus 5-FU and LMWH	Placebo: 23/87 (26.4%) postoperative PVR, 22/87 (25.3%) reoperation with 16/22 (72.7%) due to PVR 5-FU and LMWH: 11/87 (12.6%, $P = 0.02^*$) postoperative PVR, 17/87 (19.5%) reoperation with 9/17 (52.9%) due to PVR No difference in visual acuity outcomes, nor complication rates	[68]
5-FU and LMWH	200 μg/mL 5-FU and 5 IU/mL LMWH, For target, see Row 1 above	157 patients with established grade C PVR	Intravitreal inf.: placebo versus 5-FU and LMWH	Placebo: 51% ($n = 84$) retinal reattachment (RRA) at 6 months with no reoperation 5-FU and LMWH: 56% ($n = 73$) RRA at 6 months with no reoperation ($\chi^2 = 2.9$, $P = 0.59$)	[69]
5-FU and LMWH	200 μg/mL 5-FU and 5 IU/mL LMWH, For target, see Row 1 above	615 patients with unselected primary RRD	Intravitreal inf.: placebo versus 5-FU and LMWH	Placebo: 86.8% RRA, 4.9% development of PVR, ($n = 288$) 5-FU and LMWH: 82.3% RRA ($P = 0.12$), 7% development of PVR ($P = 0.072$), ($n = 327$) No difference in final mean visual acuity	[70]
Dauno-rubicin	7.5 μg/mL, inhibits cell proliferation and migration	286 patients with advanced preoperative PVR after RRD	Surgery only versus surgery with intravitreal inf. of daunorubicin	Surgery only: 73/135 (54.1%) RRA with no reoperation at 6 months Surgery and daunorubicin: 89/142 (62.7%) RRA with no reoperation a 6-month post-op ($P = 0.07$) Daunorubicin: less reoperation 1st year postop (46.1% versus 34.5%, $P = 0.005^*$)	[75]
13-cis-retinoic acid (13cRA)	10 mg orally, 2x daily, for 8 weeks, inhibits proliferation of RPE cells	35 patients with primary retinal detachment and PVR	Surgery only versus surgery and oral 13cRA	Surgery only: 12/19 (63.2%) RRA at one-year follow-up Surgery and 13cRA: 15/16 (93.8%) RRA at one-year followup ($P = 0.047^*$)	[80]

5-FU: 5-fluorouracil. LMWH: low-molecular-weight heparin. bFGF: basic fibroblast growth factor. PDGF: platelet-derived growth factor. inf.: infusion. RRD: rhegmatogenous retinal detachment. post-op: postoperative. *statistically significant difference compared to control. RRA: retinal reattachment. RPE: retinal pigment epithelium. 13cRA: 13-cis-retinoic acid. Ref: Reference number.

surgery [77]. 13-Cis-retinoic-acid (13cRA) has been found to inhibit proliferation of RPE cells *in vitro* [78, 79]. A randomized, controlled, clinical trial of 35 patients with primary retinal detachment and PVR undergoing similar surgery, assigned 16 patients to receive 10 mg of oral 13cRA twice daily for eight weeks postoperatively and the other 19 patients to the control group; the primary outcome measure was retinal attachment at one-year followup (Table 2) [80]. At one-year followup, there was a statistically significant difference in retinal attachment ($P = 0.047$) between the two groups, with 93.8% (15/16) of eyes in the 13cRA group maintaining retinal attachment compared to 63.2% (12/19) of eyes in the control group [80]. Other agents, including the cyclin-dependent kinase inhibitor roscovitine and a novel anti-angiogenic compound IMS2186, have shown promise in animal models for inhibiting the proliferation of retinal pigment epithelial cells and fibroblasts, respectively [81, 82].

13. Conclusions

Basic science and clinical studies continue to provide growing insight into the pathophysiology of proliferative vitreoretinopathy. In posttraumatic PVR, macrophages can secrete growth factors (e.g., PDGF) and can transdifferentiate into fibroblast-like cells, thereby contributing to vitreoretinal membrane formation. In animal models, the injection of cells into the vitreous, whether they are macrophages, dermal tissue, fibroblasts, or RPE-J cells, results in pathology that mimics PVR. Tumor necrosis factor alpha, a pro-inflammatory cytokine, has been identified in close association with the membranes of patients with PVR, and genetic analysis has identified a single nucleotide polymorphism at the tumor necrosis factor locus that alters protein structure. Inflammatory processes in the vitreous are accentuated by the presence of growth factors, including PDGFs, HGF, bFGF, and EGF, to name a few. These growth factors, and especially the non-PDGFs, appear to activate PDGFRs on the surface of RPE cells, retinal glial cells, and fibroblasts, leading to cell survival, proliferation, organization into a membrane, and subsequent membrane contraction. Vitreal VEGF-A appears to competitively inhibit the binding of PDGFs to PDGFR-α. This promotes activation of PDGFR-α by non-PDGFs through an indirect pathway that results in persistent PDGFRα signaling—a pathway that leads to prolonged suppression of p53 and triggers the events leading to PVR. One key difference between animal models of PVR and the disease as it occurs in humans is that in the majority of animal models, PVR is induced by injection of cultured fibroblasts. Meanwhile, in humans, PVR may follow retinal detachment or primary repair of rhegmatogenous retinal detachment; the inflammatory process in humans is more likely to involve cells local to the retina and vitreous rather than cells introduced from outside the eye. While clinical trials have thus far offered mixed results in attempting to prevent the pathogenesis of proliferative vitreoretinopathy, experiments at the bench have provided novel strategies *in vitro* and in animal models and offer new avenues clinically for future attempts to prevent this sight-threatening disease. Clinical strategies to prevent PVR will probably require a multimodal, combinatorial approach, such as ROS inhibition and blocking the direct and indirect pathway of PDGFRα activation. Furthermore, pars plana vitrectomy will remain a critical component of the treatment in rhegmatogenous retinal detachment and PVR since residual vitreous is a risk factor of PVR. Finally, attention should be given to optimizing the correct dosing and administration of drugs, since some of the past failures may be due to the manner and time of administration rather than due to lack of true efficacy of the drugs tested.

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Review Article

Usefulness of the Vitreous Fluid Analysis in the Translational Research of Diabetic Retinopathy

Olga Simó-Servat,[1,2] Cristina Hernández,[1,2,3] and Rafael Simó[1,2,3]

[1] *Department of Endocrinology, Hospital Vall d'Hebron, Barcelona, Spain*
[2] *Diabetes and Metabolism Research Unit, Vall d'Hebron Institute of Research (VHIR), Autonomous University of Barcelona, Pg Vall d'Hebron 119-129, 08035 Barcelona, Spain*
[3] *CIBER de Diabetes y Enfermedades Metabólicas Asociadas, (CIBERDEM), Instituto de Salud Carlos III, 08035 Barcelona, Spain*

Correspondence should be addressed to Rafael Simó, rafael.simo@vhir.org

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Diabetic retinopathy (DR) is the major cause of acquired blindness in working-age adults. Current treatments for DR (laser photocoagulation, intravitreal corticosteroids, intravitreal antivascular endothelial growth factor (VEGF) agents, and vitreo-retinal surgery) are applicable only at advanced stages of the disease and are associated with significant adverse effects. Therefore, new pharmacological treatments for the early stages of the disease are needed. Vitreous fluid obtained from diabetic patients undergoing vitreoretinal surgery is currently used to explore the events that are taking place in the retina for clinical research. However, several confounding factors such as vitreous haemorrhage and concentration of vitreous proteins should be considered in the analysis of the results. In this paper we will focus on the vitreous fluid as a tool for exploring the mediators of DR and in particular the molecules related to inflammatory pathways. In addition, their role in the pathogenesis of DR will be discussed. The usefulness of new technologies such as flow cytometry and proteomics in identifying new candidates involved in the inflammatory process that occurs in DR will be overviewed. Finally, a more personalized treatment based on vitreous fluid analysis aiming to reduce the burden associated with DR is suggested.

1. Introduction

Diabetic retinopathy (DR) remains the leading cause of blindness and vision loss among adults aged under 40 years in the developed world. Population-based studies suggest that about one-third of the diabetic population have signs of DR and approximately one-tenth have vision-threatening stages of retinopathy such as diabetic macular edema (DME) and proliferative diabetic retinopathy (PDR) [1–3]. DR is associated with considerable costs related to laser coagulation therapy, vitrectomy in severe cases, and eventually costs for social support when useful vision has deteriorated completely [4]. In this regard, it has been reported that the consumption of health care resources is almost double in type 2 diabetic patients with microvascular complications than in patients without it [5]. Notably, average healthcare costs increase considerably with the severity of DR, which suggests that preventing the progression of DR may alleviate the economic burden related to this complication of diabetes [6].

Current treatments for DR (laser photocoagulation, intravitreal corticosteroids, intravitreal anti-VEGF agents, and vitreo-retinal surgery) are applicable only at advanced stages of the disease and are associated with significant adverse effects [7–9]. Therefore, new pharmacological treatments for the early stages of the disease are needed.

The research in DR has three main limiting factors. First, a suitable animal model to explore both PDR and DME is needed. Among the available animal models, rodents have been studied most extensively owing to their short generation time and the inherited hyperglycemia and/or obesity that affect certain strains. In particular, mice have proven useful for studying DR and evaluating novel therapies because of their amenability to genetic manipulation. Mouse

models suitable for replicating the early, nonproliferative stages of the retinopathy have been characterized, but no animal model has yet been found to demonstrate all of the vascular and neural complications that are associated with the advanced, proliferative stages of DR that occur in humans [10]. In addition, whereas most of clinical trials have been performed on patients with advanced DR, preclinical studies target prevention. Therefore, the success of a drug in preventing the development of experimental DR can hardly be transferred to the clinical practice. Second, the length of observation is another challenge. Although there is no fixed rule, the duration of the trial must be consistent with the natural history of DR and, in consequence, at least 5 years will be required to separate the behaviour of DR in the intervention and control groups. Finally, the direct access to the retina is not possible and for this reason vitreous fluid obtained from diabetic patients undergoing vitreoretinal surgery is currently used to indirectly explore the events that are taking place in the retina for clinical research.

In this paper we will focus on the vitreous fluid as a tool for exploring the mediators of DR and in particular the molecules related to inflammatory pathways.

2. Usefulness of Vitreous Fluid Analysis in Diabetic Retinopathy Research

Regional concentrations of growth factors in the retina may be more important than systemic levels in the pathogenesis of DR. In this regard, vitreous fluid obtained from diabetic patients undergoing vitreoretinal surgery is currently used to indirectly explore the synthesis by the retina of mediators involved in the development of DR. Nondiabetic patients in whom vitrectomy is also indicated by conditions in which retina is not directly affected by neovascularization such as macular holes or idiopathic epiretinal membranes could serve as control group. However, there are two main confounding factors that could lead to misinterpretation of the results.

First, vitreous haemorrhage, which often occurs in PDR, can produce a massive influx of serum proteins, thus precluding the usefulness of the vitreous fluid when studying the intraocular production of a particular protein. This problem can be solved by either rejecting the vitreous samples in which haemoglobin is >5 mg/mL (measured by spectrophotometry) or adjusting the results using the equation proposed by Ambati et al. [11]. Second, the disruption of the blood-retina barrier (BRB) that occurs in DR produces an increase in proteins in the vitreous body of diabetic patients. Indeed, we have repeatedly detected 3-4-fold higher level of intravitreal proteins in diabetic patients than Nondiabetic subjects. Therefore, an elevated intravitreal level of a particular protein does not necessarily increase in intraocular production and might simply reflect a nonspecific increase in protein levels due to serum diffusion. This problem can be solved by either correcting the intravitreal concentration of the peptide under study for total vitreal proteins or calculating the ratio of vitreous to plasma concentration. This simple methodology has enabled us to rationalize the use of vitreous fluid as a tool for assessing the intraocular production of angiogenic, antiangiogenic factors and proinflammatory cytokines [12, 13].

Vitreous fluid obtained from PDR patients underwent vitrectomy only allows us to explore the mediators of advanced stages of DR. By contrast vitreous samples of cadaveric eyes obtained from diabetic patients without history of DR or who were free of fundoscopic abnormalities according to ophthalmologic examinations performed during the previous 2 years could be useful for exploring early stages of DR. We have used this strategy to demonstrate that downregulation of somatostatin is an early event of DR and is associated with retinal neurodegeneration [14]. Alternatively, vitreous samples obtained from diabetic patients without DR or with NPDR in whom vitrectomy is performed by a coexistent macular hole are also very useful. In fact, this approach permitted us to identify interphotoreceptor retinoid-binding protein (IRBP) as a new candidate in the development of DR [15].

3. Vitreous Inflammation in Diabetic Retinopathy

Systemic inflammation is an intrinsic response to overfeeding, obesity, and diabetes, and diabetes increases the release of retinal inflammatory mediators and activation of microglial cells in early retinopathy [16].

A large body of evidence supports the role of proinflammatory cytokines, chemokines, and other inflammatory mediators in the pathogenesis of DR leading to persistent low-grade inflammation which contributes not only to the damage of the retinal vasculature but also to DME and PDR development [17, 18]. In fact, an emerging issue in DR research is the focus on the mechanistic link between activation of subclinical inflammation and angiogenesis [19].

3.1. Cytokines/Chemokines. Interleukin 1-β (IL-1β) is a pivotal inflammatory cytokine which is mainly produced by macrophage cells and it is able to activate NF-κB [20]. Levels of IL-1β are known to be increased in retinas from diabetic rats. Intravitreal injection of IL-1β or exposure of retinal endothelial cells to the cytokine in vitro was shown to be capable of causing degeneration of retinal capillary endothelial cells [21]. In addition, IL-1β together with high concentrations of glucose (25 mM) has been used to induce the disruption of retinal pigment epithelial cells (outer blood-retinal barrier), thus mimicking what occur in DME [22]. However, the clinical relevance of these findings is not clear because the levels of IL-1β used in these in vitro experiments were much higher than those reported in vivo.

The role of IL-1β in the pathogenesis of DR has recently been more directly studied using diabetic mice in whom the enzyme responsible for IL-1β production was inhibited or in whom the IL-1β receptor was deleted. IL-1β is the predominant product of caspase-1, and the biological activity of IL-1β is mediated by binding to the cell surface receptor, IL-1R1. Recent experimental evidence suggests that

activation of caspase-1 and the subsequent production of IL-1β play an important role in the development of diabetes-induced retinal pathology [23].

Although IL-1β is essential in the inflammatory process involved in DR, there are few studies in which it has been found higher in the vitreous fluid of diabetic patients in comparison with Nondiabetic subjects [24, 25]. This is because the short half-life of the molecule and the low sensitivity of the commercial kits currently available. However, it should be noted that, as occurs with other cytokines, much of the IL-β production occurs at tissue level, where this production exerts important paracrine effects.

There are other interleukins that have been involved in the development of DR. Both interleukin-6 (IL-6) and interleukin-8 (IL-8) have been found elevated in the vitreous of patients with PDR [19, 24–29]. The role of IL-6 and IL-8 in the pathogenesis of PDR is not completely understood. However, there are reports suggesting that cytokine IL-6 can increase endothelial cell permeability in vitro by rearranging actin filaments and by changing the shape of endothelial cells [29]. IL-8 has been recognized as a potent chemoattractant and activator of neutrophils an T lymphocytes [30], and it is also a potent angiogenic factor [31]. In addition, it should be noted the mean levels for IL-8 within the vitreous fluid have been found in the same range as that reported in pleural effusions of patients with pneumonia or tuberculosis and they correlated with PDR activity [27]. Furthermore, the increased vitreous levels IL-6 and IL-8 correlated with the progression of PDR in the outcome of vitreous surgery [29]. These findings underscore inflammation as crucial in the pathogenic events that lead to PDR.

The source of high levels of IL-6 and IL-8 detected within the vitreous fluid of diabetic patients with PDR remains controversial. Plasma diffusion favoured by the breakdown of the BRB is an unlikely candidate. This is because of the strikingly higher concentrations of both cytokines detected in the vitreous fluid in comparison with serum. In addition, a relationship between plasma and vitreous concentrations of IL6 and IL-8 does not exist [27]. Thus, a possibility is that cells in the vitreous could be the main cause accounting for the high levels of these cytokines. In fact, macrophages, monocytes, retinal pigment epithelial (RPE) cells, and glial cells are found in the vitreous of patients with PDR, and the majority of these cells are capable of producing cytokines in vitro [32].

On the other hand, it is known that during the inflammatory reaction, anti-inflammatory cytokines are also produced and tend to modulate the inflammatory process. However, little information is available regarding the potential role of anti-inflammatory cytokines in PDR. Interleukin-10 (IL-10) is an anti-inflammatory cytokine with potent deactivating properties on macrophages. In addition, antitumoral effects of IL-10 have been associated with its ability to prevent angiogenesis by downregulating vascular endothelial growth factor (VEGF) expression. Our group provided evidence that this anti-inflammatory cytokine is not increased in the vitreous fluid of diabetic patients with PDR or, in other words, the enhancement of the proinflammatory cytokines is not counter-balanced by an increase of IL-10 [27].

3.2. Monocyte Chemotactic Protein-1. Monocyte chemotactic protein-1 (MCP-1) is the most common chemokine and its expression is regulated through NF-κB. MCP-1 has been found elevated in the vitreous fluid of diabetic patients and their levels are higher than in serum [19, 25, 27–29, 33]. As occurs with IL-8, MCP-1 levels has been found in the same range as that reported in pleural effusions of patients with pneumonia or tuberculosis and they correlated with PDR activity [27]. Therefore, MCP-1 is a significant component of the diabetes-induced inflammation in the retina [34]. In fact, MCP-1 plays an important role in inducing leukocyte recruitment, and it is also a potent inducer of angiogenesis and fibrosis [35, 36]. Hyperglycemia has been shown to increase the MCP-1 generation from retinal vascular endothelial cells, RPE cells, and Muller's glial cells [28, 34]. Therefore, cells within the vitreous fluid could be the main cause accounting for the high levels of MCP-1 [32]. In addition, MCP-1 is expressed in myofibroblasts and in the vascular endothelial cells of epiretinal membranes from PDR patients [33]. Finally, the MCP-1 gene polymorphism has been indicated as a potential risk factor for DR, and the BRB disruption is prevented in a diabetic mice knockout for MCP-1 gene [37]. Further research is needed to establish the relevance of inhibitors of MCP-1 for preventing DR.

3.3. Interferon Gamma-Induced Protein 10 (IP-10). IP-10 is a CXC chemokine which has been found higher in the vitreous form diabetic patients than in Nondiabetic controls [33, 38, 39], and its levels have been reported even higher than those detected in serum samples [27]. The consequence of these findings is not easy to interpret because recent evidence demonstrates that members of the CXC chemokine family can act as either angiogenic or angiostatic factors, depending on the presence of the ELR (Glu-Leu-Arg) motif in their NH2 terminus [40]. Among this family, the chemokines IP-9/ITAC (CXCL11), MIG (CXCL9), CXCL4 (PF4), and IP-10 (CXCL10) lack the canonical N-terminal ELR sequence and bind in common to the ubiquitous CXCR3 chemokine receptor [41]. CXCR3 has two isoforms: CXCR3-A and CXCR3-B. Recent studies have shown that CXCR3 isoforms differentially regulate cell function. Activation of CXCR3-A has been shown to induce chemotaxis and proliferation in various cells types [42, 43]. Alternatively, CXCR3-B activation inhibits migration and proliferation and induces apoptosis [42, 44, 45]. There is emerging evidence showing that IP-10 mainly acts as an antiangiogenic factor via its signaling through CXCR3 [46, 47]. In addition, IP-10 inhibits angiogenesis in vivo at least in part by antagonizing the functions of IL-8 [48, 49]. Finally, an IP-10-derived peptide has been recently reported as a novel antiangiogenic agent [50]. For all these reasons, the elevated IP-10 levels detected in the vitreous fluid of diabetic patients could be contemplated as a mechanism to counteract the angiogenic effect of VEGF and other proinflammatory cytokines.

3.4. Stromal Cell-Derived Factor-1 (SDF-1). SDF-1 is the predominant chemokine which is upregulated in many damaged tissues as part of the response to injury and mobilizes

stem/progenitor cells to promote repair [51]. SDF-1 acts through its receptor CXCR4 at several key steps in the process of ischemic repair, such as recruitment of endothelial progenitor cells (EPCs) from the bone marrow. Moreover SDF-1 induces VEGF expression in cells that are both hematopoietic and endothelial in origin, thus increasing the angiogenesis [52, 52].

SDF-1 works in conjunction with VEGF to promote the recruitment of endothelial progenitor cells (EPCs) from remote locations, such the bone marrow to the ischemic retina [53]. Butler et al. [52] demonstrated that SDF-1 concentration increases in the vitreous of patients with either DME or PDR, and this increase was correlated with disease severity. Notably, the levels detected within the vitreous fluid were able to induce DR in a murine model. Furthermore, the same group of investigators found a dramatic decrease in the intravitreous levels of both SDF-1 and VEGF after intravitreal injection of triamcinolone [52]. Taken together, these data demonstrate that SDR-1 plays a major role in the development of DR and may be an ideal target for future therapies.

3.5. High-Mobility Group Box-1 Protein (HMGB1). HMGB1 is a nonhistone DNA-binding that stabilizes nucleosome formation and facilitates transcription. Necrotic cell death can result in passive leakage of HMGB1 from the cell as the protein is then no longer bound to DNA. In addition, HMGB1 can be actively secreted by different cell types, including activated monocytes and macrophages, mature dendritic cells, natural killer cells, and endothelial cells. Recently, El-Asrar et al. [54] reported that HMGB1 and its receptor for advanced glycation products (RAGE) were expressed by vascular endothelial cells and stromal cells in PDR fibrovascular epiretinal membranes, and that there were significant correlations between the level of vascularization in PDR epiretinal membranes and the expression of HMGB1 and RAGE. They also demonstrated elevated levels of HMGB1 in the vitreous fluid from patients with PDR.

Extracellular HMGB1 functions as a proinflammatory cytokine. When HMGB1 signals through RAGE, it leads to activation of NF-$\kappa\beta$, thus leading to the overexpression of proinflammatory molecules such as TNF-α, MCP-1, and ICAM-1 [54].

3.6. Tumor Necrosis Factor-α (TNF-α). TNF-α is primarily synthesized by macrophages and T cells and its expression is regulated by NF-kB [55]. TNF-α is a cytokine that has been associated with the pathogenesis of several chronic inflammatory diseases including type 2 diabetes [56]. In fact, diabetic patients have higher TNF-α levels in serum than Nondiabetic patients, and a strong correlation between plasma levels of TNF-α and severity of DR has been reported [57]. However, as occurs with other cytokines, intraocular production of TNF-α could be more important than systemic levels in the pathogenesis of DR. In this regard, it should be noted that not only increased levels of TNF-α have been found in the vitreous fluid of diabetic patients [24, 26, 56, 57] but also a higher vitreous/serum ratio [26]. In addition, TNF-α has been found expressed in vascular endothelial cells and stromal cells in epiretinal membranes from PDR patients [19]. When analyzing TNF-α it should be considered its short half-life (~4 minutes), which could lead to false negative results. By contrast, soluble TNF-α receptors (sTNF-α-Rs) are more stable proteins, remaining elevated for longer periods of time and, therefore, being better markers of the activation of TNF-α system than TNF-α itself.

TNF-α is known to cause significant retinal endothelial permeability by PKCζ-mediated downregulation of tight junction proteins and it is also required for VEGF-induced endothelial hyperpermeability, thus leading to the breakdown of the BRB which is the main pathogenic event of DME [58]. It also increases leukocyte adhesion and induces NADPH oxidase and production of reactive oxygen species (ROS) leading to retinal dysfunction of neurons and endothelial cells [39]. Finally, intravitreal injections of TNF-α into normal eyes lead to retinal ganglion cell death and optic nerve degeneration [59, 60].

For all these reasons, emerging strategies to block TNF-α actions in the diabetic eye seem warranted. Preliminary studies suggest a positive effect of intravenously administered TNF-α blockers [61, 62]. Unfortunately, much of the current data raises considerable safety concerns for intravitreal use of TNF-α inhibitors, in particular, intraocular inflammatory responses have been reported after intravitreal injection of infliximab. Results of dose-finding studies and humanized antibody or antibody fragments (e.g., adalimumab) are anticipated in the coming years; these will shed light on potential benefits and risks of local and systemic TNF-α blockers for treatment of DR.

3.7. Adhesion Molecules. There is growing evidence that leukostasis (the irreversible adhesion of leukocytes to the endothelium) plays a major role in capillary nonperfusion and retinal vascular leakage in DR [18, 63, 64]. In fact, intravitreal injection of corticosteroid attenuates the breakdown of the BRB by inhibiting leukostasis [65]. Moreover, leukocytes adhered to capillary endothelial cells induce apoptotic changes to endothelial cells [63, 64]. There is emerging evidence indicating that one of the most relevant mechanism by which leukocytes lead to the apoptosis of endothelial cells and the breakdown of the BRB is through the endothelial death via Fas-Fas ligand (FasL) [66]. In fact, suppression of Fas-FasL-induced endothelial cell apoptosis prevents diabetic BRB breakdown in a model of streptozotocin-induced diabetes. These data imply that the targeting of the Fas-FasL pathway may prove beneficial in the treatment of DR.

Many of the cytokines detailed above lead to chemoattraction of inflammatory cells and consequently participates in leukostasis. Diabetic retinal vascular leakage, capillary nonperfusion, and endothelial cell damage are associated with leukocyte recruitment and adhesion to the retinal vasculature which correlates with increased expression of leukocyte adhesion molecules.

The intercellular adhesion molecule ICAM-1 is the most important adhesion molecule in DR. The levels of ICAM-1 in the vitreous of patients with PDR are increased and

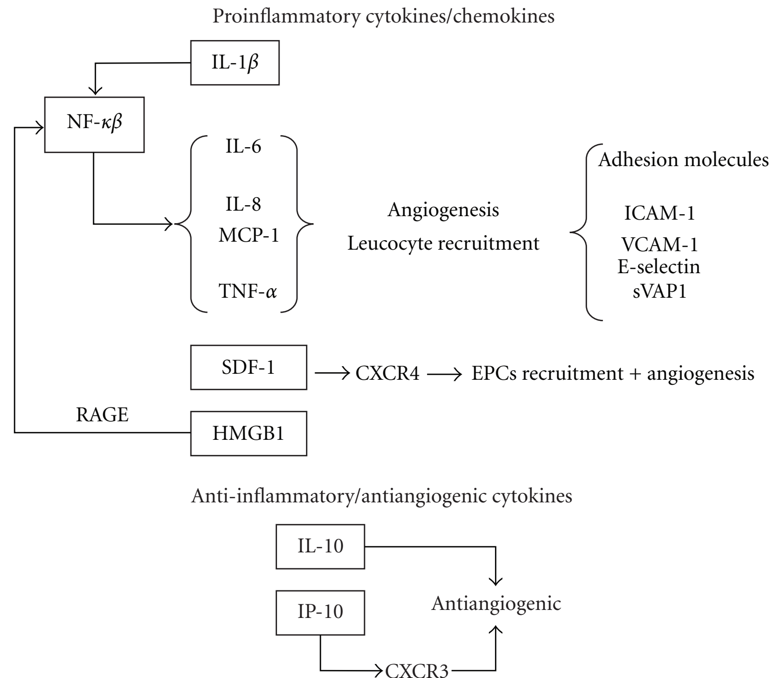

Figure 1: Main proinflammatory cytokines/chemokines increased in the vitreous fluid of diabetic patients (see text for details). Most of them participate also in the angiogenic process, which is essential for developing PDR. Anti-inflammatory/antiangiogenic cytokines also exist in the vitreous fluid of diabetic patients, but their concentration is not sufficient to counterbalance the inflammatory/angiogenic effect of proinflammatory cytokines.

the levels are higher in active PDR than inactive PDR [54]. Indeed, ICAM-1 is found to be highly expressed in the blood vessels of the retina, choroid, and fibrovascular membrane in patients with diabetes, and its expression correlates with the number of migrated neutrophils in the retina and choroid of these patients, thus indicating that elevated ICAM-1 facilitates leukocyte recruitment [67]. Furthermore, not only ICAM-1 levels are higher in diabetic patients, but also its ligands CD11a/CD18 and CD11b/CD18, specifically β-integrin and α-integrin [68]. Accordingly, the blockade of ICAM-1 or CD18 expression attenuates leukostasis, endothelial cell death, and vascular leakage in the retinal vessels of diabetic animals [69]. However, further investigation is still required to elucidate the role of integrin-ICAM-1 interaction in DR and the potential therapeutical benefits of its inhibition.

Vascular cell adhesion molecule 1 (VCAM-1) and E-selectin are also involved in the pathogenesis of DR and their soluble forms has been found increased in the vitreous of PDR patients [70–72]. Both VCAM-1 and E-selectin can act on endothelial cells as angiogenic factors and a direct correlation between VCAM-1 and VEGF levels has been reported [71]. These findings suggest that therapeutic approaches aimed to block these soluble adhesion molecules could have beneficial effects on DR.

Recently, the soluble vascular adhesion protein 1 (sVAP-1) has been found increased in the vitreous fluid and serum of patients with PDR [73]. It has been demonstrated that the retinal capillary endothelial cells produce the membrane-bound form of VAP-1 and release sVAP-1 when stimulated with high glucose or inflammatory cytokines such as TNF-α and IL-1β. The sVAP-1 seems to be involved in the pathogenesis of DR for two reasons. First, local expression of VAP-1 is involved in leukostasis and leukocyte entrapment [74]. Second, sVAP-1 has also an enzymatic function as a semicarbazide-sensitive amine oxidase which lead to the production of metabolites involved in cellular oxidative stress and advanced glycation end-product formation [73], two crucial events in the pathogenesis of DR.

In summary, an increase of several proinflammatory cytokines, chemokines, and adhesion molecules exists within the vitreous of diabetic patients which is not sufficiently counter-balanced by anti-inflammatory cytokines. This low-grade inflammation favours the angiogenic process. The main relationships among cytokines above mentioned are summarized in Figure 1.

4. New Research Approaches

4.1. Flow Cytometry. In Nondiabetic patients, the BRB has been shown to be impermeable to leucocytes. However, in the diabetic eye the migration of leucocytes into the vitreous body is favored due to leukostasis. One of the mechanisms involved is the alteration of adherent and tight junction proteins in the endothelial cells (i.e., proteolytic degradation of VE-cadherin) [75].

One of the major problems in any technique for studying the cells within the vitreous fluid is to obtain an adequate number for analysis. Many cells in the vitreous fluid are already nonviable, and the remainder can disintegrate very

quickly after collection of the sample. Flow cytometry is a laser-based method of immunocytochemistry which permits a rapid and precise cell counting and sorting. Other benefits include easier cell preparation and multiparameter analyses of specimens. Earlier shortcomings including blood contamination, errors introduced by nonviable cells, difficulty in identifying monoclonality, and slow, single-cell suspension analysis, have been overcome. The main limiting factor is that the samples should be processed immediately. However, this allows us to simulate the in vivo scenario as close as possible.

By using this method, we found T lymphocytes in most of vitreous samples from PDR patients, whereas T lymphocytes were not present in the vitreous from Nondiabetic subjects [76]. This finding supports the concept that the disruption of the BRB is crucial for permitting the access of inflammatory cells into the vitreous body of diabetic patients. In addition, T cells infiltrating the vitreous shown a different pattern than in the peripheral blood (high percentage of CD4+ CD28−). Furthermore, those patients in whom T cells were detectable showed quiescent DR and their outcome was better than in those patients in whom intravitreous T cells were undetectable [76]. Therefore, it seems that T cells infiltrating the vitreous cavity have a protective role in the outcome of PDR. In this regard, it should be emphasized that the neuroprotective effect of autoimmune cells has been reported [77, 78]. In addition to anti-inflammatory cytokines like IL-10 or transforming growth factor, neurotrophic factors could be potential candidates to explain the protective effect of T cells on PDR outcome [79, 80].

Lipopolysaccharide-binding protein and soluble CD14 (sCD14) have been also found elevated in the vitreous fluid of patients with PDR and thus may play a role in the innate immune response triggered by the inflammatory injury characteristic of PDR [81].

The different pattern of T cells identified in the vitreous fluid of diabetic patients with PDR requires further functional characterization. In addition, further studies addressed to unraveling the intraocular innate immune defences that operate in PDR are needed. This research should provide a better understanding of the events involved in the development of immune response in DR and would help us in searching for more effective treatment for this disease.

4.2. Proteomics. The volume of vitreous fluid obtained after vitrectomy is approximately 1 mL and, therefore, only a few peptides can be analysed simultaneously. The recent development of proteome analysis has made it feasible to analyse protein profiles with only a small sample.

In recent years, several proteome analyses in human vitreous fluid have been reported in the setting of DR, thus permitting us to identify new potential candidates in its pathogenesis [82–89]. Regarding mediators of inflammation, it is worthy of mentioning that several factors of the complement system have been found increased in the vitreous fluid from PDR patients in comparison with control subjects [86, 88]. Activation of the complement cascade can both compound and initiate thrombosis, leukostasis, and apoptosis, all processes involved in vascular lesions of DR. Therefore, since several ways of specifically manipulating the complement system already exist, they could represent a possible therapeutic approach. Apart from complement factors, inflammation-associate proteins such as AAT, APOA4, ALB, and TF have been found significantly elevated in the vitreous of PDR patients [89].

Most of proteomic studies have been focused on PDR whereas there are only few studies performed on samples from patients with DME [90–92]. One of the most important findings of proteomic studies on DME has been reported by Gao et al. [91] demonstrating the essential role of both extracellular carbonic anhydrase-I and the kallikrein-mediated innate inflammation in the pathogenesis of DME. In addition, we have shown four proteins differently expressed in the vitreous fluid of patients with DME in comparison with PDR and Nondiabetic subjects: hemopexin (increased); clusterin, transthyretin, and beta crystalline S (decreased) [92]. Perhaps the most interesting finding is the increase of hemopexin (Figure 2). Hemopexin is an acute phase reactant which is believed to act as a protective molecule against heme-mediated oxidative injury as well as nitric oxide-mediated toxicity. Plasma hemopexin is mainly synthesized by hepatocytes, but it is also expressed by most of the cells of neural retina including the photoreceptors and, notably, the ganglion cells [93]. Apart from the elevated levels in the vitreous fluid on diabetic patients with DME, we have recently shown that hemopexin leads to the disruption of RPE cells, thus increasing permeability, and this effect is blocked by specific antihemopexin antibodies (unpublished results). Therefore, hemopexin could be a relevant factor in the pathogenesis of DME. T-cell-associated cytokines, like TNF-α, are able to enhance hemopexin production in mesangial cells in vitro, and this effect is prevented by corticosteroids [94]. Taken together, these findings suggest that hemopexin might be a mediator of the disruption of the BRB induced by proinflammatory cytokines, but further research on this issue is needed.

5. Concluding Remarks and Future Research

Vitreous fluid is a useful tool for analyzing the pathophysiological events that are taking place in the retina of diabetic patients. However, several confounding factors such as vitreous haemorrhage and concentration of total vitreous proteins should be considered before validating the results. In addition, subjects who had undergone laser photocoagulation in the preceding 3–6 months should be excluded because a significant alteration in the balance of intravitreal growth factors and transcriptional activity in the retina has been shown following this procedure [95]. With all these caveats in mind, the analysis of key molecules involved in the pathogenesis of DR by using the vitreous fluid remains the most direct manner to explore the "in vivo" candidates involved in the development of DR. In fact, vitreous fluid analysis has been very useful in the translational research of DR. For instance, the seminal paper by Aiello et al. [96] in which was clearly demonstrated that VEGF was elevated in

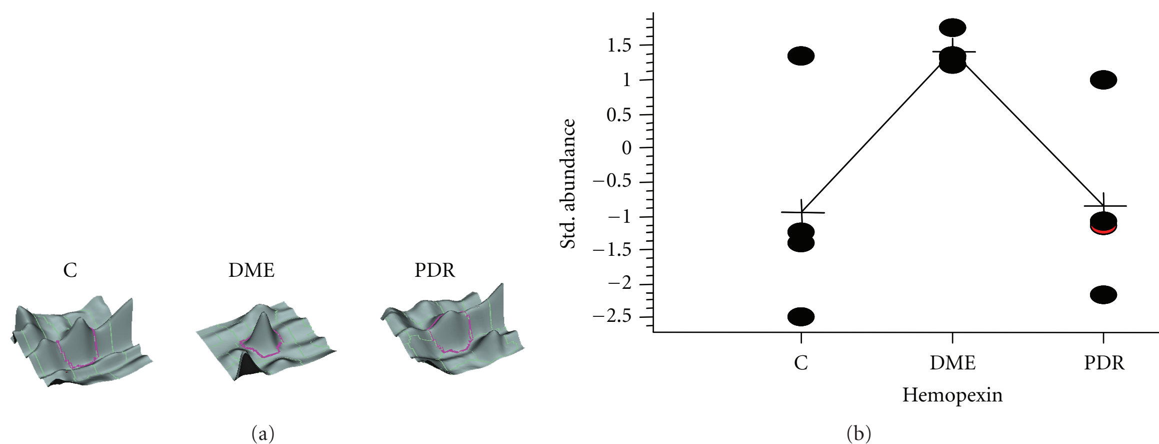

Figure 2: Results obtained by using the fluorescence-based difference gel electrophoresis (DIGE) strategy showing the higher abundance of hemopexin in the vitreous fluid of diabetic patients with DME in comparison with Nondiabetic controls and PDR patients. (a) Three-dimensional images of the hemopexin spot corresponding to the image of a control (C), DME, and PDR samples. (b) Standardised abundance plot for hemopexin displaying the log of abundance observed for the spot in each of the four gel images corresponding to control (C), DME, and PDR samples. The line links the average abundance values for each group of samples (crosses). Student's t test results in a significant increase ($P < 0.05$) in DME sample in comparison with either C or PDR sample.

the vitreous fluid of PDR and it was able to stimulate retinal endothelial cells in vitro, as did vitreous fluid containing measurable VEGF, was essential for proposing anti-VEGF therapy by intravitreal injections in advanced stages of DME or PDR. Another more recent example is the low intravitreous levels of somatostatin detected not only in advanced but also in early stages of DR [14, 97–99]. These findings together with mechanistic experiments supporting the antiangiogenic and neuroprotective role of somatostatin have led to propose somatostatin as a replacement treatment for DR [100]. In this regard, a multicentric, phase II-III, randomized controlled clinical trial (EUROCONDOR-278040) to assess the efficacy of SST administered topically to prevent or arrest DR has been approved by the European Commission in the setting of the FP7-HEALTH.2011. This trial will start in September 2012 and the results should be available in 2015.

Proinflammatory cytokines (i.e., IL-1β, IL-6, IL-8, TNF-α, and IP-10), chemokines (i.e., MCP-1, IL-8, IP-10, and SDF-1) and adhesion molecules (i.e., VCAM, ICAM, and VAP-1) have been found elevated in the vitreous fluid of diabetic patients, and the causal relationship between inflammation and angiogenesis is now widely accepted. Therapeutic strategies addressed to blocking their deleterious activity have been successfully reported in experimental models. However, the current treatment of both PDR and DME by intravitreous injections of anti-VEGF drugs or corticosteroids is not based in an individualized analysis. This is a serious limiting factor because the participation of either angiogenic factors (i.e., VEGF) or proinflammatory cytokines is highly variable in both PDR and DME. Therefore, a more personalized treatment based in the results of vitreous fluid analysis could be proposed.

New technologies such as flow cytometry and proteomics of the vitreous fluid have permitted us to gain new insights into the pathogenesis of both PDR and DME. Multiplex bead immunoassay, a type of assay that simultaneously measures multiple analytes in a single run/cycle of the assay, is also a useful tool in exploring the mediators of DR because it permits us to make the vitreous samples more profitable. Metabolomics has also allowed the obtainment a metabolic signature of PDR [101] and has the advantage of being applicable "in vivo" in the eye. With all these tools a more targeted treatment could be envisaged in the near future, thus reducing the burden associated with this devastating complication of diabetes.

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Review Article

Therapeutic Interventions against Inflammatory and Angiogenic Mediators in Proliferative Diabetic Retinopathy

Daniel Gologorsky, Aristomenis Thanos, and Demetrios Vavvas

Retina Service, Department of Ophthalmology, Massachusetts Eye and Ear Infirmary, Harvard Medical School, 243 Charles Street, Boston, MA 02131, USA

Correspondence should be addressed to Demetrios Vavvas, demetrios_vavvas@meei.harvard.edu

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The global prevalence of diabetes is estimated to be 336 million people, with diabetic complications contributing to significant worldwide morbidity and mortality. Diabetic retinopathy results from cumulative microvascular damage to the retina and inflammation is recognized as a critical driver of this disease process. This paper outlines the pathophysiology leading to proliferative diabetic retinopathy and highlights many of the inflammatory, angiogenic, and cytokine mediators implicated in the development and progression of this disease. We focus a detailed discussion on the current targeted therapeutic interventions used to treat diabetic retinopathy.

1. Introduction

The global prevalence of diabetes is estimated to be 336 million people, and this number is projected to nearly double by 2030 [1, 2]. In addition to the primary disease itself, diabetic complications are expected to have profound implications for the future of patient management. Diabetes is a disease of hyperglycemia, and diabetic retinopathy (DR) results from cumulative microvascular damage to the retina. According to the World Health Organization, DR accounts for approximately 5% of global blindness [3]. Inflammation is a critical driver of the pathophysiology of DR [4]. This paper highlights many of the inflammatory, angiogenic, and cytokine mediators implicated in the development and progression of DR and features specific and targeted therapeutic modalities to combat retinopathy.

2. Pathways to Damage

Two major studies, the Diabetes Control and Complications Trial (DCCT) of 1993 and the United Kingdom Prospective Diabetes Study (UKPDS) of 1998, have demonstrated that hyperglycemia is the causative etiology for DR [5, 6]. Hyperglycemia causes microvascular changes, that in turn results in retinopathy. At least four distinct biochemical pathways have been suggested for the mechanism leading to retinopathy. These include increased polyol pathway flux, increased advanced glycation end product (AGE) formation, activation of protein kinase C (PKC) isoforms, and increased hexosamine pathway flux. Taken together, these pathways result in oxidative stresses and inflammation that attenuate vascular wall integrity and result in increased vascular permeability, occlusion, and ischemia [7, 8]. These types of microvascular insults manifest in increased vascular leakage, as in nonproliferative retinopathy (NPDR), and retinal neovascularization secondary to ischemia, as in proliferative retinopathy (PDR) [9, 10].

There is increasing evidence that inflammation has a central role in the pathophysiology of diabetic retinopathy [4, 11]. Indeed, as early as 1964, it was noted that patients suffering from rheumatoid arthritis demonstrated less severe PDR if taking high dose aspirin [10]. In his review of the literature, Adamis similarly concludes that diabetic retinopathy is an inflammatory disease [4, 12]. He describes the orderly chronological progression of the disease process, briefly described here. Within a single week of experimental diabetes, prior to any clinical sign of diabetic retinopathy,

infiltrating leukocytes adhere to retinal vasculature. Over time, a subset of these leukocytes accumulate and transmigrate to the retina. Using their β_2 integrins, VLA-4, and CD18 surface molecules, leukocytes latch onto the local vasculature via leucocytes adhesion molecules present on the endothelium, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), PECAM-1, and P-selectin [11–14]. In fact, early DR is marked by a disorderly upregulation of these adhesion molecules, precisely when leukocyte numbers begin to increase [15]. Once leukocytes attach to the vascular epithelium, inflammatory cytokines, growth cytokines, and vascular permeability factors are released, altering endothelial junctional proteins and allowing for leukocytic diapedesis into the retina, with concurrent compromise to the blood-retinal barrier (BRB) [4] (Figure 1).

3. Mediators of Damage

The upregulation of numerous factors, both angiogenic and inflammatory, has been implicated in the pathogenesis of microvascular retinopathy. Again, the expression of vascular adhesion molecules such as ICAM-1, VCAM-1, and various selectin molecules is required for leukocytic recruitment to inflammatory sites [16]. Vascular endothelial growth factor (VEGF) is an angiogenic compound that under hypoxic or ischemic conditions (as in proliferative DR) encourages aberrant vasculature. Inflammatory factors including the interleukins, tumor necrosis factor (TNF), insulin-like growth factor (IGF), angiopoietins (Ang-2), among many others have all been studied and implicated in the pathophysiological pathways leading to clinical PDR [4, 8]. These angiogenic, adhesion, and inflammatory molecules have been the focus of targeted therapies to treat DR.

4. VEGF

VEGF is a member of a large family of angiogenic growth factors, a group consisting of six known members: VEGF-A (referred to as simply VEGF), placental growth factor, VEGF-B, VEGF-C, VEGF-D, and VEGF-E. VEGF-A is the first and major form involved in angiogenesis. It increases the rate of mitosis and migration of endothelial cells and is involved in integrin $\alpha v\beta 3$ regulation as well as creation of blood vessel lumen and fenestrations. In addition, it is chemotactic for macrophages and granulocytes and leads indirectly, via NO release, to vasodilation. VEGF-B is involved in embryonic angiogenesis, specifically in myocardial tissue. VEGF-C is a major prolymphangiogenesis factor, and VEGF-D is needed for the development of bronchiolar lymphatic vasculature. VEGF-E is found in viruses. PlGF is important in vasculogenesis, but plays a role in ischemia induced angiogenesis as well as inflammation and wound healing.

Though initially recognized as a vascular permeability factor, VEGF was subsequently recognized for its angiogenic properties and as a specific mitogen for vascular endothelial cells. In the context of PDR, these two findings suggest that VEGF could account for both the proliferation and vasopermeability witnessed in the disease progression. In addition to its involvement in DR, significant evidence implicates VEGF in the pathogenesis of diabetic retinopathy, retinopathy of prematurity, age-related macular degeneration, and corneal neovascularization. A simplified mechanism follows. Pathologic angiogenesis relies on the aberrant activation of proteases and various degratory enzymes emanating from the endothelium that allow for endothelial cells to leave the parental vasculature and proliferate in the matrix. Increased levels of ocular VEGF in PDR only reinforces the role of neovascularization in the course of this disease. Recent successes with anti-VEGF therapy for age-related macular degeneration in the MARINA and ANCHOR studies have prompted significant efforts to translate the application of anti-VEGF drugs to DR [17, 18].

Some forms of VEGF are more deleterious than others. Two major VEGF isoform splice variants, $VEGF_{120(121)}$ and $VEGF_{164(165)}$, were compared in the transparent and avascular adult mouse cornea. $VEGF_{164(165)}$ was found to be significantly more potent at inducing corneal inflammation, stimulating ICAM-1 expression on endothelial cells, and inducing monocytic chemotaxis than $VEGF_{120(121)}$. Of the two major VEGF isoforms, $VEGF_{164(165)}$ was demonstrated to be more effective in inducing inflammation, neovascularization, and angiogenesis in the cornea [19].

As early as 1994, Aiello and Cavallerano demonstrated that individuals with PDR have elevated levels of vitreal VEGF, and that laser photocoagulation therapy significantly reduces these levels [9]. Shortly thereafter, Robinson et al. showed that blocking VEGF obviated the development of proliferative retinopathy in murine models [20]. Moreover, demonstrating the opposite effect, Tolentino et al. administered intravitreal VEGF injections and reported the ability to induce iris neovascularization and retinopathy in nonhuman primates [21].

These promising bench studies prompted efforts for a clinical intervention that would target VEGF for the treatment of PDR. Knowing that the pathophysiology of DR can be explained in the context of a leukocytic invasion with a concurrent inflammatory disorder, Lu et al. found that VEGF increases retinal vascular expression of ICAM-1 *in vivo*, and subsequent studies demonstrated that VEGF provides important chemotaxis for monocytes [22]. Joussen et al. similarly showed that retinal VEGF induces ICAM-1 expression and initiates early diabetic retinal leukocyte adhesion *in vivo*, and that blocking VEGF decreases retinal leukocyte counts in experimental diabetes [23].

VEGF has been the target of numerous drugs and clinical trials for the treatment of diabetic macular edema (DME) and PDR. VEGF inhibitors include the antibody bevacizumab (Avastin, Genentech Inc., San Francisco, USA), the monoclonal antibody fragment Ranibizumab (Lucentis, Genentech Inc., San Francisco, USA), an aptamer pegaptanib (Macugen, OSI Pharmaceuticals), the soluble VEGF receptor analogs, VEGF-Trap (Regeneron Pharmaceuticals, Tarrytown, NY, USA), small interfering RNAs (siRNAs) bevasiranib (Opko Health Inc., Miami, FL, USA), and rapamycin (Sirolimus, MacuSight Inc., Union City, CA, USA) [8]. The

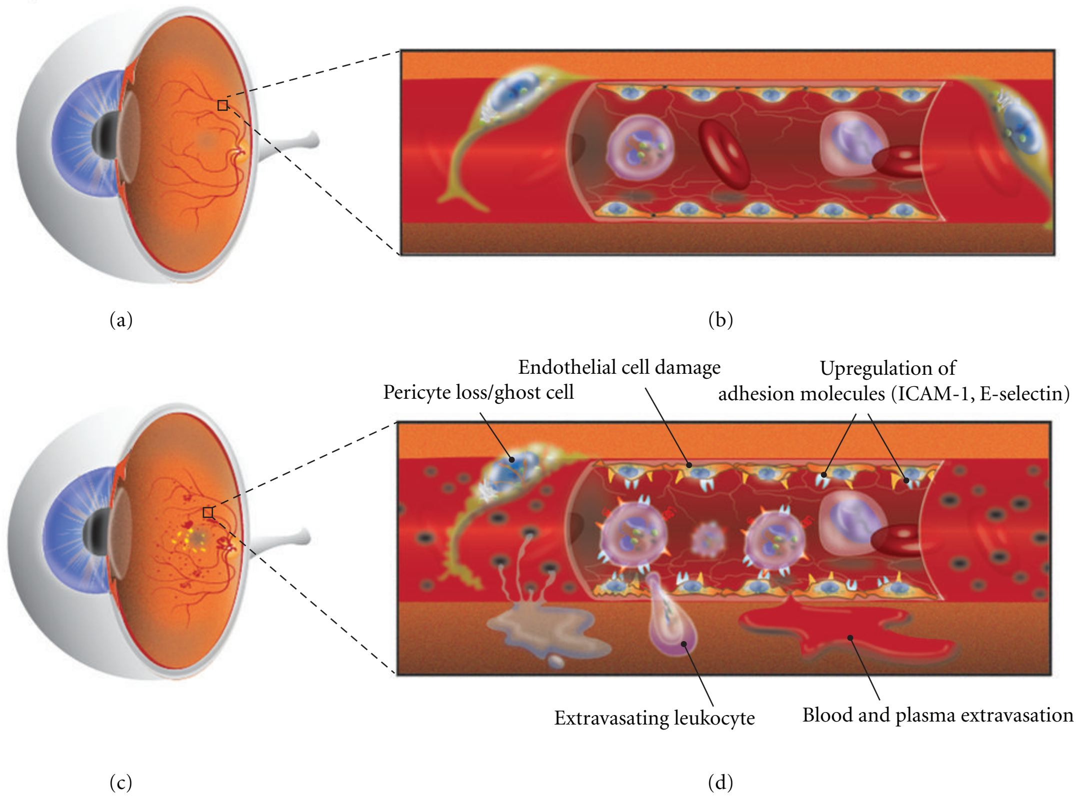

Figure 1: Normal eye (a) with intact vasculature (b). Accumulation of microvascular diabetic changes in the eye (c) manifest in adverse cellular changes with ultimate compromise to the blood-retinal-barrier (d).

application of anti-VEGF medications for PDR remains off-label, as the safety and efficacy of these drugs have not been definitively established [24].

Though anti-VEGF drugs have been studied extensively for DME; no large prospective randomized studies have been published to date for the application of these agents for PDR. A retrospective study evaluating eyes with PDR treated with intravitreal bevacizumab demonstrated that complete resolution of neovascularization of the disc (NVD) was noted in 73% of the treated eyes on fluorescein angiography (FA) [25]. In 2008, Mirshahi et al. showed that 87.5% of eyes injected with bevacizumab demonstrated complete neovascularization regression at within six weeks, though this effect was temporary, as by four months the benefits of bevacizumab were strongly attenuated [26]. In 2011, Schmidinger et al. found that a 3-monthly bevacizumab retreatment regiment may be a valid method to control persistent neovascularization in PDR patients after complete panretinal photocoagulation (PRP) [27]. Other studies confirm that intravitreal bevacizumab decreases leakage from diabetic neovascular lesions and may prove to be of utility as an adjunct when it comes to vitreous hemorrhage, post-PRP macular edema, neovascularization of the iris, pars plana vitrectomy for tractional retinal detachment, non-clearing vitreous hemorrhage, and as a prevention against exacerbation of DME after cataract surgery [24, 28]. Though anti-VEGF drugs seem promising, the lack of randomized, prospective trials, standard dosing schedules, and administration protocols limits their current role to adjunctive therapies for PDR [24].

Though promising, VEGF therapy is not without risks. While numerous studies have posited the neuroprotective role of VEGF, there is a possible neurodegenerative risk with prolonged pan-VEGF blockade. Indeed, it has been shown that VEGF demonstrates neuroprotection, neurogenesis, and angiogenesis in the ischemic brain: VEGF promotes the formation of new cerebral blood vessels in response to cerebral ischemia, reduces cerebral infarct volume and edema, reduces neurologic deficits and improves neurologic recovery outcomes, and influences cerebral neurogenesis in the adult brain [29–32]. Moreover, data suggest that VEGF is endowed with anticonvulsant properties and that VEGF protects against hippocampus neuronal loss after status epilepticus [33, 34]. Though further investigation is indicated, it has been suggested that chronic pan-VEGF blockade can have deleterious effects leading to retinal neurodegeneration and choriocapillary circulatory disturbances. Indeed, VEGF inhibition or blockade may exacerbate ischemic injury and neural damage [35]. Nishijima et al. demonstrated the important role of VEGF for retinal neural survival in ischemic-reperfusion injury [36]. The same authors also noted that chronic inhibition of VEGF in normal adult animals led to a

significant loss of retinal ganglion cells. These considerations must be taken into account when treating patients with VEGF for age-related macular degeneration or PDR.

5. ICAM

Leukocyte adhesion to the vasculature is an important initial step in the progression of endothelial cell injury and diabetic retinopathy. This initial insult is mediated through ICAM-1 and the leukocyte integrin CD18. ICAM-1 is directly involved in immune activation and inflammation through its interaction with different cytokines, including IL-1, TNF-α, and IFN-γ [37, 38]. In 1995, McLeod et al. noted enhanced expression of ICAM-1 and P-selectin in the diabetic human retina and choroid. The authors demonstrated an increase in leucocyte density in human eyes with DR, as well as an increase in retinal vascular ICAM-1 immunoreactivity [39]. Similarly, Esser et al. demonstrated higher levels of soluble ICAM-1 in PDR and in traumatic PVR, showing concentrations that were significantly elevated above total vitreal protein levels [40].

Adamiec-Mroczek and Oficjalska-Młyńczak explored variations of vitreous ICAM-1, VCAM-1, IL-6, and TNF-α concentrations in the development of PDR [41]. The authors found that both vitreous and serum soluble adhesion molecules (ICAM-1, VCAM-1) and proinflammatory cytokine (IL-6, TNF-α) levels were significantly higher in patients with PDR than in controls. Further, they found that these increases in adhesion molecule levels correlated with high vitreous concentrations of IL-6 and TNF-α in patients with PDR, providing more evidence of the inflammatory nature of PDR. A positive correlation between vitreous soluble VCAM-1 and serum HbA1c concentrations bolstered the connection between hyperglycemia and adhesion molecule proliferation.

While it had been previously established that increased serum levels of soluble ICAM-1, VCAM-1, and E-selectin may be found in patients with chronic inflammatory or ocular diseases, Limb et al. found that vitreous levels of ICAM-1, VCAM-1, and E-selectin were similarly significantly higher in eyes with PDR than in control cadaveric vitreous. Again, the connection between inflammation, ICAM expression, and DR is reaffirmed [42].

Barile et al. similarly measured vitreous levels of soluble ICAM-1 and VCAM-1 in the eyes of patients with retinal detachment (RD) due to proliferative diabetic retinopathy (PDR) or proliferative vitreoretinopathy (PVR). The authors found that soluble ICAM-1 and VCAM-1 are significantly increased in the vitreous cavity of patients with RD due to PDR or PVR when compared to control vitreous [43].

Research on ICAM-1 has highlighted its potential as a target of therapeutic intervention for the treatment of PDR. Joussen et al. treated animals with 50 mg/kg of aspirin, meloxicam (a cyclo-oxygenase-2 inhibitor), or etanercept (a soluble TNF-α receptor) [44]. The authors found that all three agents were found to reduce retinal ICAM-1 expression. Aspirin was further found to reduce the expression of CD11a, CD11b, and CD18. Each of the three agents reduced leukocyte adhesion and hindered BRB breakdown. Aspirin and meloxicam both lowered retinal TNF-α levels. None of the above three agents had any effect on VEGF levels.

Recently, Hirano et al. described a novel therapeutic option for the treatment of DR by targeting ICAM-1. Hypothesizing that control over ICAM-1 expression should prevent the earliest stages of retinopathy, the authors applied small-interfering RNA (siRNAs) through a hydrodynamics-based transfection technique (HT) and intravitreal injection (IV) to a murine retina *in vivo*. Efficient modulators of gene expression, siRNAs bind to specific mammalian RNA targets and suppress target gene expression posttranscriptionally. The authors concluded that siRNA causes specific downregulation of ICAM-1 expression, suggesting a mechanism to inhibiting leukocyte infiltration and adhesion in early stage PDR [45].

Researchers have identified other promising targets related to adhesion molecules. Fasudil, a selective ROCK inhibitor, is one prime example. The Rho/ROCK pathway promotes leukocyte adhesion to the microvasculature by increasing ICAM-1 expression and affecting the function of various adhesion molecules. Intravitreal fasudil was found to reduce ICAM-1 expression, leukocyte adhesion, and endothelial apoptosis in the retinas of diabetic rats [46]. Another example is periostin, a matricellular protein with roles in cell adhesion and migration. Periostin has been associated with the formation of preretinal fibrovascular membranes, structures that form in advanced PDR that causes blindness through intravitreal hemorrhage and tractional retinal detachment. One study has suggested that targeting periostin may be a potential therapy for inhibiting fibrovascular membranes associated with PDR [47].

6. Inflammatory Mediators

Numerous studies have demonstrated significant increases in soluble ICAM-1 and VCAM-1 levels in patients with PDR, with corresponding elevations in vitreous IL-6 and TNF-α concentrations. These observations corroborate the inflammatory and immune natures of the pathophysiology of PDR.

Two of the aforementioned studies noted an attenuation of TNF-α levels when treated with anti-inflammatory medications [41, 44]. This is significant because TNF-α plays an important role in neovascularization and vascular reactivity, in addition to its proinflammatory properties. TNF-α is directly involved in inflammation through an induction of cytokines, involvement in monocyte chemotaxis, and stimulation of adhesion molecules on retinal endothelium [48].

Focusing on TNF-α, Limb et al. measured soluble TNF-receptors (sTNF-Rs, types I and II) in patients with various retinal pathologies and found that vitreous levels of sTNF-Rs were significantly increased in eyes with PDR when compared with control eyes. Further, the authors found that the increased vitreous levels of sTNF-Rs correlate with the degree of retinopathy severity and posit that effective control of TNF-α activity by sTNF-Rs within the retinal microenvironment may determine the outcome and severity of retinal proliferative conditions [49].

Other studies demonstrate that inflammatory mediators cause gradual damage as retinopathy progresses. Gustavsson et al. measured levels of IL-1b, IL-6, and TNF-α through ELISA analysis and found that vitreous IL-6 and serum TNF-α levels were higher in diabetic patients than in nondiabetics. The authors concluded that intraocular inflammation is involved in PDR but does not seem to be prominent in nondiabetic retinopathy, nonproliferative diabetic retinopathy, or even in those progressing to early retinopathy stages. Those with PDR, however, had significantly more inflammatory activity, as evidenced by the increased serum levels of IL-6 and TNF-α [3].

Similarly, Yuuki et al. measured concentrations of IL-6, IL-8, and TNF-α via ELISA in the vitreous and serum of patients with PDR and vitreous noninflammatory retinopathies. Vitreous concentrations of IL-6 and IL-8 were significantly greater in patients with PDR than in noninflammatory retinopathies, and serum TNF-α was significantly greater in PDR than in noninflammatory retinopathies (this latter finding was limited to the serum but did not hold true in the vitreous). The authors postulated that these significant increases in IL-6, IL-8, and TNF-α may be diagnostically useful in PDR management [50]. Other studies have similarly found vitreal increases in IL-6 and IL-8 in PDR.

Inflammatory cytokines enhance leukocyte adhesion to endothelium, vascular permeability, and thrombus formation by inducing procoagulant and inhibiting anticoagulant, activity. Adamiec-Mroczek et al. collected vitreous and serum samples of patients with proven PDR in order to establish the role of inflammatory-proliferative processes of the endothelium in this disorder [51]. The authors found that vitreal and serum concentrations of endothelin-1 (ET-1), TNF-α, IL-6, vWF, and E-selectin were higher in patients with PDR than in controls. Moreover, the mean vitreous ET-1 level in the PDR patients was significantly higher than in the control group, and its serum concentration was higher in patients with PDR by a factor of seven.

While IL-6 and IL-8 hold a prominent place in the inflammatory process, other cytokines also play prominent roles. Zhou et al. measured the vitreal concentrations of IL-1B, IL-6, IL-8, IL-10, CCL2, endothelin 1 (EDN-1), VEGF, and TNF-α in patients with PDR and in controls with ELISA. The authors found that with the exception of IL-10, the concentrations of all the aforementioned factors were considerably higher in PDR patients than in controls [52]. They also found a significant positive correlation between vitreous TNF-α, EDN1, and serum HbA1c levels in PDR patients. These results add support to the role of inflammatory cytokines and angiogenic factors in the genesis of PDR.

Chemokines are yet another potential target for therapeutic intervention for PDR. Bian et al. demonstrated that one particular chemokine, CCL2, is an important factor in initiating leukocyte recruitment and activation, especially in the context of hyperglycemia. Levels of CCL2 are significantly elevated in the vitreous of patients with DR when compared to controls. CCL2 (also referred to as MCP-1) is the most common chemokine that is significantly elevated in the serum and vitreous [53]. Moreover, CCL-2 levels have been found to correlate with the severity and clinical stage of DR [54]. Various studies have identified other cytokines and chemokines significantly elevated in both the serum and vitreous of those suffering from DR. As CCR2 inhibitors are being studied in clinical trials to treat inflammatory disorders such as atherosclerosis, multiple sclerosis, rheumatoid arthritis, and systemic lupus erythematosus, similar chemokines are currently being studied under animal models as potential therapeutic targets for the treatment of PDR.

While it seems that inflammatory mediators dominate the pathogenesis of PDR, other mediators and chemokines are important in the pathophysiology of the disorder. Recognizing that any growth factors present in the inert vitreous (protected by the BRB) are likely a reflection of retinal production. Pfeiffer et al. measured and found that insulin-like growth factor I (IGF-I), IGF-II, IGF binding protein 2 (IGFBP-2), and IGFBP-3 were elevated 3–13 fold in nondiabetic retinal ischemia and 1.5–3 fold in PDR [55]. Though clearly not specific to one disorder,these changes suggest that BRB breakdown and subsequent serum leakage into the vitreous is an important aspect of the pathogenesis of PDR and is a promising target for intervention.

Moreover, the same authors investigated vitreal TGF-β2, as it is a proposed antiangiogenic factor in the eye [55]. While the authors noted that total TGF-β2 levels were not altered, the active fraction of TGF-β2 was decreased by 30% in PDR patients. As plasmin is thought to control TGF-β2 activation, the authors demonstrated that serum protein α2-antiplasmin was significantly elevated in PDR patients to 150% of control values. This finding suggests that the flow of serum markers into the vitreous due to microvascular alterations is another potential target for therapeutic intervention.

Microvascular changes and damage to the BRB contribute to the pathogenesis of PDR. Shiels et al. furthered the hypothesis that there is a direct relationship between plasma leakage from damaged retinal vasculature and the proliferation and phenotypic change of RPE cells with fibroblasts. These latter cells, once damaged, contribute to retinopathies by secreting matrix molecules such as fibronectin and expressing deviant surface antigens. The authors posit that control of this inflammation-induced vascular leakage would prove an important future target against microvascular damage.

Angiopoietins, inflammatory growth factors that bind to tyrosine kinase receptors, are yet other potential targets for the treatment of BRB compromise [8]. Patel et al. attributed BRB compromise as the reason for the elevated levels of angiopoietin-2 (Ang-2) in the vitreous of patients with clinically significant macular edema (CSME) [56]. In the same vein, Rangasamy et al. found that intravitreal injection of Ang-2 in non-diabetic rats resulted in a multifold increase in retinal vascular permeability, and that Ang-2 leads to a loss of VE-cadherin function as well [57]. Fiedler et al. demonstrated that Ang-2 sensitizes endothelial cells to TNF-α induced expression of ICAM-1, the critical player in the pathogenesis of inflammation-induced retinopathy [58].

Proteinases are yet another class of factors involved in the progression of PDR. Parks et al. demonstrated that metalloproteinases (MMPs) are important modulators of

innate immunity and inflammation, both acute and chronic [59]. Specific MMPs have been implicated in PDR. Giebel et al. found that the retinas of diabetic animals demonstrated elevated levels of MMP-2, MMP-9, and MMP-14 mRNA, and that the production of MMP-9 was especially increased in cells exposed to a hyperglycemic environment. Ultimately, cells treated with purified MMP-2 or MMP-9 demonstrated degradation of occludin, a tight junction protein [60]. Jin et al. also found that vitreal levels of MMP-9 were higher in diabetic patients with retinopathy than in controls [61]. Navaratna et al. found that the proteolytic degradation of VE-cadherin, a cell-to-cell junction protein, alters the blood-retinal barrier in diabetes and decreases vascular permeability [62]. The ramification of these studies suggests that MMPs are an important potential target for the control of PDR progression.

Many other factors related to inflammation play a role in PDR. Augustin et al. found that lipid peroxide levels and myeloperoxidase activity was elevated in patients with PDR, suggesting the role of oxygen free radicals complementing the inflammatory pathogenesis of diabetic retinopathy [63].

7. Other Targets

Another target to prevent retinal angiogenesis and neovascularization in the context of PDR has been aimed at protein kinase C (PKC). The PKC enzymes, especially the beta isoforms, are found in high levels in the retina. Activator molecules, often induced by tissue hypoxia, result in increased VEGF expression. Thus, efforts have been aimed at inhibiting PKC beta enzymes, those specifically found in the retina, with low systemic toxicity. Selective inhibition of the PKC beta isoform prevents VEGF-mediated cell growth inutero and has been shown to reduce ischemia-related retinal neovascularization *in vivo* [64]. Indeed, Ishii et al. demonstrated that oral administration of a PKC-beta inhibitor reduces diabetes related vascular permeability and changes in retinal blood flow [65]. Various PKC inhibitor compounds have already been developed, such as ruboxistaurin, and several are in phase III clinical trials [66–72]. While the initial results of the multicenter randomized trial from the Protein Kinase C β Inhibitor Diabetic Retinopathy Study (PKC-DRS) group noted no statistically significant effect of ruboxistaurin at any of the three treatment doses for the progression of DR by their primary outcome measurements after a minimum followup of 3 years, they did note the effects of the PKC inhibitor on their secondary outcome, moderate visual loss (MLV), and sustained moderate visual loss (SMVL) [67]. Indeed, the PKC-DRS2 group subsequently studied this latter effect in more detail and concluded that ruboxistaurin reduces the occurrence of SMVL by 40% in patients with moderately severe to very severe nonproliferative diabetic retinopathy, while increasing the likelihood of visual improvement by a factor of two [66].

Fenofibrates and statins have recently been suggested to be a therapy for PDR, due to their secondary anti-inflammatory and oxidative properties rather than their primary effects on lipid levels. Studies have demonstrated that simvastatin treatment of diabetic rats resulted in the retinal suppression of superoxide formation and decreased expression of VEGF, angiopoietin 2, and erythropoietin [73]. Two recent major randomized clinical trials suggest the important role of fenofibrate for the treatment of PDR: the FIELD (Fenofibrate Intervention and Event Lowering in Diabetes) study and ACCORD (Action to Control Cardiovascular Risk in Diabetes)-Eye study. These trials included an aggregate of 11,388 patients with diabetes mellitus type II, of which 5,701 were treated with fenofibrate (±statin) for up to 5 years. In the FIELD study, retinopathy progression was defined as laser treatment for PDR or macular edema or an increase by ≥2 steps on the Early Treatment Diabetic Retinopathy Study (ETDRS) scale. Disease progression in the ACCORD-Eye study was defined as an increase of ≥3 steps on the ETDRS scale or proliferative disease requiring laser or vitrectomy treatment. In FIELD, fenofibrate (200 mg/day) reduced the requirements for laser therapy and was shown to arrest disease progression in patients with preexisting diabetic retinopathy. In ACCORD-Eye, fenofibrate (160 mg/day) taken with simvastatin yielded a 40% reduction in the odds of retinopathy progression when compared with simvastatin alone over 4 years. Fenofibrate reduced first laser treatment by 31% ($P = 0.0002$) and progression of diabetic retinopathy with absolute reductions of 5.0% over 5 years ($P = 0.022$, FIELD) and 3.7% over 4 years ($P = 0.006$, ACCORD-Eye) [74–77].

While the benefits of fenofibrates and statins have been discussed as they apply to PDR, the benefits of statins on those with cardiovascular disease have been previously established. In the randomized and double-blinded JUPITER trial, 17,603 men and women without diabetes or established cardiovascular disease were randomly assigned to rosuvastatin 20 mg or placebo and followed for up to 5 years as a primary endpoint. The trial demonstrated that rosuvastatin significantly reduced the incidence of major cardiovascular evidence in otherwise healthy individuals with elevated high-sensitivity C-reactive proteins [78]. Although the results suggested that rosuvastatin could also result in a small but significant risk of diabetes (as of February 2012, the USA Food and Drug Administration added a new warning to statin medications reflecting this risk), a subsequent study analyzing the data from the JUPITER trial determined that the risk of developing diabetes from statin therapy was limited to those subjects with baseline high risk of developing diabetes, including those with evidence of impaired fasting glucose, metabolic syndrome, severe obesity, or raised HbA1c. The authors emphasize that the cardiovascular and mortality benefits of statin therapy exceed the risk of diabetes in the trial population as a whole as well as in participants at increased risk of developing diabetes. Indeed, even among those with high risk of diabetes, rosuvastatin was estimated to prevent 134 heart attacks, strokes, or deaths, with an additional 54 cases of diagnosed diabetes. In the low-risk group, 86 heart attacks, strokes, or deaths were prevented, with no new cases of diabetes [79].

The role of better blood pressure control on the progression of PDR is less clear. Though the ACCORD-Eye and ADVANCE studies did not demonstrate any significant benefits of intensive blood pressure control on progression of

diabetic retinopathy, the UK Prospective Diabetes Study did [75, 80–82].

Coumarin has also recently been investigated as a potential treatment for PDR. Mazzon et al. recognized the essential role of retinal microvascular compromise in the pathophysiology of PDR and investigated the effects of cloricromene on diabetes elicited by injection of streptozotocin in rats [83, 84]. An antiplatelet drug with vasodilatory and endothelial-preserving properties, cloricromene (ethyl 2-(8-chloro-3-(2-diethylaminoethyl)-4-methyl-2-oxochromen-7-yl)-oxyacetate), is a semisynthetic coumarin derivative that has been shown to mitigate chronic inflammation and resultant tissue damage associated with arthritis in rats [53]. The authors found that cloricromene significantly lowered retinal TNF-α, ICAM-1, VEGF, and nitric oxide synthase (eNOS) levels and suppressed diabetes-related BRB breakdown by 45%.

Many inhibitors have been developed to arrest the progression of glycation and advanced glycation end products (AGE). It has been shown that prolonged oxidative stresses in the context of diabetes result in the production and accumulation of AGEs, receptor-independent agents promoting vascular damage, fibrosis, and inflammation. Aminoguanidine, pyridoxamine, OPB-9195, LR-90, and alagebrium chloride are all agents developed to address AGE related damages [85, 86]. These have all demonstrated variable levels of therapeutic efficacy in diabetic complications. Most recently, Li et al. described the use of RAGE inhibitors on early diabetic retinopathy and tactile allodynia [87]. RAGE is the receptor for AGE, one of the pathway mechanisms of microvascular damage due to hyperglycemia in DR. The authors reported that RAGE fusion protein inhibited capillary degeneration, albumin accumulation in the neural retina, retinal protein nitration, tactile allodynia, diabetes-related retinal leukostasis, and ICAM-1 expression, although the effects of the latter two were not statistically significant at low doses.

This paper serves as a review of the pathophysiology leading to PDR and highlights the major interventional opportunities for the treatment of this disorder. A literature search for clinical markers associated with PDR is replete with inflammatory factors and numerous mediators currently being investigated for their roles in PDR. As unique markers associated with the pathophysiology of PDR are discovered, further studies will be needed to evaluate efficient and effective interventions targeted at deleterious mediators and pathways in order to obviate microvascular disease from progressing to fulminant PDR.

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Review Article

Systemic Treatment of Vitreous Inflammation

John B. Christoforidis, Susie Chang, Angela Jiang, Jillian Wang, and Colleen M. Cebulla

Retina Division, Department of Ophthalmology, Wexner Medical Center at The Ohio State University, 915 Olentangy River Road, Suite 5000, Columbus, OH 43212, USA

Correspondence should be addressed to John B. Christoforidis, jbchristo@hotmail.com

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Non infectious vitreous inflammation is often vision threatening and can be associated with potentially life-threatening systemic conditions. Treatment is often challenging as it involves systemic medications that can be associated with adverse effects. The classes of drugs are ever expanding and include corticosteroids, antimetabolites, alkylating agents, T-cell and calcineurin agents, biologic agents, and interferons. Each class of systemic therapy for non-infectious vitreous inflammation is reviewed. We discuss the mechanisms of action, usual clinical dosages, the specific conditions that are treated, the adverse effects, and usual course of treatment for each class of therapy.

1. Introduction

Vitreous inflammation is a hallmark of posterior uveitis which accounts for 9–38% of uveitis cases [1]. The sequelae of posterior inflammation include visual loss from vitreous opacities, cystoid macular edema, serous retinal detachment, retinal ischemia, neovascularization, retinal pigment epithelium (RPE) changes, and subretinal fibrosis, as well as glaucoma and cataract. Vitreous inflammation may be infectious in origin as in cases of toxoplasmosis, syphilis, *Bartonella*, or infectious endophthalmitis. Many vision and potentially life-threatening causes of vitreous inflammation are noninfectious in origin and include sarcoidosis, Vogt-Koyanagi-Harada (VKH), Behçet's disease, sympathetic ophthalmia, intermediate uveitis, Wegener's granulomatosis and systemic lupus erythematosus (SLE). This paper will focus on systemic treatment of non-infectious causes of vitreous inflammation.

2. Corticosteroids

Corticosteroids are the primary treatment for non-infectious uveitis. Most treatments commence with the use of topical drops or ointments. However, penetration to the posterior segment is limited [2]. Higher local concentrations into both the anterior and posterior segments without significant systemic side effects are achieved by injecting or implanting steroid compounds, such as triamcinolone acetonide, either periocularly or intravitreally [3]. Intravitreal delivery can also be achieved with placement of slow-release devices such as Ozurdex and fluocinolone implants. Although these local treatments are often efficacious, inflammation may recur as the intravitreal steroid concentration decreases. While these local delivery routes have minimal systemic consequences, local side effects such as increased intraocular pressure and glaucoma, cataract, ptosis, and rarely herpetic retinitis can occur.

For patients with uveitis who do not respond adequately to local forms of steroid treatment or have severe disease, systemic steroid therapy is required to gain control of vitreous inflammation [4]. Oral prednisone is typically the first systemic therapeutic agent used at a dose of about 1 mg/kg/day followed by gradual tapering [5]. Patients on oral corticosteroid therapy are monitored for both response to treatment and adverse effects. Potential complications resulting from systemic steroid therapy include increased intraocular pressure and cataract formation, osteoporosis, hyperglycemia, aseptic bone necrosis, gastrointestinal ulcers,

pancreatitis, myopathy, psychosis, delayed wound healing, Cushingoid features, secondary infection and reactivation of latent herpes simplex or tuberculosis. In successful systemic corticosteroid therapy, inflammation subsides and steroids are slowly tapered. If inflammation recurs during tapering, a higher corticosteroid dosage is instituted until inflammation resolves followed by tapering. If reactivation occurs and inflammation persists for over 4 weeks with therapy, or if the patient develops systemic adverse effects, adding locally delivered steroids and/or systemic immunosuppressive therapy should be considered [6].

3. Immunosuppressive Medications

Systemic immunosuppressive treatments are critical to adequately manage certain underlying causes of inflammation (e.g., Wegener's granulomatosis) and are used as "steroid-sparing" agents when long-term treatment is indicated. Immunosuppressive therapy can replace or supplement corticosteroid therapy. More importantly, early immunosuppressive therapy is helpful in reducing blindness in conditions such as Behçet's disease, VKH, sympathetic ophthalmia, Wegener's granulomatosis, juvenile idiopathic arthritis-associated uveitis, rheumatoid necrotizing scleritis, and ocular cicatricial pemphigoid [6]. Table 1 lists uveitis conditions for which immunosuppressive chemotherapy is indicated.

Immunosuppressive agents are classified generally as antimetabolites, alkylating agents, T-cell inhibitors/calcineurin inhibitors, and biologic agents. Ophthalmologic conditions for which immunosuppressive agents are used are summarized in Table 2. Individual therapeutic agents within each class are discussed below.

3.1. Antimetabolites

3.1.1. Methotrexate. Methotrexate is a folic acid analog that competitively binds with and inhibits dihydrofolate reductase, thereby, reducing production of thymidylate and purine which are essential for DNA replication [7].Highly metabolic inflammatory mediator cells such as leukocytes are suppressed thereby reducing inflammation [8]. Folinic acid supplementation is used with high doses of methotrexate to protect more slowly dividing cells by restoring thymidylate and purine biosynthesis [7].

Methotrexate can be administered orally, subcutaneously, intramuscularly or intravenously at doses ranging between 7.5 to 25 mg per week. It has been reported to reduce vitreous inflammation as well as other inflammatory conditions such as vasculitis, scleritis, anterior uveitis, orbital pseudotumor and sarcoidosis [9–14]. Three to eight weeks are required for the anti-inflammatory effects of methotrexate to take full effect.

Methotrexate is commonly used as a steroid-sparing agent, allowing steroids to be tapered. Its side effect profile is preferable to that of high-dose steroids for long-term treatment and includes fatigue, nausea, vomiting and anorexia (5–25%), which usually improve following dosage reduction [15]. Less common but serious side effects include hepatotoxicity (15%), bone marrow suppression, cutaneous vasculitis and urticaria [15]. Methotrexate is a teratogen and is contraindicated for use during pregnancy [7]. Laboratory monitoring for side-effects must be performed during methotrexate therapy. Baseline labs include complete blood count (CBC), serum chemistry, blood urea nitrogen, serum creatinine, liver function tests (LFTs), urinalysis and pregnancy test. Maintenance laboratory testing is conducted at 4-week intervals and consists of CBC and LFTs. The methotrexate dose is reduced if liver enzymes double on 2 subsequent measurements and is discontinued if the liver enzymes remain elevated after dose reduction [16]. Therapy is continued for 2 years following ocular quiescence to avoid recurrence of inflammation [17].

3.1.2. Azathioprine. Azathioprine (Imuran, GlaxoSmithKline, London, UK) is an imidazolyl derivative that metabolizes to thioinosine-5-phosphate, a purine analog that interferes with DNA and RNA replication and transcription. It suppresses lymphocyte proliferation and antibody production [18]. It also suppresses natural killer cells and the delayed type hypersensitivity reaction [19]. Azathioprine has been effective in the treatment of serpiginous choroiditis, multifocal choroiditis, panuveitis, ocular cicatricial pemphigoid, and juvenile idiopathic arthritis [20–22].

Azathioprine is administered orally at an initial dose of 2 to 3 mg/kg/day and then adjusted based on clinical response and adverse effects. The most common adverse effect is gastrointestinal upset followed by hepatotoxicity, bone marrow suppression (leucopenia and thrombocytopenia), alopecia and pancreatitis [19, 23].Baseline labs should include CBC and LFTs. In addition, Foster and Vitale recommend obtaining blood thiopurine methyltransferase enzyme activity levels at baseline and to withhold treatment if the enzyme activity is low or absent [24]. CBC and liver function tests are repeated at 4 to 6 week intervals during treatment. Treatment dose should be decreased in cases of mild abnormalities and temporarily discontinued and resumed at lower doses in the presence of major abnormalities [25]. Treatment is continued for 2 years following ocular quiescence [24].

3.1.3. Mycophenolate Mofetil. Mycophenolate mofetil (MMF) (CellCept, Roche, Basel, Switzerland) is a reversible inhibitor of the enzyme inosine monophosphate dehydrogenase that is involved in guanosine nucleotide synthesis. It disrupts DNA synthesis that is used by B and T cells for purine synthesis [26]. MMF also interferes with cellular adhesion to vascular endothelium and disrupts lymphocytic chemotaxis [27]. MMF has been found to be effective as monotherapy in the treatment of chronic ocular inflammatory disease [28]. It has also been found to be effective in combination with cyclosporine and methotrexate for the treatment of scleritis and uveitis [29].

Mycophenolate mofetil is administered as an initial dose of 500 mg twice a day and adjusted after monitoring for side effects. The dose is increased to 1 g twice a day if it is

Table 1: Diseases indicated for immunosuppressive chemotherapy.

Strong indications	Relative indications	Questionable indications
Behçet's disease with retinal involvement	Intermediate uveitis	Intermediate uveitis in children
Sympathetic ophthalmia	Retinal vasculitis with central vascular leakage	Sarcoid-associated uveitis inadequately responsive to steroid
Vogt-Koyanagi-Harada syndrome	Severe chronic iridocyclitis	Keratoplasty with multiple rejections
Rheumatoid necrotizing scleritis or peripheral ulcerative keratitis		
Wegener's granulomatosis		
Relapsing polychondritis with scleritis		
Juvenile idiopathic arthritis-associated iridocyclitis unresponsive to conventional therapy		
Ocular cicatricial pemphigoid		
Bilateral Mooren ulcer		

Table 2: Uses of immunosuppressive agents in ophthalmologic conditions.

Drug	Diagnosis
Antimetabolites	
Methotrexate	Chronic non-infectious uveitis, sarcoidosis, and non-infectious ocular inflammation
Azathioprine	Chronic uveitis, Behcet's disease, choroidal neovascularization, OCP, retinal vasculitis, serpiginous choroiditis, and neuroretinitis
Mycophenolate mofetil	Chronic uveitis, non-infectious ocular inflammation, intermediate and posterior uveitis, refractory uveitis, and scleritis
Leflunomide	Sarcoidosis uveitis
Alkylating agents	
Cyclophosphamide	Refractory uveitis, peripheral uveitis, Wegener, OCP, scleritis, Behçet's disease, non-infectious ocular inflammation, and optic neuropathy (SLE)
Chlorambucil	Serpiginous choroiditis, severe chronic uveitis, uveitis, and Behçet's disease
T-cell inhibitors/calcineurin inhibitors	
Cyclosporine	Serpiginous choroidopathy, Behçet's disease, endogenous uveitis, chronic idiopathic uveitis, scleritis, rheumatoid arthritis, and non-infectious uveitis
Tacrolimus	Refractory uveitis (limited experience)
Rapamycin	Refractory uveitis (limited experience)
Biologic agents	
Etanercept	Juvenile idiopathic arthritis, non-infectious uveitis, and ocular inflammatory disease
Infliximab	Refractory uveitis, childhood uveitis, Behcet's disease, and refractory uveitis
Adalimumab	Refractory uveitis, ankylosing spondylitis, and juvenile idiopathic arthritis
Daclizumab	Juvenile idiopathic arthritis, recalcitrant ocular inflammation, and birdshot retinochoroidopathy
Rituximab	Primary Sjogren syndrome, thyroid eye disease, and Wegener
Tocilizumab	Refractory uveitis (limited experience)
Other	
Interferons	Behcet's disease, non-infectious uveitis

well tolerated [24]. Adverse effects are most commonly gastrointestinal including nausea, vomiting and diarrhea. Less frequent side effects include leukopenia, lymphocytopenia, and hepatotoxicity [7]. CBC and LFTsshould be obtained at baseline and followed with weekly CBC for the first 4 weeks, twice monthly for 2 months and monthly thereafter. Liver function tests are obtained monthly for the duration of treatment [30]. Treatment is continued for 2 years following ocular quiescence [24].

3.1.4. Leflunomide. Leflunomide (Arava, Sanofi-Aventis) inhibits the enzyme dihydro-orotate dehydrogenase which is involved in pyrimidine synthesis. Consequently, it interferes with B and T cell proliferation and suppresses the inflammatory response [31]. Suppression of tyrosine kinase and possibly cyclo-oxygenase and histamine release may further potentiate its anti-inflammatory effects [32, 33]. Although ocular use of leflunomide is limited, it has shown promise in the treatment of ocular inflammation associated with sarcoidosis [34].

Leflunomide therapy for systemic conditions such as rheumatoid and psoriatic arthritis is typically administered orally with a loading dose of 100 mg followed by 10 to 20 mg daily. The loading dose is necessary because of the long plasma half-life of leflunomide (15–18 days) [35]. The most serious adverse effect is hepatotoxicity which can range from jaundice to fulminant hepatitis. Other adverse effects include headache, paresthesias, leucopenia, anemia, thrombocytopenia and interstitial lung disease [36]. Concurrent use with methotrexate is discouraged because of potential hepatotoxicity. Patients should have baseline CBC and LFTs every 2 weeks for the first 6 months and then every 8 weeks following commencement of treatment. Leflunomide is teratogenic and contraindicated for use during pregnancy [35]. The optimal duration of therapy is currently not certain.

3.2. Alkylating Agents

3.2.1. Cyclophosphamide. Cyclophosphamide (Cytoxan, Bristol-Myers Squibb) is an alkylating agent derived from mustard gas. It is cytotoxic to rapidly dividing cells such as T and B lymphocytes by alkylating DNA bases and disrupting DNA cross-linking, and antibody production and delayed-type hypersensitivity is suppressed [37]. Cyclophosphamide has been found to be effective in the treatment of ocular inflammation associated with Wegener's granulomatosis, polyarteritis nodosa and Behcet's disease. It is also used to treat bilateral Mooren ulcer and severe ocular cicatricial pemphigoid and scleritis secondary to rheumatoid arthritis and relapsing polychondritis [38–43].

Cyclophosphamide is preferably administered intravenously rather than orally as induction is more rapidly achieved and bladder exposure is reduced [44]. It is initially administered intravenously at a dose of 1 g/m^2 and adjusted based on the response, serial CBCs and the presence of any adverse events [30]. Treatment is commenced every 2 weeks and once stabilized treatment intervals are reduced to 3 to 4 weeks.Adverse effects are reversible bone marrow suppression, hemorrhagic cystitis, secondary cancers such as bladder cancer and acute myeloid leukemia, testicular atrophy and ovarian suppression. Cyclophosphamide is teratogenic and contraindicated in pregnancy. Baseline CBC with platelets, liver function tests and urinalysis are obtained. CBCs and urinalysis are repeated weekly initially and all labs are repeated monthly when the blood counts are stabilized. The dose is lowered if there is mild bone marrow suppression and interrupted and restarted at a lower dose if there is severe bone marrow suppression. The dose is discontinued if there is hematuria and consultation with urology is recommended if persistent after 3 weeks [30]. Treatment is continued for 1 year following ocular quiescence [1].

3.2.2. Chlorambucil. Chlorambucil (Leukeran, GlaxoSmithKline, London, UK) is an alkylating agent derived from nitrogen mustard. It causes cross linking within DNA strands interfering with DNA replication and transcription [45]. Chlorambucil has been used to treat Behçet's disease, sympathetic ophthalmia and serpiginous choroiditis [46, 47].

Chlorambucil is administered orally as it does not have the deleterious bladder effects seen with cyclophosphamide. Initial dosing is typically 0.1 mg/kg/day with incremental adjustments based on response, clinical labs and adverse effects to a maximum of 12 mg daily. Treatment is continued for 1 year following ocular quiescence [24]. Another treatment algorithm is short-term high-dose oral therapy for 3 to 6 months [30]. Adverse events related to chlorambucil include reversible myelosuppression, bone marrow aplasia, male sterility, amenorrhea, gastrointestinal distress, hepatotoxicity, central nervous system effects such as seizures, secondary cancers, and reactivation of latent herpes simplex virus [30, 48, 49]. Baseline labs include CBC with differential and LFTs. CBC is followed weekly initially and monthly along with LFTs once the dose is stabilized. It is especially important to monitor these patients for myelosuppression since the effect of chlorambucil on the bone marrow is cumulative [30].

3.3. T-Cell Inhibitors/Calcineurin Inhibitors

3.3.1. Cyclosporine. Cyclosporine (Neoral, Novartis) is a fungal byproduct that binds cyclophilin and calcineurin and inhibits T lymphocytes and their ability to produce lymphokines such as interleukin-2 [50]. Although it was originally used to suppress rejection following solid organ transplantation, its ophthalmologic use is well established. It has been found to be effective in the treatment of Behçet's disease, VKH, sarcoidosis, sympathetic ophthalmia and birdshot retinochoroidopathy [51, 52].

Cyclosporine is administered orally with an initial dose of 2.5 mg/kg/day and increased in increments of 50 mg to a maximum dose of 5 mg/kg/day based on clinical response [24]. Side effects include hypertension, renal toxicity, hypertrichosis, gingival hyperplasia, myalgia, tremor, paresthesia, and lymphoma. Side effects are more likely to occur with

doses higher than 10 mg/kg/day or with prolonged use. Baseline labs should include CBC with differential, blood electrolytes with BUN and creatinine, LFTs, urinalysis and blood pressure. Blood pressure and blood electrolytes with BUN are monitored every 2 weeks and then monthly along with liver function tests, creatinine, and CBC once therapy is stabilized [30]. Treatment is continued for 2 years following ocular quiescence [24].

3.3.2. Tacrolimus (FK506). Tacrolimus (Prograf, Astellas, Tokyo, Japan) is a macrolide antibiotic that like cyclosporine, inhibits calcineurin and suppresses T-cell signal transduction and interleukin-2 transcription [53]. Although clinical experience for treatment of uveitis is more limited than that of cyclosporine, tacrolimus has been found to be effective in the treatment of intraocular inflammation usually in conjunction with systemic corticosteroids [54]. It has also been found to be effective in treatment failure with cyclosporine [55, 56].

Tacrolimus is administered orally at a dose of 0.10 to 0.15 mg/kg/day. It is also available for intravenous use. Adverse events are similar to those seen with cyclosporine. In addition to hypertension and nephrotoxicity, side effects include hyperglycemia, hyperkalemia, hypomagnesemia, loss of appetite and neurologic symptoms such as insomnia, confusion, depression, catatonia, tremors and seizures, and increased risk of non-Hodgkin's lymphoma. The more severe symptoms are seen at higher doses when given intravenously in transplant patients [57–59]. Laboratory evaluation is similar to that for cyclosporine and includes baseline CBC, LFTs, and electrolytes with BUN and creatinine. Blood pressure and blood electrolytes with BUN are monitored every 2 weeks and then monthly along with liver function tests, creatinine, and CBC once therapy is stabilized.

3.3.3. Rapamycin (Sirolimus). Rapamycin (Rapamune, Phizer, New York, NY USA) is a macrolide antibiotic that inhibits cellular response to interleukin-2 to block B and T lymphocyte activation. Unlike tacrolimus and cyclosporine, rapamycin inhibits the mammalian target of rapamycin (mTOR) and is not a calcineurin inhibitor. Thus it is thought to be less nephrotoxic. Early studies suggest that rapamycin may be most useful in combination with other immunosuppressive agents [60, 61].

Rapamycin is administered orally at a loading dose of 6 mg followed by a daily dose of 2 to 6 mg/day [60]. Baseline and follow-up laboratory and blood pressure monitoring is similar to that of cyclosporine and tacrolimus. Described side effects include elevated liver enzymes, thrombocytopenia, anemia, hypercholesterolemia, nausea, abdominal pain and eczema. All high-dose immunosuppressants given to transplant patients carry an elevated risk of malignancy, and the risk to uveitis patients is likely proportional to dose and duration.

3.4. Biologic Agents. Biologic response modifiers, also known as biologics, are a newer class of therapeutic proteins used to treat uveitis by inhibiting bioactive mediators or cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin-2. These agents were developed for the treatment of systemic inflammatory diseases and for the prevention of solid organ transplant failure. They target specific molecules in the inflammatory process and may be an alternative in treating uveitis refractory to conventional treatment. Because of the integral immunologic role of TNF, its suppression increases the risk of latent and opportunistic infections such as tuberculosis, histoplasmosis, coccidiomycosis, and herpes viruses. In addition, there are rare reports of lymphoma and other malignancies with the use of TNF blockers. Use of biologics for the treatment of uveitis is considered off-label in the USA.

There are 2 major groups of biologic agents, monoclonal antibodies and fusion proteins. Types of monoclonal antibodies are identified by their suffix. Antibody sequences are human if the suffix is "-umab," human-murine (human constant and murine variable regions) if the suffix is "-ximab" and humanized (human constant and murine/human variable regions) if the suffix is "-zumab." Fusion proteins are created by joining two or more genes originally coded for separate proteins. They are composed of a receptor with specificity to the molecule of interest fused with another protein fragment such as a portion of an antibody. The suffix ending in "-cept" denotes fusion proteins.

3.4.1. Etanercept. Etanercept (Enbrel, Pfizer, New York, NY USA) is a fusion protein composed of a TNF receptor and the Fc fragment of human IgG antibody. It inhibits the binding of TNF-α and TNF-β to the surface TNF receptors, inactivating TNF and suppressing neutrophil migration and pro-inflammatory cytokine synthesis. Clinical studies have been indeterminate regarding the efficacy of etanercept for the treatment of ocular inflammation [62–65].

Etanercept is administered subcutaneously, 25 mg twice a week for 2 years. Contraindications to Etanercept include a history of latent tuberculosis (TB) and exposure to hepatitis B. Adverse effects of etanercept include infection, reactivation of latent TB and hepatitis B, and rare reports of pancytopenia, central nervous system demyelination, congestive heart failure, and lymphoma [66, 67]. Tuberculin skin testing and hepatitis B serologic testing are performed at the time of screening. CBC and LFTs are also performed at baseline and repeated monthly [30, 68].

3.4.2. Infliximab. Infliximab (Remicade, Janssen, Beerse, Belgium) is a monoclonal antibody that binds and inhibits both bound and circulating TNF-α [69]. It has shown encouraging responses in patients with treatment-resistant ocular inflammation including Behçet's disease, Wegener's granulomatosis, sarcoidosis, and juvenile inflammatory arthritis [70–74].

Infliximab is administered intravenously with loading infusions at weeks 0, 2, and 6. The doses are 5 mg/kg for monotherapy and 3 mg/kg in patients receiving concurrent noncorticosteroid immunomodulatory treatment. Maintenance infusions are then performed every 8 weeks [74]. Adverse events include infections including upper

respiratory and urinary tract, cough, rash, nausea, vomiting, abdominal pain, headache, lupus-like illness, vasculitis, anemia, and thrombocytopenia [74–76]. Tuberculin skin testing is performed at the time of screening. CBC and LFTs are also performed at baseline and repeated monthly. Treatment is maintained for 2 years after ocular quiescence is achieved [17].

3.4.3. Adalimumab. Adalimumab (Humira, Abbott) is a recombinant humanized monoclonal antibody that binds and inhibits TNF-α [77]. Adalimumab has been used with increasing frequency and found to be effective for treatment of Behçet's disease, VKH, birdshot retinochoroidopathy, juvenile inflammatory arthritis, and scleritis due to rheumatoid arthritis [78–82].

Adalimumab is administered subcutaneously at a dose of 40 mg every two weeks [83]. Adverse effects are similar to those of infliximab and etanercept and include the reactivation of latent infections such as tuberculosis and opportunistic infections. The most common side effects are injection site reactions, upper respiratory and urinary tract infections, headache, confusion and rare reports of central nervous system demyelination, hepatotoxicity, congestive heart failure and lymphoma [84, 85]. As with other TNF-α inhibitors, tuberculin skin testing is performed at the time of screening. CBC and LFTs are also performed at baseline and repeated monthly. Treatment is maintained for 2 years after ocular quiescence is achieved [17].

3.4.4. Daclizumab. Daclizumab (Zenapax, Genentech/Roche) is a humanized monoclonal antibody to the interleukin-2 receptor on T lymphocytes [86]. In several small case series, daclizumab has been found useful in treating birdshot retinochoroidopathy, posterior uveitis and juvenile inflammatory arthritic uveitis [87–89].Daclizumab, which was discontinued by Hoffman La Roche on Sept 01, 2009, is no longer available to the US market and is primarily used through participation in clinical trials.

Daclizumab is administered intravenously at 1 mg/kg every 2 weeks with or without other immunomodulators. The dose is then adjusted based on the clinical response to a maximum daily dose of 200 mg [90]. Daclizumab is generally better tolerated than TNF-α inhibitors. Adverse effects include rash, lymphadenopathy, chest discomfort, and fever [91]. Baseline laboratory evaluation includes CBC and LFTs, which are repeated prior to each infusion. Treatment is maintained for 2 years after ocular quiescence is achieved [87].

3.4.5. Rituximab. Rituximab (Rituxan, Biogen Idec, Weston, MA) is a chimeric monoclonal antibody that binds to CD20 antigen on the surface of B cells and suppresses B-cell differentiation resulting in reduced IgG and IgM production [92]. It has been found to be effective in treatment of Behçet's disease, Wegener's granulomatosis uveitis and retinal vasculitis [93, 94]. It has also been used in conjunction with intravenous IgG in the treatment of ocular cicatricial pemphigoid [95]. It is important to emphasize that rituximab treatment is associated with a risk of death from severe side effects, such as *pneumocystis* infection, toxic epidermal necrolysis, and progressive multifocal leukoencephalopathy [96, 97]. Other adverse events include severe infusion reaction, infection, and acute renal failure [98].

3.4.6. Tocilizumab. Tocilizumab (Actemra, Roche, Basel, Switzerland) is a humanized monoclonal antibody against the interleukin-6 (IL-6) receptors on T-and B-cells and monocytes and hinders IL-6 expression. It is primarily used for the treatment of rheumatoid arthritis and received FDA approval in April 2011 for treatment of systemic juvenile idiopathic arthritis [99]. IL-6 has been found to be elevated in the vitreous of patients with active posterior uveitis [100]. Tocilizumab for ophthalmic use has been limited to date. It has recently shown promise in the treatment of refractory uveitis [101].

Common side effects include upper respiratory tract infections, nasopharyngitis, hypertension, headache, and transient increases in alanine transaminase [102]. Less common side effects include neutropenia, thrombocytopenia gastritis, gastrointestinal perforations, and opportunistic or recurrent infections such as tuberculosis and fungal infections [103].

3.5. Other

3.5.1. Interferons. Interferons (IFNs) are endogenous cytokines that are released by a variety of cells in response to the presence of external pathogens such as viruses, bacteria, and tumor cells. There are multiple classes of interferons but IFN-α 2a and 2b and IFN-β 1a and 1b are used therapeutically for high-risk cutaneous melanoma, hepatitis C, and multiple sclerosis [104, 105]. IFN-α 2a has been used successfully in the treatment of Behçet's disease [106, 107]. IFN-β 1a has been used to reduce recurrences of uveitis in patients with multiple sclerosis [108, 109].

IFN-α 2a is typically administered at a dose of 3 to 6 million international units each day to 3 times weekly [110]. The most common adverse effects include fever, chills, myalgias, fatigue, alopecia, and depression [111]. Interferon retinopathy has been reported and should be evaluated. Baseline laboratory tests include CBC, LFTs and thyroid function. CBC and LFTs are repeated at 4 week intervals, while thyroid function tests are performed every 3 months. The goal of treatment is to achieve ocular inflammatory quiescence for 2 years before stopping therapy [6].

4. Conclusion

In summary, a heterogeneous group of non-infectious inflammatory diseases result in vision-threatening vitreous inflammation. The goal of treatment is to eliminate intraocular inflammation rapidly while closely monitoring drug side effects. There are now many classes of drugs which may be used as monotherapy or in combination to achieve this goal. Many of these inflammatory disorders require long-term steroid-sparing agents to adequately control disease, usually

beyond two years. Immunomodulatory therapy requires close monitoring due to potential adverse effects and varied individual responses. Biologic agents may be an alternative for patients with refractory uveitis. Further studies are required to determine their efficacy.

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Review Article

Intravitreal Devices for the Treatment of Vitreous Inflammation

John B. Christoforidis,[1] Susie Chang,[2] Angela Jiang,[2] Jillian Wang,[2] and Colleen M. Cebulla[2]

[1] *Retina Division, Department of Ophthalmology, The Ohio State University Wexner Medical Center, Columbus, OH 43212, USA*
[2] *Department of Ophthalmology, The Ohio State University Wexner Medical Center, Columbus, OH 43212, USA*

Correspondence should be addressed to John B. Christoforidis, jbchristo@hotmail.com

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The eye is a well-suited organ for local delivery of therapeutics to treat vitreous inflammation as well as other pathologic conditions that induce visual loss. Several conditions are particularly challenging to treat and often require chronic courses of therapy. The use of implantable intravitreal devices for drug delivery is an emerging field in the treatment of vitreous inflammation as well as other ophthalmologic diseases. There are unique challenges in the design of these devices which include implants, polymers, and micro- and nanoparticles. This paper reviews current and investigational drug delivery systems for treating vitreous inflammation as well as other pathologic conditions that induce visual loss. The use of nonbiodegradable devices such as polyvinyl alcohol-ethylene vinyl acetate polymers and polysulfone capillary fibers, and biodegradable devices such as polylactic acid, polyglycolic acid, and polylactic-co-glycolic acid, polycaprolactones, and polyanhydrides are reviewed. Clinically used implantable devices for therapeutic agents including ganciclovir, fluocinolone acetonide, triamcinolone acetonide, and dexamethasone are described. Finally, recently developed investigational particulate drug delivery systems in the form of liposomes, microspheres, and nanoparticles are examined.

1. Introduction

The eye is a model organ for the implantation of devices that provide long-lasting infusion of a therapeutic agent. It is easily accessible for implantation of such a device and success of therapy is measurable objectively by direct visualization of the intraocular structures and by patient responsiveness. The treatment of posterior uveitis and vitreous inflammation usually involves a chronic course of therapy often over a period of years. Topical agents require frequent administration which is often impractical for patients. Sub-Tenon's and intravitreal steroid injections also can require frequent retreatment to adequately control disease. Treatment with powerful systemic corticosteroid and immunomodulatory agents most often have poor vitreous penetration and can be associated with severe systemic side effects [1]. Implantable devices offer an alternative therapeutic approach that can circumvent many challenges of these other modes of therapy.

The first implantable device for clinical use was developed in 1992 [2]. Vitrasert, a nonbiodegradable implant, delivers ganciclovir into the eye for the treatment of acquired-immunodeficiency-syndrome (AIDS)-related cytomegalovirus (CMV). Newer biodegradable implantable devices can provide sustained release of pharmacologics. More recently, there are serious investigations of biodegradable polymers that encapsulate particulate systems for long-lasting delivery of therapeutic nanoparticles which can be injected intravitreally.

In this review, current and investigational drug delivery systems for treating vitreous inflammation are described. These are summarized in Tables 1 and 2.

2. Drug Delivery Implant Polymers

2.1. Nonbiodegradable Devices. Nonbiodegradable devices require surgical implantation and contain a drug reservoir within a permeable polymer membrane. Although useful in some clinical settings, nonbiodegradable implants are not without limitations. Due to their large size a relatively large incision is required for implantation. Furthermore, these devices typically require removal and reimplantation of a second device once the drug has been completely released.

Table 1: Drug delivery implant polymers.

Material	Properties	Clinical application
	Nonbiodegradable devices	
Ethylene vinyl acetate	Nonpermeable, hydrophobic	Vitrasert implant, intravitreal dexamethasone, and cyclosporine
Polysulfone capillary fiber	Water impermeable; increases surface area for drug release	Used experimentally for carboxyfluorescein dye release and daunomycin in rabbit eyes
Polyvinyl alcohol	Permeable	Vitrasert implant, intravitreal dexamethasone, and cyclosporine
	Biodegradable devices	
Polyanhydrides	Degrade by surface erosion into biocompatible monomers	5-fluorouracil, taxol, and etoposide
Polycaprolactone	Semicrystalline, hydrophobic	5-fluorouracil, dexamethasone, and triamcinolone implants
Polyglycolic acid	Semicrystalline; synthesized using toxic solvents	
Polylactic acid	Hydrophobic; degrades more slowly than polyglycolic acid	
Polylactic-co-glycolic acid	Copolymer (adjustable ratio) of polyglycolic and polylactic acid	Dexamethasone (Ozurdex), indomethacin

Table 2: Characteristics of intravitreal devices.

Device	Materials	Active agent	Duration of drug release	Diseases
		Nonbiodegradable devices		
I-vation	Drug-polymer-coated nonferrous alloy helix (polybutyl methacrylate/polyvinyl alcohol; bravo drug delivery polymer matrix)	Triamcinolone acetonide (1–3 μg/day)	2 years	Investigational: diabetic macular edema phase 2b trial suspended in 2008
Illuvien/medidur	Polyvinyl alcohol (with silicone bioadhesive in low-dose version)	Fluocinolone acetonide (0.59 mg; 0.2–0.5 μg/day)	18–30 months	Investigational: diabetic macular edema (phase 3)
Retisert	Silicone/polyvinyl alcohol	Fluocinolone acetonide (0.59 mg)	Up to 3 years	FDA approved for the treatment of uveitis. Investigational: diabetic macular edema, retinal vein occlusion
Vitrasert	EVA/polyvinyl alcohol	Ganciclovir (4.5 mg)	5 to 8 months	Implantable reservoir system
		Biodegradable devices		
Ozurdex	Polylactic-co-glycolic acid	Dexamethasone (0.7 mg)	6 months	DA approved for the treatment of macular edema following branch or central retinal vein occlusion. Investigational: diabetic macular edema, uveitis
Surodex	Polylactic-co-glycolic acid, hydroxypropyl methylcellulose	Dexamethasone (60 μg)	7–10 days	Investigational in the USA: postoperative inflammation following cataract surgery (phase 3). Regulatory approvals in Singapore, China, Mexico

The rate of drug release can be slowed by decreasing the surface area or increasing the thickness of the permeable membrane [3]. Complications associated with these devices include retinal detachment, vitreous hemorrhage, epiretinal membrane formation, and dissolution of the implant [4].

2.1.1. Polyvinyl Alcohol-Ethylene Vinyl Acetate Polymers. These devices are made of polyvinyl alcohol (PVA), a permeable polymer functioning as a structural component, and ethylene vinyl acetate (EVA), a nonpermeable hydrophobic polymer used to restrict drug release. These devices are essentially inert, almost devoid of intraocular inflammatory

response when implanted, but must be removed to prevent fibrous encapsulation after drug delivery is complete. The initial device of this type was originally formulated to contain 5-fluorouracil and placed subconjuctivally to prevent scarring following glaucoma filtration [5]. Subsequent devices have been used for intravitreal dexamethasone and cyclosporine implantation [6, 7]. The major clinical application of this device type was the ganciclovir (Vitrasert, Bausch and Lomb) implant, which was used frequently prior to the development of highly active antiretroviral therapy for HIV.

2.1.2. Polysulfone Capillary Fiber. These devices are water impermeable and contain deep macrovoids which increase the surface area for drug release. It is permeable to both lipophilic and hydrophilic compounds and is well tolerated [7]. Polysulfone capillary fiber implants have only been used experimentally for carboxyfluorescein dye release and daunomycin in rabbit eyes [8, 9].

2.2. Biodegradable Devices. Biodegradable devices are particularly useful as intraocular implants since they do not need to be removed and have increased flexibility in their shape. They can be formulated as rods, discs, and microparticles [3].

2.2.1. Polylactic Acid, Polyglycolic Acid, and Polylactic-Co-Glycolic Acid. Polylactic acid (PLA), polyglycolic acid (PGA), and polylactic-co-glycolic acid (PLGA) are the most studied synthetic biodegradable polymers. They are biocompatible, biodegradable and are FDA approved for drug delivery [10]. These polymers are widely used as suture materials, bone screws and pins, vascular grafts and stents, and surgical scaffolds for tissue regeneration.

PGA is a semicrystalline polymer that is synthesized using toxic solvents limiting its potential for clinical use since any residual solvent may react with the drug or tissue [11]. PLA is a hydrophobic polymer that degrades more slowly than PGA. PLGA is a copolymer of PLA and PGA and is the most widely used biodegradable polymer for drug delivery. The ratio of PLA to PGA can be adjusted to modulate the rate of polymer degradation. The rate of drug release depends on the total surface area of the device, the percentage of loaded drug, the water solubility of the drug, and the speed of polymer degradation for human immunodeficiency virus [12].

There are three phases of drug release in these types of polymers.

(1) Initial burst from the surface of the implant.

(2) Diffusion phase during biodegradation of the polymer.

(3) Final burst from the disintegration of the implant.

The initial burst is followed by a longer steady drug release and is well suited for diseases that require an initial loading dose followed by tapering. However, the first and last phases release higher drug concentrations and potential toxic effects at these levels need to be considered. Blending polymers with different molecular weights can reduce the final drug burst and result in a more stable agent release [13, 14]. Examples of PLGA devices include dexamethasone (Ozurdex) and indomethacin.

2.2.2. Polycaprolactones. Polycaprolactone (PCL) is a polymer of ε-caprolactone, a semi-crystalline and hydrophobic compound that is biodegradable and biocompatible. It is widely used in the biomedical industry (e.g., Monocryl suture, artificial skin, and osteosynthetic material). It is very slowly degraded in the human body by hydrolysis of its ester linkages and its fragments are phagocytized [15]. When the implants are immersed in water, there is dissolution leaving pores in the PCL, allowing for a long-term, well-controlled steady release rate over a period of greater than one year [16, 17]. Intravitreal PCL implants with 5-fluorouracil has been investigated for the prevention of proliferative vitreoretinopathy [18]. Intravitreally-placed PCL devices containing dexamethasone delivers the drug in a controlled and prolonged manner for at least 55 weeks. At 55 weeks, 79% of drug was still present in the implant. It was found to be very well tolerated in rabbit eyes with no sign of anterior or posterior segment inflammation [19]. PCL devices containing triamcinolone acetate have also been implanted in the subretinal space of rabbit eyes and was found to be well- tolerated by retinal tissue, releasing the drug for at least 4 weeks without an inflammatory response [20]. PCL can also be mixed with other polymers, usually more hydrophilic than PCL, to form copolymers which degrade at faster rates. These have been used experimentally for drug delivery of cyclosporine and tacrolimus [21, 22].

2.2.3. Polyanhydrides. Polyanhydrides are a class of biodegradable polymers that degrade by surface erosion into biocompatible monomers that are then metabolized and removed from the body [23]. Surface erosion provides a more controlled drug release compared to drugs that are released by bulk erosion, making them useful as drug delivery devices. There are several classes of polyanhydrides including aliphatic, unsaturated, and aromatic. Aliphatic polyanhydrides degrade in a few days while some aromatic polyanhydrides degrade over few years. Degradation rates of copolymers of aliphatic and aromatic polyanhydrides vary between these extremes and this feature of polyanhydrides gives an opportunity for making a drug delivery system which can provide the release of drugs for a desired time length of treatment [24].

The most frequently used is a copolymer of the 1,3-bis(carboxyphenoxypropane) (PCPP) and sebacic acid (SA). PCPP is aromatic and hydrophobic and by itself has a long lifetime of over 3 years, while SA is aliphatic and hydrophilic with a lifetime of a few days. Copolymerization with SA reduces the lifetime to a few days [25]. The 80 : 20 copolymer has been FDA approved for intracranial delivery of carmustine (Gliadel) for treatment of brain tumors, and intravenous delivery for treatment of recurrent Hodgkin's lymphoma and multiple myeloma. In ocular use they have been investigated in the delivery of 5-fluorouracil, taxol, and etoposide for experimental glaucoma filtration surgery in a non-human primate model [26, 27].

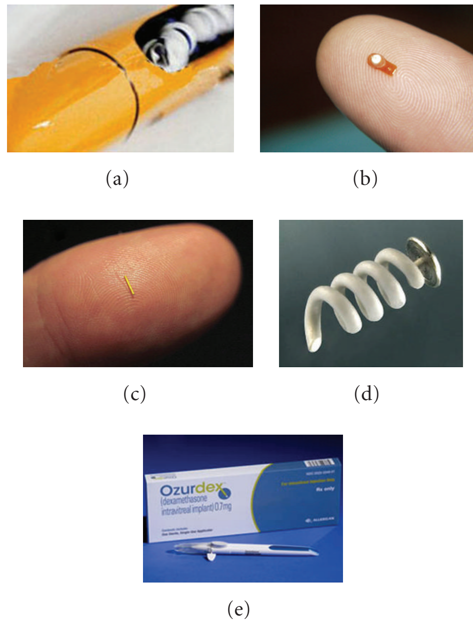

Figure 1: Intravitreal devices. (a) Vitrasert, image courtesy of Bausch & Lomb. (b) Retisert, image courtesy of pSIVIDA. (c) Medidur, image courtesy of pSIVIDA. (d) I-vation, image courtesy of SurModics, Inc. (e) Ozurdex, image courtesy of Allergan, Inc.

2.3. Clinically Used Intravitreal Implants

2.3.1. Ganciclovir. Vitrasert (Bausch & Lomb, Rochester, NY) is a PVA-EVA reservoir implant consisting of a pellet containing at least 4.5 mg of ganciclovir as the active ingredient and 0.25% magnesium stearate as the inactive ingredient with a ganciclovir release rate of 1 mcg/hour (Figure 1(a)). The EVA limits the surface area of ganciclovir. A 5-6 mm scleral incision is made at pars plana and after trimming away any prolapsed vitreous, the device is implanted into the vitreous cavity. It is sutured in place on the sclera prior to closing the sclera and overlying conjunctiva. It is removed if another ganciclovir implant is placed (usually after 6 months) or if there are any complications such as endophthalmitis or retinal detachment. Vitrasert offers superior control of retinitis over systemic ganciclovir therapy [28]. The Vitrasert disc is composed of outer and inner permeable PVA layers surrounding a discontinuous hydrophobic EVA film. The device allows diffusion of fluid into the device dissolving the drug pellet, which then diffuses into the vitreous at a constant rate [2].

2.3.2. Fluocinolone Acetonide. Retisert (Bausch & Lomb, Rochester, NY) is a tablet containing 0.59 mg of fluocinolone acetonide that is coated with nonbiodegradable PVA and silicon laminate (Figure 1(b)). It is 5 mm long, 2 mm wide and 1.5 mm thick with a release rate of 0.3–0.6 mcg/d over a period of 30 months. It is inserted into the vitreous cavity and sutured to the sclera through a pars plana surgical technique similar to Vitrasert. In April 2005 it became the first FDA-approved device for use in the treatment of chronic noninfectious posterior uveitis [29].

In clinical studies Retisert was found to significantly reduce inflammation and lower intravitreal vascular endothelial growth factor (VEGF) levels. In patients with noninfectious posterior uveitis treated with Retisert, the recurrence rate of uveitis was reduced from 62% before treatment to 4%, 10%, and 20% at 1, 2, and 3 years, respectively following treatment [30, 31]. Despite the excellent reduction of uveitis, the complication rate was high. At 34 weeks, 51% of patients had an increased intraocular pressure (IOP) that required pressure lowering agents. At 3 years, 78% required pressure lowering agents and approximately 40% required glaucoma filtering surgery. In addition, 100% of phakic patients developed cataract formation within 3 years of implantation. Other side effects included hypotony (6.1%), retinal detachment (2.9%), endophthalmitis (0.4%), and the need for explantation at 2 years (3.6%) [31, 32].

Recently, the Multicenter Uveitis Steroid Treatment (MUST) trial compared the relative effectiveness of systemic therapy and fluocinolone acetonide implant for the treatment of noninfectious uveitis in 479 eyes over 2 years. It found that both treatment groups were effective and neither group was superior to the other in improving visual acuity. Systemic therapy was well tolerated while the implant group had an 80% risk of cataract surgery and 61% required treatment for elevated intraocular pressures [33].

2.3.3. Iluvien (Fluocinolone Acetonide). Illuvien (Alimera Sciences Inc., Alpharetta, GA; pSivida Inc., Watertown, MA) is an injectable nonbiodegradable intravitreal implant containing fluocinolone acetonide (Figure 1(c)). It is 3.5 mm long and 0.37 mm wide and releases fluocinolone acetonide at a rate of 0.2 mcg or 0.5 mcg per day over 18–36 months. It is inserted with a 25 gauge needle. Phase III clinical trials for diabetic macular edema (DME) were recently concluded.

The fluocinolone acetonide for diabetic macular edema (FAME) study group tested the low dose 0.2 mcg insert and the high dose 0.5 mcg insert against a sham implant. At 24 months, 28% of those receiving either dose had an improvement of ≥15 in best-corrected visual acuity (BCVA) letters compared to 16% of those in the control. At 36 months, it was 33.0% in the low dose and 31.9% in the high dose compared with 21.4% in the sham. Increased incidence of cataracts was seen in implanted eyes but long-term vision was not compromised. Increase in intraocular pressure was also a concern with implantation of the device. At 36 months, 4.8% of those receiving the low dose implant required glaucoma surgery but visual outcome was not impacted when compared to those who did not require incisional surgery. These results show promise in DME patients who otherwise have limited effective treatment options [34]. In addition to its use in DME patients, phase II trials for the treatment of exudative age-related macular degeneration (AMD) and retinal vein occlusion are also being conducted.

2.3.4. Triamcinolone Acetonide

(1) I-vation. (SurModics, Eden Prairie, MN) is a 0.4 mm × 0.21 mm titanium helical nonbiodegradable implant that

Table 3: Comparison of corticosteroid properties.

Steroid	Water solubility (μ/mL)	Half-life	Relative potency
Triamcinolone acetonide	21	18 days	1
Fluocinolone acetonide	50	1.3–1.7 hours	0.4 x
Dexamethasone	100	3–5 hours	3–5 x

contains 0.925 mcg triamcinolone acetonide (Figure 1(d)). Triamcinolone is coated with polybutyl methacrylate and polyEVA. It is intended for a sustained delivery of 2 years. The helical design increases the surface area for drug release and stabilizes the device onto the sclera [35]. It was recently found to be effective in the treatment of diabetic macular edema after 24 months in a Phase I clinical trial although all phakic patients developed visually significant cataracts and increases in intraocular pressure occurred in 50% of eyes [36].

(2) Verisome. Verisome (Ramscor, Inc., Menlo Park, CA) is a nonpolymer-based intraocular drug delivery system that provides long-acting intravitreal drug therapy. It can be injected through the pars plana using a 30 gauge injector. Triamcinolone has been used investigationally providing a mean vitreous level of 1.1 mcg/mL for up to 1 year [37].

2.3.5. Dexamethasone

(1) Ozurdex. Ozurdex (formerly Posurdex Allergan Inc., Irvine CA) is a rod-shaped 6.5 × 0.45 mm pellet composed of a mixture of dexamethasone as the active pharmaceutical ingredient (API) and biodegradable PLGA (Figure 1(e)). Although dexamethasone has a short half-life relative to triamcinolone, it is 20 and 5 times more potent than fluocinolone and triamcinolone, respectively (Table 3) [38].

Ozurdex is placed intravitreally through the pars plana with an injector using a 22-gauge needle device. The insert contains 0.7 mg dexamethasone and provides peak doses for 2 months initially followed by lower doses for up to 6 months. Ozurdex received FDA approval in June 2009 for the treatment of macular edema associated with retinal vein occlusion, and in September 2010, it became the second FDA-approved therapeutic agent for the treatment of noninfectious posterior uveitis.

In a 26-week, multicenter, double-masked, randomized clinical study in which 229 patients were randomized in a 1 : 1 : 1 ratio receiving 0.70 mg Ozurdex ($n = 77$), 0.35 mg Ozurdex ($n = 76$), or sham injection ($n = 76$). Eighty-one percent of patients had intermediate uveitis. At the eighth week primary endpoint, 47%, 36% and 12% of patients had no vitreous inflammation. The response was maintained at week 26. In addition, both treatment groups achieved a 3-line improvement in visual acuity and reduced central macular thicknesses on ocular coherence tomography at 8 weeks that was statistically significant compared to the sham group. The complication rates were not found to be significant. Twenty-three percent of eyes in the 0.7 mg Ozurdex group required IOP-lowering agents and none needed surgical intervention for glaucoma. Cataract formation was seen in 15% in the 0.7 mg group, 12% in the 0.34 mg group, and 7% in the sham group [39, 40].

(2) Surodex. Surodex (Oculex Pharmaceuticals, Sunnyvale, CA) is a 1.0 × 0.4 mm PLGA pellet that provides sustained release of dexamethasone after insertion into the anterior chamber. It is primarily targeted to reduce post-cataract surgery inflammation for 7–10 days [41].

2.4. Experimental Intravitreal Implants

2.4.1. Cyclosporine. Cyclosporine A placed in the deep sclera adjacent to the suprachoroidal space has been found to be effective in controlling uveitis in an equine recurrent uveitis model [42]. In a chronic uveitis rabbit model, 2 mg cyclosporine A conjugated to a PCL/PLGA copolymer was found to be significantly more effective than oral cyclosporine [20].

2.4.2. Indomethacin. PLGA discs containing 7 mg of indomethacin released over 3 weeks was evaluated in a rabbit model. Although postoperative inflammation was decreased there was no significant decrease in posterior capsular opacification of the lens [43].

2.4.3. Particulate Drug Delivery Systems. The long-term drug delivery of small scale biodegradable devices has been recently investigated in experimental studies. These include liposomes, microspheres, and nanoparticles.

(1) Liposomes. Liposomes are spherical liposomal structures, about 0.01 to 10 μm in diameter (Figure 2). They are formed of a vesicular lipid bilayer separated by water or an aqueous buffer compartment [44]. They can circumvent cell membrane barriers and protect drugs from metabolic or immune attack. Since the phospholipid bilayers are naturally occurring, they are biocompatible and minimize toxicity and immunogenicity.

Liposomes are colloidal particles made of phospholipids that encapsulate hydrophobic or hydrophilic therapeutic agents. They often contain inner aqueous spaces where hydrophilic enzymes remain soluble and hydrophobic outer layers that allow passage through natural membrane barriers.

Currently verteporfin (Visudyne, QLT Inc. Vancouver BC, Canada) is the only liposomal drug that is FDA approved for use in the eye for the treatment of predominantly classic wet AMD. Liposomal amphoterecin B (AmBisome, Gilead Sciences, Foster City, CA) is used off-label for the treatment of fungal endophthalmitis and has been found to exhibit fewer side effects than the nonliposomal forms allowing for higher dosages (up to 30 micrograms) to be injected intravitreally [45].

(2) Particulate Ocular Drug Delivery Systems. Particulate Ocular Drug Delivery Systems include nanoparticles and microparticles. Although the distinction is often not consistent, nanoparticles are considered to be between 10 and

Table 4: Comparison of intravitreal implants for the treatment of noninfectious uveitis [40].

	0.59 mg fluocinolone acetonide (FA) implant	0.7 mg dexamethasone (DEX) implant
Administration	Operating room	Officebased
Matrix	Non-biodegradable	Biodegradable
Duration of effect	30 months	6 months
Improvement of >15 letters (% eyes)	21% by week 34	38% by week 26
Rescue medications (% eyes)	25.4% by week 34	22% by week 26
Glaucoma surgery (% eyes)	30.6% by month 24	0.5% by month 6
Cataract surgery (% eyes)	89.4% by month 24	4% by month 6

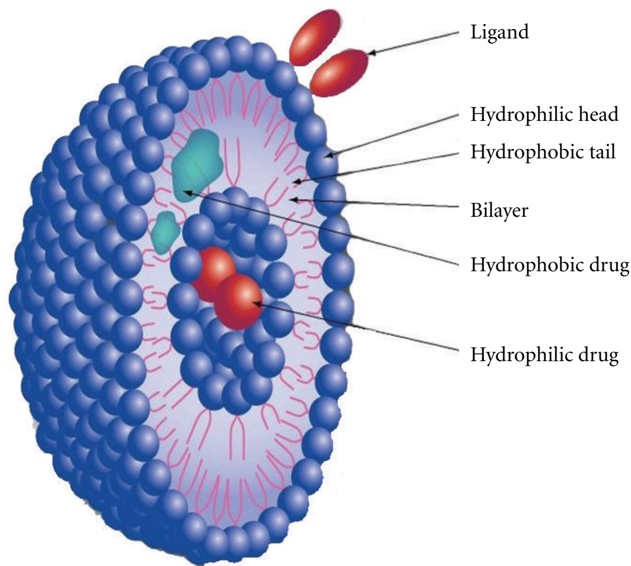

Figure 2: Liposome and its different drug-loading and surface functionalization modalities. (Courtesy of Nanomedicine (2010) Future Medicine Ltd).

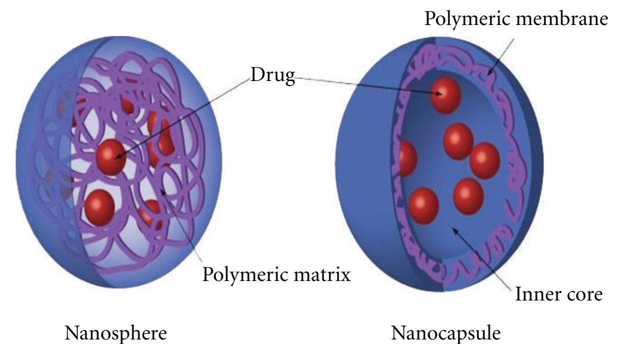

Figure 3: The two main types of polymeric nanoparticles known as nanosphere (matrix system) and nanocapsule (reservoir system) with different drug-loading modalities. (Courtesy of Nanomedicine (2010) Future Medicine Ltd).

1,000 nm in size and microparticles 1 to 1,000 μm in diameter [46]. Nanoparticles and microparticles are subdivided into nanospheres and microspheres which are a polymer-drug combinations where the drug is homogenously dispersed in the polymeric matrix, and nanocapsules/microcapsules, in which the drug particles or droplets are entrapped in a polymeric membrane (Figure 3).

(3) Microparticles. Microparticles are similar to liposomes in shape, size, and route of administration. However, nanoparticles offer several advantages over microsomes such as higher stability and larger drug-loading capabilities. Polymers such as PLGA and PLA are widely used for nanoparticle drug delivery systems. Surface polymer modifications also provide greater protection of the drug against degradation and phagocytosis by macrophages [47]. Although there are no currently used FDA approved microparticle devices, a wide variety of therapeutic agents are being investigated to improve the cellular penetration and allow long-term delivery using microsphere and nanosphere technology (Table 4).

(4) Microspheres. Microspheres have been developed for sustained ocular delivery of therapeutic agents such as progesterone, adriamycin, and pegaptanib [48–50]. Microspheres composed of chitosan, a natural biodegradable polymer, have been used for transcorneal acyclovir delivery [51]. A sustained release of microsphere-encapsulated cyclosporine was found to be present compared to cyclosporine solution [52].

(5) Nanoparticles. Nanoparticles have been used experimentally with several agents. Tamoxifen (PEG coated) was found to be effective in the treatment of experimental autoimmune uveitis in a rat model [53]. Intravitreally injected nanoparticles containing ganciclovir and acyclovir have been studied in a rabbit model with steady drug concentrations, but were found to be associated with cataract formation and flare [54]. Scleral injections of pigment epithelium-derived factor (PEDF) resulted in increased PEDF expression in the retina and retinal pigment epithelium and resulted in significant reductions of choroidal neovascularization in mouse and pig models [55, 56]. In a Phase I clinical trial, recombinant adeno-associated viral mediated expression of (rAAV-PEDF) was administered intravitreally in patients with exudative AMD. Although this resulted in transient intraocular inflammation (25%) and IOP elevations (21%), no other adverse events were seen and the majority of the patients achieved stable or improved visual acuity [57].

3. Summary

In conclusion, the eye is well suited for local delivery of therapeutics to treat vitreous inflammation as well as other pathologic conditions that induce visual loss. However, there are some unique challenges in designing local ocular drug delivery devices, which include implants, polymers, and micro- and nanoparticles. An integrated approach involving biomedical engineering, molecular biology, immunology, pathology, and pharmacology will continue to be critical to designing optimal devices for ocular inflammation and other diseases.

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Review Article

Role of Inflammation in Endophthalmitis

J. L. Vallejo-Garcia,[1] M. Asencio-Duran,[2] N. Pastora-Salvador,[2] P. Vinciguerra,[1] and M. R. Romano[1]

[1] *Ophthalmology Department, Istituto Clinico Humanitas, Rozzano, Milan, Italy*
[2] *Universitary Hospital La Paz, Madrid, Spain*

Correspondence should be addressed to J. L. Vallejo-Garcia, jvallejogarcia@gmail.com

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Inflammation originating from infection of the vitreous cavity is called endophthalmitis. Attention has been focused on the epidemiologic, microbiologic reports, and treatment options; unfortunately, the role of the host immune reaction in the visual function damage is still not well understood. Endophthalmitis occurs most frequently after cataract surgery. In this paper we review the published literature regarding inflammatory mediators and apoptosis during the course of endophthalmitis. Toll-like receptors, cytokines, high-mobility group box 1 proteins, aB-crystallin and apoptosis have been studied during clinical and experimental cases of endophthalmitis. Further understanding of the host-immune reaction to vitreous infection is essential for the development of new therapies. The use of intravitreal antibiotics and corticosteroids, vitrectomy and systemic antibiotics for the preservation of visual function is still discouraging.

1. Role of Inflammation in Endophthalmitis

Endophtalmitis is defined as inflammation originating from infection of the vitreous cavity. The specific features of the cellular damage that is created from the excessive immune response are still not well understood. The inflammatory cascade activated by the specific toxic effects of the pathogen ultimately determines the final anatomical and functional visual outcome. Treatments available to neutralize the infection and to diminish the inflammatory damage are intravitreal antibiotics, intravitreal corticosteroids and vitrectomy. Although systemic antibiotics did not demonstrate any added treatment benefit in the Endophthalmitis Vitrectomy Study (EVS) [1], systemic therapeutic agents are currently widely used as their intraocular penetration and spectrum range has significantly improved. It is not clear whether the most severe damage to the visual function is caused by the infectious process or by the host immune response. Endophthalmitis is classified according to its origin as exogenous (postsurgical, after penetrating trauma, or contiguous infection) and endogenous or metastatic. It is also classified according to its presentation as acute, if it occurs within 6 weeks of surgery, or chronic, more than 6 weeks following surgery. Endophthalmitis after cataract surgery is responsible for 90% of endophthalmitis cases [2]. The incidence of endophthalmitis after cataract surgery ranges between 0.087 and 0.265% [3, 4].

Bacteria are responsible for the majority of endophthalmitis cases and the prevalence is higher in tropical locations. Isolated outbreaks have also been reported due to contamination of ocular irrigation fluids during surgery [5, 6]. Positive cultures were obtained in 69% samples in the EVS and coagulase-negative staphylococci. Most frequent germs are coagulase-negative staphylococci, accounting for more than 50% of the positive cultures, followed by other gram-positive germs like *Streptococcus* spp. and *Staphylococcus aureus*. Gram-negative organisms were responsible for 6% of cases and 2 or more organisms were found in 2.4 to 4% of cases [1, 7].

The ability of bacteria to cause endophthalmitis is related to the bacterial load and to the virulence of the organism such as rapid replication in the eye and the production of toxins that produce inflammatory reaction and cellular necrosis. Gram-positive cell wall components such as peptidoglycan, lipoteichoic acid and capsular polysaccharide have intraocular proinflammatory properties even if the

organisms themselves are inactive [8, 9]. Gram-negative cell walls contain lipopolysaccharides, which are also proinflammatory [10]. The production of different types of bacterial enzymes such as hemolysins, lipases, enterotoxins, proteases, collagenases and hyaluronidases damages the host tissue. In addition to bacterial growth and direct toxicity, excessive host inflammatory response is responsible for impaired visual outcome due to photoreceptor toxicity; as these cells do not replicate, it is essential to minimize the collateral damages caused by inflammation. Ocular tissue evolution has developed an immune-privileged microenvironment to suppress the destruction of its cells, as it is critical to preserve the integrity and functionality of retinal cells and the clarity of the visual axis. This requires the preservation of specific anatomic characteristics such as the blood-ocular barrier and soluble immunosuppressive factors.

Suppressor immunity is expressed by the induction of antigen-specific efferent suppressor CD8 T cells and afferent suppressor CD4 T cells also known as T-reg cells [11]. The ocular microenvironment is rich in immunosuppressive molecules that influence the activity of immune cells, such as neuropeptides-like α-Melanocyte-stimulating hormone (α-MSH), vasoactive intestinal peptide (VIP) and somatostatin (SOM), the cytokines-like transforming growth factor beta-2 (TGF-β2), indoleamine 2,3-dioxygenase enzyme (IDO), prostaglandin E2, and surface expression of FasL to suppress the activation of Th1 cells [12]. The presence of migration inhibitory factor (MIF) in aqueous humor prevents natural-killer-cell activation [13]. They not only suppress endotoxin-induced inflammatory activity, but also induce an anti-inflammatory cytokine production by macrophages. Breakdown of these mechanisms that preserve immune privilege from inflammatory eye diseases such as endophthalmitis, uveitis and keratitis can result in destruction of host tissue and loss of vision. This breakdown can be triggered by both infectious and immune mechanisms, toll-like receptors (TLRs), cytokines are part of the initiation of the eye immune response. Direct cellular damage and apoptosis are the consequences of the bacterial attack and host immune reaction.

2. Toll-Like Receptors

TLRs are a family of receptors that recognizes microbial-associated molecular patterns from diverse organisms, including bacteria, viruses, fungi and parasites (Table 1) [14]. Activation of TLRs on immune cells by pathogens or their products initiates the innate response characterized by the expression of proinflammatory mediators and antimicrobial effector molecules, responsible for recruiting immune cells to the site of infection, mediating host inflammatory response to injury and stress and tissue repair [14, 15]. Initial inflammatory responses mediated by TLRs are required for host defense against invading pathogens.

TLRs have been identified in many cells throughout the eye, including retinal pigment epithelial (RPE) cells, astrocytes, corneal epithelium, iris epithelium, retinal microglia and Muller cells [14, 16, 17]. The presence of TLRs in microglia and Muller glial cells constitute an important feature in the recognition and initiation of the innate response to live pathogens and other microbial products such as lipopolysaccharids, other lipoproteins, peptiglicans, hemolysins, phospholipases, enterotoxins and proteases [14, 17].

Table 1: Toll-like receptors and their known ligands.

TLR	Principal exogenous ligand(s)
TLR2	Lipoproteins/lipopeptides (various pathogens) Peptidoglycan and lipoteichoic acid (gram-positive bacteria) Zymosan (fungi)
TLR3	Double stranded RNA (viruses)
TLR4	LPS (gram-negative bacteria) Bacterial HSP6 Respiratory syncytial virus coat protein
TLR5	Flagellin (flagellated bacteria)
TLR7	Imidazoquinolone antiviral drug Double stranded RNA (viruses)
TLR8	Single stranded RNA (viruses) Imidazoquinolone antiviral drug
TLR9	Unmethylated cytidine-phosphate-guanosine CpG
TLR10	Unknown

Experimental models of TLR attenuated animals have demonstrated that the damage induced is lower than in normal TLR models. Novosad et al. [15] demonstrated that TLR2 deficient mice model of endophthalmitis resulted in decreased intraocular proinflammatory cytokine/chemokine levels and altered recruitment of inflammatory cells into the eye, resulting in less intraocular inflammation and preservation of retinal architecture, and a slightly greater degree of retinal function. Kumar et al. [18] demonstrated that a TLR2 ligand, Pam3Cys, injected intravitreally previously to an *S. aureus* endophthalmitis murine model attenuated the clinical inflammation, reduced the bacterial load in the retina, and preserved intact retinal architecture with normal electroretinogram (ERG) response. They also mentioned that intravitreal injection of Pam3Cys, alone or with antibiotics (vancomycin and ceftazidime) 24 h after *S. aureus* infection significantly improved the outcome of endophthalmitis in B6 mice (unpublished data).

In another similar study Kochan et al. [17] also demonstrated the benefit of the TLR2 ligand in a mouse model of *S. aureus* endophthalmitis. They studied the behaviour of retinal microglia, normally present in the inner and outer plexiform layers, and found that TLR2 expression in this population was increased and activated cells presence was frequent in the ganglionar cell layer of the infected animals. They also demonstrated that pre-endophthalmitis treatment with TLR2 ligand significantly increased their phagocytic activity and reduced the inflammatory response.

3. Cytokines

Cytokines are polypeptides that act as intercellular messengers that play an important role in mediating processes of

inflammation and repair. They are secreted by macrophages, lymphocytes, natural killers, endothelial cells, in vitro RPE cells and other immune cells [19, 20]. The cytokines include tumour necrosis factors (TNFs), interleukins (ILs), interferons (IFNs), and a number of growth factors. They have been grouped into four categories or phases of inflammatory reaction.

(a) Recognition (mainly IL-1 and TNF-α); they are rapidly expressed and basic for the establishment of cytokine networks.

(b) Recruitment (called "chemokines"—human IL-8); they are essential for the elicitation of leukocytes.

(c) Removal (mainly IFN-γ, IL-2, and IL-6); their function is the activation of macrophages (IFN-γ) or lymphocytes (IL-2 and IL-6).

(d) Repair (growth factors); necessary to restore tissue structures.

The endophthalmitis immune response generates cell activation and cytokine secretion to suppress the infectious process. Petropoulos et al. [19] used an animal model of endophthalmitis caused by *S. epidermidis* to serially measure the levels of TNF-α, IL-1β, and IFN-γ. TNF-α and IL-1β behaved in a similar fashion, they peaked earlier, at 12 h after injection, while IFN-γ reached its maximum levels later, at 48 h. At day 7 after injection there was no statistically significant difference in cytokine levels between the experimental and the control groups. Clinical inflammation behaved in a similar way with the peak occurring slightly after the cytokines. TNF-α, IL-1β, and IFN-γ were not detected systemically, suggesting only local production. Clinical signs of endophthalmitis peaked at 24 h and by day 7 they were virtually nonexistent. TNF-α and IL-1, both produced by macrophages, are considered to be early initiators of this inflammatory process [16].

However it is not clear how the intraocular immune reaction starts. Rosenbaum showed in 2 studies that administration of a human IL-1 receptor antagonist (IL-1ra) did not block endotoxin-induced uveitis (EIU) in rabbits and that inhibitors of TNF-α also failed to block EIU [21, 22]. In another uveitis model, Brito et al. [23] demonstrated reverse passive Arthus reaction (RPAR), that mice deficient for the 2 known TNF-α receptor (TNFR) or IL1 receptor type I or both had a significantly reduced infiltration by inflammatory cells. The difference was the greatest in mice deficient for both receptors.

A specific endophthalmitis model in mice deficient for TNF-α with *B. cereus* resulted in reduced inflammation, more rapid bacterial replication, retinal function loss, and compensating proinflammatory cytokines. Chemokines were synthesized in the eye in the absence of TNF-α, resulting in less inflammation but an equally devastating course of infection [24].

High-mobility group box 1 (HMGB1) proteins are another class of molecules that have been identified in high concentrations in endophthalmitis and experimental uveitis [25, 26]. They are an abundant nonhistone nuclear protein with a dual function dependent upon its cellular location. In the nucleus, HMGB1 binds to DNA and is critical for proper transcriptional regulation. HMGB1 can also be passively released into the extracellular milieu by necrotic cells and secreted by activated macrophages, acting as a necrotic marker of tissue damage and a proinflammatory cytokine-like mediator.

Arimura et al. [25] studied the presence of HMGB1 in cases of human endophthalmitis using idiopathic macular holes as controls, and found that the HMGB1 levels in the vitreous were significantly elevated in eyes with endophthalmitis, especially those with a longer disease duration. Interestingly, HMGB1 concentration was significantly correlated with visual acuity; patients having the higher concentrations had lower visual acuity (VA). They also analysed one enucleated eye because of endophthalmitis secondary to corneal ulcer. HMGB1 was present in the cytoplasm and nuclear region of the choroid and retina, diffusely in all retinal layers including outer segments, outer plexiform layer, and especially in the damaged ganglion cell layer with infiltrating inflammatory cells. In a control enucleated eye due to a malignant conjunctival melanoma, HMGB1 was observed predominantly in the nuclei of retinal and choroidal cells suggesting that reduced HMGB1 concentration during endophthalmitis may reduce inflammatory induced retinal damage. Interestingly HMGB1 serum levels are elevated in sepsis, and an experimental sepsis model with specific inhibition of HMGB1 activity demonstrated an improvement in the clinical course and survival rate [27, 28].

4. Apoptosis

Apoptosis, or programmed cell death for controlled deletion of unwanted cells, involves a sequence of events including blebbing, cell shrinkage, nuclear fragmentation, condensation of nuclear chromatin and DNA fragmentation. Finally apoptotic bodies are produced, and these are engulfed and quickly removed by phagocytic cells before the contents of the cell can spill out onto surrounding cells and cause damage. Apoptosis is controlled by a wide range of cell signals that may originate either intracellularly (intrinsic or mitochondrial pathway) or extracellularly (extrinsic pathway). Internal cellular damage upregulates the Bax protein which pricks the mitochondrial membrane forming high-conductance channels that allow release of cytochrome C from the mitochondria to the cytosol and activate caspases, which ultimately lead to cell death [29]. Extracellular signals may include toxins, hormones such as glucocorticoids, growth factors, nitric oxide, or cytokines [30]. When extrinsic aggression occurs, apoptosis can be directly initiated by TNF receptors or Fas receptors, both activating the caspase enzymes and leading to cell death.

In endophthalmitis, infection and inflammation involve many of these apoptosis-signaling molecules, and several experimental models have demonstrated that retinal cells apoptosis is increased [31, 32]. Pharmakakis et al. [31] created a model of *S. epidermidis* experimental endophthalmitis and found that there was an increased rate of apoptosis in

correlation with upregulation of the expression of proapoptotic proteins Bax and Fas mainly within the ganglion cells, bipolar cells, and photoreceptors. Inflammation peaked at 24 hours after injection, Bax and Fas expression peaked at 48 hours after injection, and apoptotic rate peaked at 72 hours.

In a study to alter the normal apoptosis rate, Engelbert and Gilmore [32] tested the behaviour of FasL deficient mice in an *S. aureus* endophthalmitis model. They found that FasL expression on ocular tissues was essential for efficient clearance of *S. aureus.* Deficient FasL mice recruited less phagocytic cells and lost retinal function earlier with lower bacterial loads as compared with wild-type mice. Apoptosis through Fas is considered an important mechanism of maintenance of the immune privilege of the eye and contributes to the regression of the inflammatory process after the elimination of the etiologic factor [33].

aB-crystallin is a small heat shock protein that plays a critical role in protecting against apoptosis. It is expressed in long-lived tissues, such as muscle, brain and lens [34, 35]. It prevents apoptosis by inhibiting the activation of caspase 3 directly or indirectly by binding to the proapoptotic factors Bax and Bcl-X(S), by preventing their translocation into the mitochondria and by restricting the release of cytochrome C [36]. Recent studies have demonstrated that aB-crystallin is expressed in the retina (in the ganglion cells, inner and outer nuclear layers, inner segments and retinal pigment epithelium), where it is upregulated and prevents apoptosis in response to oxidative stress [37, 38]. Whiston et al. [39] analysed this protein in an *S. aureus* endophthalmitis model. They used wild and aB-crystallin knockout mice. Their results demonstrated that in the early response aB-crystallin is upregulated; however they also found that *S. aureus* produces a protease that cleaves and inactivates aB-crystallin. Deficient mice showed the same ability to clear the infection as wild mice but interestingly retinal function was significantly reduced and took more time to recover. Histological analysis demonstrated higher levels of apoptosis and retinal damage in the deficient mice.

5. Conclusions

Endophthalmitis often has a devastating effect on the eye and on visual function. Currently, vitrectomy, intravitreal antibiotics and corticosteroids are our main treatment options. Corticosteroids are the only available anti-inflammatory treatment used. The EVS advocated oral prednisone treatment (1 mg/Kg day) for 5 to 10 days, starting one day after intravitreal antibiotics [1]. More recently the use of intravitreal corticsteroids has been examined in several studies. Although they clearly seem to diminish the inflammatory reaction [40], their ultimate effect on the VA is contradictory as some studies have found a beneficial trend [41, 42], while others demonstrated no relation with the outcomes in eyes treated with intravitreal dexamethasone [43, 44]. Experimental endophthalmitis models have not clearly demonstrated the benefit of intravitreal dexametasone. Some studies have shown better electroretinogram function, less tissue destruction and reduced clinical inflammation scores in the groups treated with the combination of intravitreal antibiotics and dexametasone compared to intravitreal antibiotics alone [45, 46], while others demonstrated no benefit [47, 48]. Unfortunately, corticosteroids have not been demonstrated to successfully control the host immune reaction.

TLR2 ligand, Pam3Cys, has demonstrated encouraging results when administrated pre-endophthalmitis, but also when injected at 24 hours of the infection in combination with intravitreal antibiotics. In clinical practice, prevention of endophthalmitis is crucial; Pam3Cys properties are both prophylactic and therapeutic; if the results obtained by Kumar et al. [18] and Kochan et al. [17] are furtherly validated, a new treatment option that increases bacterial clearance and protects retinal function may be available. aB-crystallin upregulation during endophthalmitis seems to protect retinal functionality as it reduces apoptosis in retinal cells [39]; understanding better how this happens and managing to induce this upregulation can be an important step to achieve better functional results after endophthalmitis.

In order to improve the outcomes, it is essential the better understanding of the host immune reaction and the cellular pathways leading to tissue damage. Different types of research involving all the above mentioned mediators of inflammation are going on but we are still at initial stages. More efficient elimination of microorganisms, modulation of inflammation prior to retinal tissue damage and cellular protection are the pathways for the development of new therapeutic options that will help us to improve the final functional outcomes of this devastating condition.

Disclosure

The authors have no proprietary or commercial interest in any materials discussed in this paper.

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Review Article

Sterile Endophthalmitis after Intravitreal Injections

Joaquín Marticorena,[1] Vito Romano,[2] and Francisco Gómez-Ulla[1, 3]

[1] *Retina and Vitreous Unit, Instituto Oftlamológico Gómez-Ulla, 15705 Santiago de Compostela, Spain*
[2] *Department of Ophthalmology, Second University of Naples, 81100 Naples, Italy*
[3] *Department of Ophthalmology, University Hospital Complex of Santiago de Compostela, 15706 Santiago de Compostela, Spain*

Correspondence should be addressed to Joaquín Marticorena, jmarticorena@gmail.com

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Sterile endophthalmitis appears as an infrequent complication of intravitreal injections and seems to develop mainly in the context of the off-label use of drugs that have not been conceived for intravitreous administration. The aetiology of sterile endophthalmitis, independently of the administered drug, remains uncertain and a multifactorial origin cannot be discarded. Sterile inflammation secondary both to intravitreal triamcinolone acetonide and to intravitreal bevacizumab share many characteristics such as the acute and painless vision loss present in the big majority of the cases. Dense vitreous opacity is a common factor, while anterior segment inflammation appears to be mild to moderate. In eyes with sterile endophthalmitis, visual acuity improves progressively as the intraocular inflammation reduces without any specific treatment. If by any chance the ophthalmologist is not convinced by the sterile origin of the inflammation, this complication must be treated as an acute endophthalmitis because of the devastating visual prognosis of this intraocular infection in the absence of therapy.

1. Introduction

It was Rycroft in 1945 who first described the intravitreal injection of penicillin for the treatment of endophthalmitis [1]. Intravitreal injections give the opportunity of administering the drug straight where it is necessary. The vitreous cavity offers the great advantage of being a reservoir where high levels of drugs can be maintained for long periods, exceeding by far the concentrations obtained by the administration of drugs through other ways (i.e., topical, intravenous) and minimizing possible systemic side effects due to the small dose given and the little amount of drug that may escape from the eye into the systemic circulation. All these advantages and the presence of novel drugs designed specially for intravitreal use have produced an enormous increase in the number of intravitreal injections administered. The safety profile of intravitreal injections depends not only on the surgical technique, but also on the characteristics of the administered drug. Probably, the most feared and potentially devastating complication of intravitreal injections is endophthalmitis. Once the diagnosis of acute infectious endophthalmitis is suspected, vitreous tap for microbiological study and administration of intravitreal antibiotics must be done, while pars plana vitrectomy will be necessary in a subgroup of patients [2]. Prompt diagnosis and treatment of this entity are crucial for obtaining the best visual prognosis. On the other hand, certain intravitreal-administered therapies can produce an acute and sterile intraocular inflammation that can mimic a true endophthalmitis, but the former is related to good visual prognosis with resolution without the need of intravitreal antibiotics or surgical treatment. For the ophthalmologist it is crucial to know the potential inflammatory reaction that can be associated with the use of certain therapies, as well as to distinguish sterile endophthalmitis from infectious endophthalmitis in order to establish the adequate treatment. The purpose of this paper is to describe the clinical features of sterile endophthalmitis and to discuss the possible mechanisms involved in the development of inflammation after the administration of different drugs by intravitreal injection.

2. Definition of Sterile Endophthalmitis

For the purpose of this paper, we have defined sterile endophthalmitis as the acute intraocular inflammation of the vitreous cavity that resolves without the need of intravitreal antibiotics and/or vitreoretinal surgery. Necessarily, if vitreous microbiological study has been done, it needs to be negative culture proven. Patients treated with intravitreal antibiotics or vitrectomy, despite having negative cultures, were excluded from the analysis since an infectious origin of the inflammation cannot be ruled out [2]. The administration of topical antibiotics alone or in combination with intravenous antibiotics was not considered an exclusion criterion for being a sterile endophthalmitis since these treatments would not resolve by themselves a true acute infectious endophthalmitis. A review of the literature published in Pubmed between 1945 and June 2012, searching for keywords endophthalmitis, pseudoendophthalmitis, sterile endophthalmitis, and pseudohypopyon in combination with intravitreal injection, was done. Results were restricted to articles in English and Spanish. The search retrieved 334 articles that were analysed. Other articles referenced in the literature obtained through the initial search were also included.

3. Triamcinolone Acetonide

Triamcinolone acetonide is a white-colored, crystalline steroid. Almost insoluble in water, triamcinolone has an anti-inflammatory power 5 times greater than hydrocortisone. Because of the antiangiogenic and antioedematous properties of triamcinolone acetonide, it has been widely used as an off-label treatment for numerous eye diseases that have new vessels or an alteration of the blood-eye barriers. The development of sterile endophthalmitis after intravitreal triamcinolone acetonide (IVTA) has been described by numerous authors [3–12], and it is supposed to occur between 0.20% and 6.73% of the injections [4–7]. However, these numbers need to be interpreted cautiously since most of the reports are based on retrospective studies or small case series; therefore distinguishing sterile endophthalmitis from endophthalmitis can be difficult. Some cases catalogued in the literature as sterile endophthalmitis were treated with intravitreal antibiotics or vitrectomy, making it impossible to discard a true endophthalmitis. Other cases have been catalogued as sterile endophthalmitis just because of negative cultures. In other occasions vitreous haze secondary to dispersion of triamcinolone particles is difficult to differentiate from a real inflammatory process affecting the vitreous [5].

Sterile endophthalmitis secondary to IVTA has been described as a decrease in visual acuity that occurs more frequently within the first 3 days from the injection. Patients usually do not complain of eye pain. Slit-lamp examination may show some signs of mild-to-moderate intraocular inflammation in the anterior chamber such as flare, cells, and keratic precipitates [3, 4, 6]. Usually, hypopyon is not present [3, 4, 10, 11] and fundus examination typically reveals deep vitreous haze obscuring the retina. Nevertheless, it seems necessary to mention that, in the series described by Nelson et al. [5], eye pain was present in 4 cases while 7 cases had a severe inflammatory reaction in the anterior chamber with hypopyon. In the absence of specific treatment, vitreous haze can disappear between 2 weeks to 2 months [3, 4, 6]. Visual prognosis does not seem to be deteriorated and only some few cases have experienced a decrease of visual acuity despite clearing of the media. In these patients, visual decrease was most probably secondary to the underlying pathology than to the temporal inflammatory process.

The aetiology of sterile endophthalmitis is not fully understood. Contamination of triamcinolone vials with endotoxins has been postulated as a possible cause [4]. However, in the context of a cluster of sterile endophthalmitis, no endotoxins were found in the commercial vials of triamcinolone tested [12]. A toxic effect of the triamcinolone itself as well as the preservatives present in the vial (benzyl alcohol, polysorbate 80 and carboxymethylcellulose sodium) has been suggested. Retinal pigment epithelium and glial cells damage [13–15], together with an alteration in the morphology of rabbit photoreceptors, have been observed after the exposure to benzyl alcohol or commercial triamcinolone acetonide given at doses slightly higher than those used in human eyes [16, 17]. On the other hand, other studies in rabbits have not observed signs of cellular toxicity on morphologic or electrophysiologic tests [18–20]. Removal of benzyl alcohol by filtering the commercially available triamcinolone has been proposed as a possible method to reduce the rate of sterile endophthalmitis [9], but a couple of cases have been described even though triamcinolone was filtered and benzyl alcohol almost completely removed before IVTA [11]. An immune response to triamcinolone or any of the preservatives of the commercial vial has been also suggested as a possible cause of sterile endophthalmitis due to the development of intraocular inflammation after a second intravitreal injection [4]. Allergic reactions to triamcinolone have been described, but most possibly these cases corresponded to a reaction to any of the preservatives [21–23]. We observed a repeated episode of sterile endophthalmitis in a patient treated in 2 consecutive occasions with combined photodynamic therapy with verteporfin and IVTA [10]. In that patient, systemic and cutaneous allergic tests were negative; therefore, hypersensitivity reaction type 1 and type 4 were ruled out. However, non-IgE-mediated reactions have been observed with polysorbate 80. Considering that there are no systemic allergic reactions that would be necessary to prevent in patients with sterile endophthalmitis secondary to IVTA, the performance of allergy tests is of doubtful utility. Furthermore, negative allergy tests do not discard a future episode of inflammation.

4. Pseudoendophthalmitis and Pseudohypopyon after Triamcinolone Acetonide

Pseudoendophthalmitis is an infrequent complication of IVTA and occurs in about 0.74–0.8% of the injections [6, 24]. The term pseudoendophthalmitis has been used previously as synonymous of sterile endophthalmitis, but

most of the authors use it to the describe the dispersion of triamcinolone crystals and their passage from the vitreous cavity to the anterior chamber [25], more frequently in eyes with posterior capsule impairment or suspected zonular defect after being vitrectomized [8, 24, 26–29]. The settling of the crystals in the inferior angle of the anterior chamber produces the appearance of a "pseudohypopyon." This has been observed to happen immediately after the intravitreal injection, but usually occurs within the first 3 days. Patients typically do not present eye pain, conjunctival hyperemia, or any sign of intraocular inflammation [6, 24, 26, 29–34]. Pseudohypopyon usually can be differentiated from true inflammatory hypopyon on the slit lamp. Chen et al. [26] recommend to distinguish pseudohypopyon from true inflammatory hypopyon by tilting the patient's head and observing the shifting of the crystals upon the new position. Despite the amount of triamcinolone occupying the angle, no changes in the intraocular pressure have been associated with pseudohypopyon. Washout of the anterior chamber has been described in two cases of high-dose IVTA injections [29, 33], while all other cases resolved spontaneously between 4 days and 2 months [6, 24, 27, 30–32, 34]. No alterations of the anterior segment structures have been described once the triamcinolone reabsorbed.

5. Antivascular Endothelial Growth Factor Drugs

Bevacizumab (Avastin, Genentech, Inc., San Francisco, California, USA) is a full-length humanized monoclonal nonselective antibody against vascular endothelial growth factor approved by the Food and Drug Administration for the treatment of glioblastoma and of metastatic colorectal cancer, advanced nonsquamous non-small-cell lung cancer and metastatic kidney cancer in combination with chemotherapy. Rosenfeld et al. described for the first time the use of intravitreal bevacizumab (IVB) for the treatment of macular oedema secondary to retinal vein occlusion and exudative age-related macular degeneration [35, 36]. Since then, several studies have described the off-label use of IVB for the treatment of numerous vascular and oedematous eye diseases. The incidence of acute cultured proven endophthalmitis appears to be very low, ranging from 0.02% to 0.16% [37–39], while the incidence of sterile endophthalmitis has been described between 0.09% and 1.1% of IVB injections [37, 40–43].

An early and acute decrease in visual acuity appears as the most common symptom in patients with sterile endophthalmitis secondary to IVB. This can occur during the first 48 hours after the intravitreal injection and in all cases seems to be within the first week [40–42, 44]. Despite the intraocular inflammation, ocular pain seems to be infrequent [42]. Of the 44 cases observed by Chong et al. [40] blurred vision was present in 73% of the patients, floaters in 43%, and pain in 34%. Most of the patients had signs of inflammation in vitreous cavity (80%) as well as in the anterior chamber (77%). Considering just those eyes of this series that presented signs of inflammation that did not receive intravitreal antibiotics/vitrectomy, inflammation was mild to moderate in the anterior chamber in 7 out of 9 cases and mild to moderate in the vitreous cavity in 8 out of 9 cases. Interestingly, Georgopoulos et al. [42] observed a "pseudogranulomatous" inflammation of the vitreous because of the presence of large cellular aggregates. None of the reported cases with sterile inflammation presented fibrin or hypopyon [40–42, 44]. In the internet-based survey done by Fung et al. [41] all 10 cases of inflammation were catalogued as mild or moderate and lasted no longer than a week while sterile endophthalmitis cases reported by Chong et al. [40] resolved after 37 ± 5 days. These authors observed that mean time for visual acuity recovery was 53 ± 18 days and there was no difference between visual acuity observed at the end of the inflammatory process compared with pretreatment visual acuity [40]. A similar situation was observed in the 8 cases described by Georgopoulus et al. [42] where all patients but one recovered initial visual acuity.

It is necessary to mention that different degrees of acute anterior segment inflammation have been described after 0.25% of IVB injections [45]. Sterile intraocular inflammation has been described in patients with a severe inflammatory reaction in the anterior segment of the eye. Ocular pain and hypopyon were present in some of these patients, whereas vitreous inflammation was mild to moderate [46–49].

Diverse hypotheses have been proposed to explain the inflammatory response secondary to IVB. The solution of bevacizumab for intravenous administration comes in vials of 100 mg/4 mL or 400 mg/16 mL; therefore, obtaining different 0.1 mL or 0.05 mL doses for intravitreal use implies the manipulation and possible risk of contamination of the solution. As Wickremasinghe et al. mentioned in their report [43], although contamination of individual aliquots of bevacizumab with bacterial endotoxins during preparation may occur, this theoretical situation could explain clusters of sterile endophthalmitis in patients treated with injections coming from the same batch [44], but seems unlikely to be the cause of sporadic cases. Bacterial endotoxins are frequent and recalcitrant contaminants of antibody preparations during the production phase of the drug [50]. Preparations of bevacizumab that are originally designed for intravenous use may contain traces of endotoxin at levels that incite intravitreous inflammation, even though they are of no significance when the drug is administered systemically [43]. A specific immune reaction to the anti-VEGF antibody could also explain the development of sterile inflammation. Different authors have highlighted the presence of sterile endophthalmitis after repeated intravitreal bevacizumab injections [40, 43]. However, sterile endophthalmitis can develop after the first IVB. Another important fact is that the manufacture of bevacizumab recommends to keep it refrigerated between 2 and 8°C and protected from light [51]. Fluctuation of the temperature has been proposed as a factor that may increase immunogenic properties of bevacizumab [43]. Temperature fluctuation has been demonstrated to increase the immunogenicity of therapeutic proteins [52]. This may be due to protein degradation creating novel antigenic epitopes not found in the parent molecule [53].

Ranibizumab (Lucentis, Novartis Pharma AG; Genentech USA Inc.) is a recombinant, humanized monoclonal antibody Fab that neutralises all active forms of VEGF-A. Ranibizumab is approved for the treatment of exudative age-related macular degeneration, diabetic macular oedema, and macular oedema secondary to retinal vein occlusion. Pseudoendophthalmitis was reported to occur in 1 out of 599 patients (0.16%) treated with ranibizumab in the CATT study [54]. Unfortunately, there is no detailed information regarding the characteristics of this episode. Fauser et al. described 2 consecutive episodes of intraocular inflammation in the same patient. It occurred 24–48 hrs after the injection and visual acuity decrease, eye pain, hypopyon, and moderate vitreous cells were present [55]. The first episode was treated with intravitreal antibiotics, but no specific treatment was given for the second episode. Interestingly, there was no recurrence of the inflammation after a subsequent injection of ranibizumab. Sharma et al. described 1 patient (1/891 injections, 0.11%) with mild anterior chamber inflammation together with mild vitritis 3 days after ranibizumab injection [56]. There was spontaneous resolution of the inflammation and improvement of visual acuity. As far as the authors are aware, there are no other cases in the literature describing the development of sterile endophthalmitis secondary to intravitreal ranibizumab. The very low frequency of this adverse event may be related to the characteristics of the molecule, but the ultimate cause remains to be elucidated.

6. Methotrexate

Sterile endophthalmitis has been also described after the intravitreal injection of methotrexate in patients with primary central nervous system lymphoma involving the eye. Usually, multiple intravitreal injections of 200–400 μg/0.1 mL of methotrexate are required to observe the remission of the disease. In the literature there are some few cases of acute intraocular inflammation that developed after intravitreal methotrexate, but the majority of these cases lack detailed description of the ocular signs and evolution. In a series of 16 patients treated with intravitreal methotrexate, 1 patient developed intraocular inflammation that was catalogued as sterile endophthalmitis [57]. Microbiologic cultures were negative and the inflammation remitted rapidly after the administration of intravitreal antibiotics in combination with topical and systemic corticosteroids. In another series of 44 eyes from 26 patients, 2 patients developed severe intraocular inflammation that responded to topical steroids; the first one was catalogued as sterile endophthalmitis while the second was assumed as a toxic anterior segment syndrome [58]. However, it is important to mention that this entity is characterized by an early and intense postoperative inflammation after anterior segment surgery accompanied by minimal or no pain, fibrin formation, corneal edema, and the absence of vitreous involvement [59]. The mechanism of inflammation after intravitreal methotrexate remains uncertain.

7. Conclusions

Sterile endophthalmitis appears as an infrequent complication of intravitreal injections and seems to develop mainly in the context of the off-label use of drugs that have not been conceived for intravitreous administration. Sterile inflammations secondary to IVTA and to IVB share many characteristics such as the acute and painless vision loss present in the big majority of the cases. Dense vitreous opacity is a common factor, while anterior segment inflammation appears to be mild to moderate. Hypopyon is a very infrequent sign in the context of sterile inflammation after intravitreal injections. In eyes with sterile endophthalmitis, visual acuity improves progressively as the intraocular inflammation reduces without any specific treatment. In this study eyes treated with intravitreal antibiotics or vitrectomy were not included. This may constitute a bias by excluding severe cases that presented signs such as ocular pain or hypopyon. If by any chance the ophthalmologist is not convinced by the sterile origin of the inflammation, this complication must be treated as an acute endophthalmitis because of the devastating visual prognosis of this intraocular infection in the absence of therapy. The aetiology of sterile endophthalmitis, independently of the administered drug, remains uncertain and a multifactorial origin cannot be discarded.

Conflict of Interests

The authors declare that there is no competing/conflict of interests related to any topic of this paper.

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Review Article

Proteomic Analyses of the Vitreous Humour

Martina Angi,[1] Helen Kalirai,[1] Sarah E. Coupland,[1] Bertil E. Damato,[1] Francesco Semeraro,[2] and Mario R. Romano[3]

[1] *Liverpool Ocular Oncology Research Group, Department of Molecular and Clinical Cancer Medicine, Institute of Translational Medicine, University of Liverpool, Liverpool, UK*
[2] *Department of Ophthalmology, Spedali Civili di Brescia, University of Brescia, Brescia, Italy*
[3] *Department of Ophthalmology, Istituto Clinico Humanitas, Milan, Italy*

Correspondence should be addressed to Martina Angi, m.angi@liverpool.ac.uk

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The human vitreous humour (VH) is a transparent, highly hydrated gel, which occupies the posterior segment of the eye between the lens and the retina. Physiological and pathological conditions of the retina are reflected in the protein composition of the VH, which can be sampled as part of routine surgical procedures. Historically, many studies have investigated levels of individual proteins in VH from healthy and diseased eyes. In the last decade, proteomics analyses have been performed to characterise the proteome of the human VH and explore networks of functionally related proteins, providing insight into the aetiology of diabetic retinopathy and proliferative vitreoretinopathy. Recent proteomic studies on the VH from animal models of autoimmune uveitis have identified new signalling pathways associated to autoimmune triggers and intravitreal inflammation. This paper aims to guide biological scientists through the different proteomic techniques that have been used to analyse the VH and present future perspectives for the study of intravitreal inflammation using proteomic analyses.

1. Introduction

The human vitreous humour (VH) is a transparent, highly-hydrated gel, which occupies the posterior segment of the eye between the lens and the retina [1]. It is comprised almost entirely of water (99%) with the remainder consisting of a mixture of collagen fibres, hyaluronic acid, hyalocytes, inorganic salts, and lipids [2]. The average protein concentration of the healthy VH is 0.5 mg/mL, consisting largely of albumin (60–70%). Further components are globulins, coagulation proteins, complement factors, and low-molecular-weight proteins [3]. The ciliary body provides a constant fluid exchange by diffusion, ultrafiltration, and active transport of aqueous fluid into the posterior segment [4]. Proteins may accumulate in the vitreous by local secretion (e.g., glycoprotein), filtration from blood (e.g., albumin), or diffusion from the surrounding tissues [5]. Because of the close contact between the vitreous and the inner retina, physiological and pathological conditions of the retina affect both the proteome and the biochemical properties of the VH. Various vitreoretinal diseases induce changes in specific vitreous proteins, especially when the blood-retinal barrier is disrupted [6].

Because VH can be totally or partially removed without marked detriment to the eye [1], surgical vitrectomy and vitreous biopsies are performed as part of routine clinical practice, providing abundance of human VH samples for analysis. Many earlier studies investigated levels of individual proteins in VH from healthy and diseased eyes, using biochemical or immunological techniques, in particular enzyme-linked immunoabsorbent assay (ELISA) [7–10]. This approach, however, is not suitable for the discovery of networks of functionally related proteins; hence it can further our understanding of the pathophysiology of a disease only to a limited degree.

Proteomics is the large-scale study of the entire complement of proteins, the so-called proteome, present in a cell, tissue, biofluid, or organism in any given state [11]. A novel hypothesis can be generated from global protein expression analysis of disease tissue, which can then be addressed with

cellular and in vivo functional studies. Proteomic analyses of healthy and diseased VH have been performed [5, 6, 10, 12–24] to scrutinize the protein profile of vitreoretinal diseases, with the ultimate aim of identifying disease markers that could become the diagnostic and pharmaceutical targets of the future. The search so far has not been conclusive, but as proteomics is still an evolving field, better technologies and deeper understanding of the peculiar nature of the VH bear promising potential.

This paper aims to guide biological scientists through the different proteomic techniques that have been used to analyse the VH. It will discuss their findings and limitations. A second objective is to present future perspectives for the study of intravitreal inflammation using proteomics.

2. Proteomic Workflow

Proteomics experiments are categorised according to their objective: assay or discovery. Assay or targeted studies typically seek to quantify a predefined set of proteins or peptides, whereas discovery experiments aim to analyse larger, "unbiased" sets of proteins [11]. All proteomic analyses conducted on VH have used mass spectrometric discovery techniques to facilitate the identification and quantification of the many proteins occurring in the VH, expanding the spectrum of suitable candidates for targeted analyses.

Of the discovery methods that have been developed, all involve a multistep process, which includes sample acquisition, digestion of the protein sample into peptides, fractionation of the peptide mixture (or prefractionation of the proteins, depending on the technique chosen), protein identification by mass spectrometry, and data analysis. The various methods differ in their requirements for sample preparation, the extent and the level of sample fractionation (proteins or peptides), the type of MS, and the data processing tool used [25].

Each step will be described, reporting the different experimental strategies used for analysis of the VH and discussing their advantages and limitations.

3. Sample Acquisition

3.1. Anatomical Considerations. Anatomically, the vitreous body can be subdivided into three main regions: the vitreous core, the vitreous base, and the vitreous cortex. The vitreous core (or central vitreous) comprises the main bulk of the VH and is a highly hydrated extracellular matrix, which is normally a cellular. The vitreous base and cortex both contain a low concentration of cells, named hyalocytes, and dense bundles of collagen fibrils [1].

Skeie and Mahajan recently demonstrated by one-dimensional (1D) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) that the different substructures of the human vitreous, when individually isolated from post-mortem eyes, are characterised by an unique protein profile [26]. Hence, the dissection technique and the size of the sample are likely to influence the proteome composition.

3.2. Vitreous Sample Collection. For ethical reasons, it is not possible to obtain human vitreous samples from healthy eyes. Vitreous surgery necessitates a pathological state, even in retinal conditions such as macular pucker or macular hole. For this reason, some authors [5] argue that examining VH from a carefully selected biobank eye is more representative of the "normal" vitreous proteome. Whilst such opinion is debatable because of the postmortem changes that can occur, being able to harvest the entire vitreous body offers a definite advantage over the small sample produced by a core vitreous biopsy.

VH can also be extracted from eyes enucleated because of a trauma or an ocular malignancy. In such cases, it is important to preserve the integrity of the globe for pathological examination. In the authors' experience, the majority of the VH can be harvested anyway using a 23 G needle on a 10 mL syringe, which is inserted transclerally in the posterior segment of the intact globe. This yields at least 3 mL (out of the 4 mL total volume of the vitreous body). It is not advisable to harvest the VH following sectioning of an enucleated eye, as on opening the globe the more liquid part of the VH tends to spill, leaving the scientist with a highly viscous residue, which is nonrepresentative.

In the vast majority of studies undiluted core vitreous biopsies are taken at the time of surgical vitrectomy for an underlying vitreoretinal disease, most often proliferative diabetic retinopathy. Approximately 1 mL of undiluted VH can be obtained at the onset of pars plana vitrectomy, with closed infusion line, by manual aspiration with cutting on through the vitrectomy probe into a 2.5 mL syringe connected along the aspiration line. Core vitreous biopsies from patients undergoing vitrectomy for macular hole (MH) have been often used as "normal" controls, as MH is an idiopathic condition that develops as the result of vitreofoveal traction and is therefore unlikely to affect the protein composition of the VH [27].

Most proteomic studies have been conducted on vitreous fluid obtained from diabetic patients undergoing surgery for proliferative diabetic retinopathy (PDR), which is a major cause of vitreous haemorrhage. This is an important element to consider when collecting vitreous fluid for proteomic analyses, as the haemorrhage can cause a massive influx of serum proteins into the VH, confounding results. For this reason, Simó and colleagues have measured vitreous haemoglobin levels with a spectrophotometer and excluded all samples containing more than 5 mg/mL of haemoglobin [14, 19].

The preservation of biological state and sample quality prior to proteomic processing and analysis are extremely important. The proteins should be protected against loss or change as a consequence of proteolytic degradation. Ideally, VH should be snap-frozen in liquid nitrogen immediately and stored at −80°C until used [28]. Some authors recommend adding protease inhibitor cocktail to the VH sample prior to freezing [18].

3.3. Vitreous Sample Preparation. The ability to extract proteins is the key limiting factor in all subsequent proteomic

identification and profoundly influences differential protein identification associated with diseased states [29]. The main problem when handling VH specimens is the viscous nature of such samples.

The collagen fibrillar network and associated surface macromolecules maintain the VH in a gel state. With age, the vitreous undergoes progressive liquefaction, starting in the vitreous core as pockets of fluid that then coalesce [30]. Neal et al. have measured the viscosity coefficient of different regions of the human VH in phakic and pseudophakic donor eyes. In phakic eyes, viscosity is higher near the lens than near the retina, whilst this trend is reversed in pseudophakic ones [17]. Hence, the macromolecular composition and the viscosity of VH samples differ according to the anatomical region where the sample is taken, the age of the patient, the state of the lens, and the presence of any vitreous pathology.

Viscosity prevents accurate pipetting, posing a problem when small accurate aliquots are needed for antibody-based assays or for assessing the protein content of a large specimen (e.g., Bradford assay) prior to proteomic analyses. Various preanalytical treatments have been proposed to reduce viscosity, including boiling, high-speed centrifugation, microfiltration, dilution, and hyaluronidase treatment [31, 32]. The effect of these treatments on the VH has been investigated in forensic science for the postmortem analysis of chemical analytes such as glucose, urea, and creatinine, but there is no comparative study on the effect of such pretreatments on proteins.

High-speed centrifugation (12000 rpm for 15 minutes) is the most common technique that is used to separate the liquid component of the VH from its structural one [22]. Centrifugal filters, such as the 0.22 μm GV DURAPORE filter (Millipore, Carrigtwohill, Cork, Ireland) have also been used to clarify vitreous samples [15].

4. Fractionation

Because proteomes are very complex mixtures, a number of techniques have been employed to extract them prior to analysis.

Protein fractionation is an important first step in facilitating access to the low abundant proteins of interest for clinical research. The most common techniques for this purpose are affinity chromatography for protein depletion and gel electrophoresis for protein separation.

Peptide fractionation is used in "shotgun proteomics" where the entire proteome is digested into peptides, which are then fractionated and identified by MS. This approach is thought to introduce less bias into a biological sample; hence it is most frequently used in quantitative protein expression profiling. Column chromatography plays a major role in this phase.

4.1. Depletion of Highly Abundant Proteins. Albumin and immunoglobulin account for over 80% of the whole-vitreous protein content, possibly preventing the detection of less abundant proteins. This is particularly relevant in 2D-PAGE experiments, when large spots of albumin and immunoglobulin can overlap small spots, thereby obscuring less abundant proteins. Affinity chromatography is frequently used in proteomic studies of body fluids to deplete highly abundant proteins and enhance the detection of low abundance ones. In VH, IgG removal prior to electrophoresis has been achieved using Protein A Sepharose 4 Fast Flow (Amersham Pharmacia Biotech) [21] or with the ProteoExtract Albumin/IgG Removal Kit (Calbiochem, San Diego, CA, USA) [15].

Immunoaffinity subtraction (IS) is an alternative approach that allows bounding and retrieval of the 12 most abundant plasma proteins (HSA, IgG, fibrinogen, transferrin, IgA, IgM, apolipoprotein A-I, apolipoprotein A-II, haptoglobin, α1-antitrypsin, α1-acid glycoprotein, and α2-macroglobulin) from biological fluids using a commercially available system (Beckman Coulter ProteomeLab IgY-12 column, Beckman Coulter, Fullerton, CA, USA). Kim et al. treated VH samples from eyes with PDR using IgY-12 columns and subsequently compared the low and high abundance protein fractions obtained by 2-DE [15]. Forty-seven spots were excised from the low abundance protein gel and 5 proteins were identified, while 116 spots were excised from the high abundance protein gel and 25 proteins were identified. The identification rate was low in the low abundance protein gel, hence the authors abandoned this prefractionation technique suggesting that high abundance proteins account for the most protein in VH and that low abundance proteins of interest may have also been removed by the IS column, as verified in other studies [33].

4.2. Protein Separation by Gel Electrophoresis. SDS-PAGE separates proteins according to their electrophoretic mobility. The sample is first denatured with a buffer containing SDS, which charges each protein with a negative charge, identical per unit mass, so that the electrophoretic run leads to fractionation based solely on size. Depending on gel size and resolution, SDS-PAGE enables separation of proteins into about 10–50 fractions, which are recovered by excision and digested into peptides for sequencing by MS.

For separation of complex protein mixtures with a higher resolution, SDS-PAGE has been combined with isoelectric focusing (IEF), which separates proteins based on isoelectric points. This is called two-dimensional (2D) gel electrophoresis and has been used for several decades in proteomics. The use of immobilised pH gradient strips for IEF is an improved technique that allows resolution of hundreds of denatured proteins in a single 2-DE gel [34]. After electrophoresis, the proteins in the gel are stained for visualisation, quantification, and comparison. The various detection methods (radioactivity, dyes, fluorescence, and silver) as well as the data analysis issues that must be taken into account when quantitative comparative analysis of 2D gels is performed have been critically reviewed in a recent work [35].

2-DE has been the prefractionation technique of choice in the majority of proteomic studies on VH conducted to date [5, 6, 15, 17, 18, 21, 23, 24]. The stain and detection

software used evolved over time, moving from Coomassie Brilliant Blue (CBB) for global protein detection [23] to fluorescent dyes with higher sensitivity and dynamic range such as SYPRO Ruby protein stain [18]. Relative quantification of protein expression levels between samples was estimated based on the assumption that the optical density of the spots (OD%) had to be proportional to the protein concentration. Differences in apparent protein expression levels between the VH samples were considered potentially significant when matched spots exhibited at least a twofold difference in their averaged OD%. Using this technique, Ouchi et al. performed the first quantitative comparison of 2D gel protein expression in vitreous from patients with and without diabetic macular oedema (DMO), detecting 72 spots from DMO VH and 64 spots from non-DMO VH. The intensity of 8 spot was significantly different, leading to the identification of six proteins (PEDF, apolipoprotein A4, apolipoprotein 1, thyroid hormone receptor interacting protein-11, plasma retinol-binding protein, and vitamin D-binding protein) with higher expression in the DMO group [18].

A more reliable and reproducible method of relative protein quantitation from two or more samples is 2D fluorescence difference gel electrophoresis (DIGE), a version of 2D-PAGE where the proteins of each sample are labelled with a different fluorophore prior to electrophoresis [36]. Gels are scanned at wavelengths unique to each fluorescent label and the images are analysed for differences in protein patterns such as spot density or mass shift.

Using DIGE, Hernández et al. compared VH from eight diabetic patients with DME and eight nondiabetic controls and detected 1300 protein spots. The analysis of spots of differing intensity leads to the identification of 25 proteins, four of which were specifically associated with DMO [37]. García-Ramírez et al. had been the first to apply DIGE for analysis of the VH. Using this technique, they identified 11 proteins as differentially produced in the VH of PDR patients in comparison with VH from non-diabetic subjects; 8 were overproduced (ZAG, apolipoprotein A1, apolipoprotein H, fibrinogen A, C4b, factor B, C3, and C9) and 3 were significantly under produced (PEDF, IRBP, and ITIH2) [14]. The higher expression of apoliprotein A1 and H in PDR patients has been confirmed in a later study by the same group by DIGE and Western blot of VH samples, as well as mRNA expression in the retina [19].

5. Protein Identification

Mass spectrometry (MS) is the key analytical technique in proteomics for the identification and, increasingly, for the quantification of proteins. The principle of MS is to measure the mass (m) to charge (z) ratio of ions in the gas phase, hence the peptides need to be first transferred into the gas phase and ionised.

The two relevant techniques for ionization of peptides, proteins, and protein-like molecules (e.g., glycoproteins) are matrix-assisted laser desorption/ionization (MALDI) [38] and electrospray ionization (ESI) [39]. For MALDI, the analyte is dissolved and cocrystallised with a matrix on a probe surface, which is then irradiated by a UV laser pulses. The laser evaporates and converts analyte into gas phase at the ion source. The ionised analyte is then separated by the time-of-flight (TOF) analyser, most commonly employed in MALDI-MS. The m/z value of peptides is measured by recording the time ions require to travel over a fixed distance inside the mass analyser. In ESI, the peptide mixture is dissolved in a liquid solvent system instead of the matrix. Highly charged analyte droplets from a fine spray outlet are ionised at atmospheric pressure in the presence of a strong electric field, to generate a series of charged gas-phase ions. The charged ions are then emitted and focused into the high-vacuum region of the mass analyser, which records the various charge states of the molecule separated according to their m/z ratios. There are a number of mass analysers in addition to the above-described TOF: quadrupole, ion trap, orbitrap, and fourier transform cyclotron ion resonance (FT-ICR). Each one works differently, having their own strengths and weaknesses and can be used alone or in combination [40].

The mass spectra can be directly compared with protein databases for matching the molecular weights using appropriated scoring algorithm (peptide mass fingerprinting) [41]. This technique, however, is limited by the database (as it should contain prior information on the protein for matching) and by the complexity of the protein mixture (as it becomes difficult to select the right peptide mass from a lot of peaks) [42]. Tandem mass spectrometry (MS/MS) involves two consecutive steps: peptide mass determination and generation of partial amino acid sequence information for a particular peptide based on further fragmentation. The m/z values of the fragments are then recorded in the tandem mass spectrum. Tandem MS can be done by two separate analysers (e.g., TOF-TOF) or inside the same mass analyser (e.g., ion trap).

To enhance detection of proteins from very complex mixtures, frequently used platforms are the LC-MS/MS instruments, where ion-pair reversed chromatography or nanohigh performance liquid chromatography (HPLC) is used prior to tandem MS [43]. Advances in LC-MS/MS have greatly improved the dynamic range and sensitivity for analysis of complex protein mixtures [44]. Large-scale proteome profiling has been verified for different organisms, as well as mammalian tissues and cell lines by using multidimensional LC-MS/MS [45]. By adopting this technique, Yu et al. have scrutinised the protein profiles of VH from 24 patients undergoing vitrectomy for proliferative vitreous retinopathy (PVR) and 8 biobank eyes, identifying 363 proteins [22]. An even better example of how proteomics is strictly dependent on the technology employed has been provided by Kim et al., who could identify 49 proteins using 2-DE and 531 proteins using LC-MS/MS on the same set of VH from PDR eyes [15].

6. Data Analysis

Algorithms have been developed for amino acid sequence and protein identification by matching the information contained in mass spectra against a database of theoretical

or previously identified spectra. Algorithms can generate both false-positive and false-negative assignments, which are influenced by the stringency of spectra to sequence criteria. Discerning a true match from a false match is critical in proteomic data analysis. The most common tools for MS/MS-based peptide identification and data analysis have been comprehensively reviewed elsewhere [46].

Because of the complexity of the proteomic workflow and data analysis, it is essential to validate the identified candidate proteins using independent techniques, such as Western blot. Moreover, the experimental design needs to take into consideration the influence of technical and biological variabilities, which are particularly relevant in biological samples like the VH.

7. Previous Studies of the Vitreous Proteome

Fifteen studies conducted over the last decade have used a range of proteomic methodologies including 2DE, DIGE, ESI-MS, MALDI-MS, and LC-MS/MS to compare the vitreous proteome of patients with various stages of diabetic retinopathy (DR) and PVR to that from non-diabetic patients and those with MH [5, 6, 10, 12–16, 18–24, 37]. One other study investigated the proteome of VH from human phakic and pseudophakic donor eyes [17]. In general, the total protein content reported for the vitreous of patients with DR is higher than that measured in the non-diabetic and control samples. As already discussed above, this may be due, however, to an influx of serum due to vitreous haemorrhage and/or disruption of the blood-retinal barrier, leading to elevated levels of proteins not associated with intravitreal protein production. Indeed, in the study of Simó et al., a comparison of proliferative vitreoretinopathy and normal vitreous demonstrated upregulated levels of intraocularly produced lipoproteins in the former [19]. Overall, studies analysing the vitreous proteome in patients with DR have varied greatly both in terms of the total number of proteins identified and the number of proteins differentially expressed between the test group(s) and controls, as well as the specific proteins then proposed to play a role in the pathogenesis of vitreoretinal disease states. Although a detailed discussion of the specific proteins identified by these studies is beyond the scope of this chapter, it is clear that as proteomic technologies have evolved over this period, so the number of identified proteins has increased. Whether any of these proteins and the pathways that they regulate is of importance in the pathogenesis of DR remains a very interesting translational question, which is being investigated by more quantitative targeted approaches.

8. Future Perspectives: Proteomics for Intravitreal Inflammation

Intraocular inflammation accounts for 10–15% of bilateral and 22% of unilateral blindness in the United States, and 10% of visual impaired registration in the UK [47]. Many efforts are being made to deepen our understanding of the different aspects of the inflammatory process, evaluate new therapeutic strategies, and ultimately be able to deliver personalised care for patients with intraocular inflammatory diseases [48]. Animal models play a fundamental role in this process [49]. Proteomics analyses of intravitreal inflammation have not yet been performed on human samples, whilst they have been successfully performed on VH from animal models.

Endotoxin-induced uveitis (EIU) is an animal model of acute ocular inflammation. To characterize the mechanism of EIU, Bahk et al. analysed the infiltration of proteins in the vitreous bodies of rats with EIU and normal rats using 2-DE-MALDI-TOF/MS and micro LC/LC-MS/MS, identifying specific modifications in the crystallin family proteins [50].

Spontaneous equine recurrent uveitis (ERU) is a recurrent uveitis that develops in the horse and results in blindness [51]. It is the only spontaneous disease model for human autoimmune uveitis. The vitreous is the body fluid closest to the disease-affected tissue and possibly also an effector of pathological processes relevant for ERU. Surgical removal of the VH can lead to a considerable decrease in the frequency and severity of relapses, therefore vitreous composites are likely to contribute to disease progression [52]. Deeg and coworkers have been systematically comparing VH from healthy and disease-affected equine eyes by proteomic profiling [53, 54]. In an earlier study, they applied 2-DE-MALDI-TOF/MS, identifying a total of 42 proteins, 9 of which differentially expressed in ERU. These are functionally related to immune response, inflammation, and maintenance of the blood-retinal barrier [52]. More recently, they identified ERU-related functional protein networks and affiliated molecular signalling pathways using LC-MS/MS-based label-free quantification followed by pathway enrichment analyses [54]. The increased sensitivity gained by omitting gel-based prefractionation resulted in overall detection of 119 different proteins. A large fraction of these proteins were differentially expressed in ERU samples as opposed to controls (26 upregulated, 44 downregulated). Pathway enrichment analyses were performed using the ConsensusPathDB program, suggesting the participation of the Wnt pathway in the pathogenesis of the uveitis.

This shows how the development of MS-based methods significantly improved quantitative proteomic analyses of the VH, enabling comprehensive identification of differentially regulated proteins and detection of novel molecular pathways that could become the therapeutic targets of the future.

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