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REVIEW

- 1210 Role of pregnane X-receptor in regulating bacterial translocation in chronic liver diseases

Mohandas S, Vairappan B

ORIGINAL ARTICLE

Basic Study

- 1227 Liver atrophy after percutaneous transhepatic portal embolization occurs in two histological phases:
Hepatocellular atrophy followed by apoptosis

Iwao Y, Ojima H, Kobayashi T, Kishi Y, Nara S, Esaki M, Shimada K, Hiraoka N, Tanabe M, Kanai Y

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World Journal of Hepatology (*World J Hepatol*, *WJH*, online ISSN 1948-5182, DOI: 10.4254), is a peer-reviewed open access academic journal that aims to guide clinical practice and improve diagnostic and therapeutic skills of clinicians.

WJH covers topics concerning liver biology/pathology, cirrhosis and its complications, liver fibrosis, liver failure, portal hypertension, hepatitis B and C and inflammatory disorders, steatohepatitis and metabolic liver disease, hepatocellular carcinoma, biliary tract disease, autoimmune disease, cholestatic and biliary disease, transplantation, genetics, epidemiology, microbiology, molecular and cell biology, nutrition, geriatric and pediatric hepatology, diagnosis and screening, endoscopy, imaging, and advanced technology. Priority publication will be given to articles concerning diagnosis and treatment of hepatology diseases. The following aspects are covered: Clinical diagnosis, laboratory diagnosis, differential diagnosis, imaging tests, pathological diagnosis, molecular biological diagnosis, immunological diagnosis, genetic diagnosis, functional diagnostics, and physical diagnosis; and comprehensive therapy, drug therapy, surgical therapy, interventional treatment, minimally invasive therapy, and robot-assisted therapy.

We encourage authors to submit their manuscripts to *WJH*. We will give priority to manuscripts that are supported by major national and international foundations and those that are of great basic and clinical significance.

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Role of pregnane X-receptor in regulating bacterial translocation in chronic liver diseases

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Abstract

Bacterial translocation (BT) has been impeccably

implicated as a driving factor in the pathogenesis of a spectrum of chronic liver diseases (CLD). Scientific evidence accumulated over the last four decades has implied that the disease pathologies in CLD and BT are connected as a loop in the gut-liver axis and exacerbate each other. Pregnane X receptor (PXR) is a ligand-activated transcription factor and nuclear receptor that is expressed ubiquitously along the gut-liver-axis. PXR has been intricately associated with the regulation of various mechanisms attributed in causing BT. The importance of PXR as the mechanistic linker molecule in the gut-liver axis and its role in regulating bacterial interactions with the host in CLD has not been explored. PubMed was used to perform an extensive literature search using the keywords PXR and bacterial translocation, PXR and chronic liver disease including cirrhosis. In an adequate expression state, PXR acts as a sensor for bile acid dysregulation and bacterial derived metabolites, and in response shapes the immune profile beneficial to the host. Activation of PXR could be therapeutic in CLD as it counter-regulates endotoxin mediated inflammation and maintains the integrity of intestinal epithelium. This review mainly focuses PXR function and its regulation in BT in the context of chronic liver diseases.

Key words: Pregnane X receptor; Bacterial translocation; Chronic liver disease; Intestinal permeability; Inflammation; Tight junctions

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Core tip: Translocation of bacteria at pathological levels is a major driving factor in the progression of chronic liver diseases (CLD). However, it remains to be known whether it is the CLD condition that triggers leaky gut, or if translocation of bacteria plays an etiological role in the pathogenesis of CLD. Dysregulation of homeostasis in the gut-liver axis is considered as a crucial element that underlies the pathogenesis of BT. The nuclear receptor, pregnane X receptor (PXR) is widely expressed in gut and liver axis and is implicated in maintenance of equilibrium in the gut-liver axis. This

review will summarize the various studies that have highlighted the importance of PXR as the mechanistic linker molecule in the gut-liver axis and its role in regulating bacterial translocation in the pathogenesis of cirrhosis.

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INTRODUCTION

Pregnane X receptor

Pregnane X receptor (PXR) is an adopted orphan nuclear receptor (NR) that is part of a broad nuclear receptor superfamily. Specifically, PXR is encoded by *NR1I2* gene and is categorized as the 2nd member of group I (nuclear receptor subfamily 1) which also comprises VDR (NR1I1) and CAR (NR1I3)^[1,2]. Consistent with the majority of class 1 NR's, PXR behaves as a transcription factor that is present in the cytosol and is activated only post ligand binding. After ligand activation, these NR's form complexes with retinoid X receptor (RXR) and bind to DNA response elements of the genes that they regulate^[3]. PXR was initially characterized as a xenobiotic receptor that senses and responds only to exogenous toxic substances and prescription drugs. Over the last two decades, PXR is widely recognized for its additional roles in sensing a range of endobiotic compounds such as bilirubin, bile acids, dietary lipids and steroid hormones, and hence is also referred to as steroid and xenobiotic receptor (SXR)^[4]. PXR has been shown to be expressed in various tissues including stomach, placenta, kidney, lung, uterus and ovary, but is predominantly expressed in small intestine, colon and liver. Its increased expression in intestinal epithelium and hepatocytes also highlight its vital role in adaptive defense against xenobiotics and endobiotics exposure in intestine and liver^[3,5]. A recent study has shown evidence that hepatic stellate cell (HSC) expressed PXR and its activation lead to attenuation of HSC's differentiation and proliferation^[6]. A study using mice knock out model also highlighted the expression of PXR in monocyte/macrophage cells and their role in countering inflammatory profile^[7]. PXR expression has also been observed in other immune cells including T-cells and dendritic cells^[8].

Identical with other NR's, PXR contains a conserved DNA binding domain (DBD) and a flexible ligand-binding domain (LBD). Upon activation by a ligand, PXR binds to its response elements only as heterodimeric complexes that it forms with 9-cis retinoic acid receptor (RXR or NR2B) and others co-activators such as SRC-1^[9]. The structural feature that makes PXR stand out from the other NR's is its voluminous and flexible ligand-binding pocket. This enables PXR to bind and be

activated by a wide range of hydrophobic ligands. Indeed, its spherical shaped ligand binding pocket has a volume greater than 1150 angstrom, making its ligand cavity one of the largest to be characterized so far^[3], and on par with the NR PPAR- γ 's ligand pocket^[10]. It is also important to highlight the unselective nature of the PXR LBD, enabling it to sense and respond to chemicals within a broad molecular weight range (about 250-850 kDa)^[3]. An impressive range of bioactive components from herbal sources, such as Hyperforin, Paclitaxel and Guggul have also been added to the increasing list of naturally occurring PXR ligands^[11].

Functions of PXR

The primary and the most conceded function of PXR is to activate genes encoding drug metabolizing and drug transporter enzymes. It acts as sensors that monitor any alteration in the levels of foreign compounds or endobiotics^[3,12]. The genes that PXR activate take the responsibility of metabolizing and elimination of exogenous chemicals, and thus form the primary line of defense against toxicity challenge^[12,13]. In humans, among drug metabolizing cytochrome P450 (CYP) enzymes, CYP3A is the most copiously expressed isoform in the liver and intestine^[14]. Furthermore, transgenic rodent knock out models (KO)^[15,16] have established beyond doubt that PXR's are master regulators of *CYP3A* genes^[17], which encode proteins responsible for the metabolic oxidation of more than half of the known prescription drugs^[3]. Classic PXR activators such as pregnenolone 16 α -carbonitrile (PCN) and Rifampicin have also been used to validate the same. PXR also controls the expression of phase 2 conjugating enzymes such as SuT1a and UGT-1A which are primarily responsible for sulfate conjugation or glucuronidation of steroid hormones, bile acids and bilirubin^[13,18-21]. Following conjugation, PXR controlled phase 3 drug transport proteins like P-glycoprotein and MRP-2 are then involved in efflux transport and elimination of the toxic metabolites^[13,22].

The function of PXR extends beyond metabolism of drugs and endobiotics, which has made it a considerable area of research over the last decade. NR's, in general, are rising as major targets for drug discovery and the identification of additional roles of PXR has given a new perspective in freshly approaching already known disease pathologies. Apart from its most researched role in inflammatory bowel disorders (IBD), PXR dysregulation has been implicated in CLDs^[6,23] various cancers^[24] and metabolic disorders like obesity^[25]. PXR play a crucial role and aids hepatocytes in uptaking endobiotics or xenobiotics and is further involved in their metabolism and elimination^[26,27]. Anti-fibrogenic activity has also been documented, wherein PXR activation by its ligand PCN prevented the transdifferentiation of hepatic stellate cells into myofibroblasts^[6]. Similar observations were made when using another established PXR ligand Rifampicin, where PXR was associated with inhibition of major pro-fibrogenic factors such as transforming growth

factor- β (TGF- β) and Alpha smooth muscle actin^[6]. Interestingly, a significantly increased expression of PXR was observed in various tumor tissues when compared to non-neoplastic tissues, and a positive correlation was found between cell proliferation and PXR positive cells^[24].

PXR has also been attributed to playing a role in energy metabolism and has been linked with diseases such as type 2 diabetes, obesity and hyperglycemia. Activation of PXR has been observed to produce a suppressive effect on hepatic gluconeogenesis. However, it has also been reported to cause hepatic steatosis by increasing lipogenesis and fatty acid uptake^[25]. Increased skin inflammation was reported in PXR null mice when challenged with hapten and was associated with increased interferon gamma (INF- γ) and reduced anti-inflammatory cytokine interleukin-10 (IL-10)^[28]. The numerous roles of PXR in interaction with microbial metabolites, maintaining innate immunity, epithelial integrity, countering inflammation, bile acid trafficking and detoxification are discussed in detail in the upcoming sections.

GUT MICROBIOTA AND PATHOLOGICAL TRANSLOCATION

Gut microbiome and gut-liver crosstalk

The presence of bacteria in the gut gains significant importance because of the monumental level of interaction that happens between the gut microbiome and the host. In the human body, almost 100 trillion bacteria are in constant communication with the intestinal epithelium, which spans almost 400 m² in surface area-which is the largest in humans^[29]. It does not come as a surprise that, at such a level of interaction, some of the important physiological functions of the human host including digestion, energy metabolism, maintenance of intestinal integrity and innate immune homeostasis depend largely on the balance of host-microbiome interaction^[30]. These physiologic events are dependent on the extensive arsenal of microbial metabolites, which are yet to be characterized completely.

In physiological state, the commensal bacteria have influence beyond the intestine. In this context, Björkholm *et al.*^[31] have reported that more than 100 genes in the liver are differentially expressed between germ-free mice and their conventionally raised wild counterparts. However, the most gripping evidence put forth by this study was that majority of these genes that varied in expression in GF mice, were in fact related to xenobiotic metabolism. This study highlights the significance of xenobiotic sensors PXR and CAR as mechanistic links between microbes and host^[31]. Thus any alterations in the gut microbiome or its sensors will be reflected in the liver functionality. Accordingly, ulcerative colitis patients have been observed to have increased susceptibility to develop primary sclerosing cholangitis^[32]. Interestingly, any abnormalities in liver

function are also reflected as alterations in the quality and quantity of intestinal bacteria in the gut. Patients with intrahepatic cholestasis have been shown to manifest overgrowth of bacteria in the small intestine^[33]. Similar observations were made in NAFLD patients, where fat induced bile acid abnormalities were linked with bacterial dysbiosis^[34]. Moreover, studies in NAFLD and chronic alcohol feeding models have observed the manifestation of intestinal inflammation, which indirectly compromises gut integrity^[35,36].

Bacterial translocation

Bacterial translocation (BT) is defined as the passage of viable indigenous bacteria and bacterial products, such as endotoxin, from the intestinal lumen through the mucosa into mesenteric lymph nodes (MLNs) and other organs^[13,14,37,38]. It is a common physiological event in the healthy individuals and is tightly regulated by various levels of immune and physical barriers^[39]. However, BT is seen to happen physiologically at minor levels, where it is considered as a beneficial event to the host, especially in priming the host immune system^[38,39]. The mucus and tightly bound intestinal epithelial lining comprise the physical barrier, while gastric acid, antimicrobial peptides (AMPs), IgA antibodies and innate immune cells form the chemical/immune barriers^[38]. Physiologically dendritic cells constantly sample bacteria through their processes and help in priming the B-cells to secrete IgA. The DCs also aid in the transport of the translocating microbes to mesenteric lymph node (MLN), which serves as a central hub between the gut and rest of the body. MLN are also the location where the microbes are killed *via* local immune response^[38]. However, when the physiologic barriers are compromised, or if the quality or quantity of bacteria is altered in the gut due to other external abnormalities such as alcohol abuse and high fructose diet, the translocation of bacteria or its products is persistent and pathologic. This phenomenon results in chronic induction of both systemic and hepatic inflammation^[38,40].

Bacterial infections in patients with cirrhosis are correlated with a poor prognosis and an increased risk of mortality^[40]. BT can also exacerbate the hepatic and systemic hemodynamic abnormalities of liver cirrhosis. Pathological BT is an important and emerging mechanism for the pathogenesis of CLDs. Emerging research has also shown clear evidence of increased intestinal permeability, mucosal inflammation and detection of bacteremia and endotoxemia in patients with CLD^[41,42]. Indeed, spontaneous bacterial peritonitis (SBP) is considered the most evidenced clinical expression of BT, which responsible for 25%-40% of overall mortality in cirrhotic patients^[43]. Pathological BT has been implicated in a range of other complications that arise in CLD, including acute-on-chronic liver failure, hepatorenal syndrome and hepatic encephalopathy (HE)^[38]. The exact mechanisms of increased intestinal

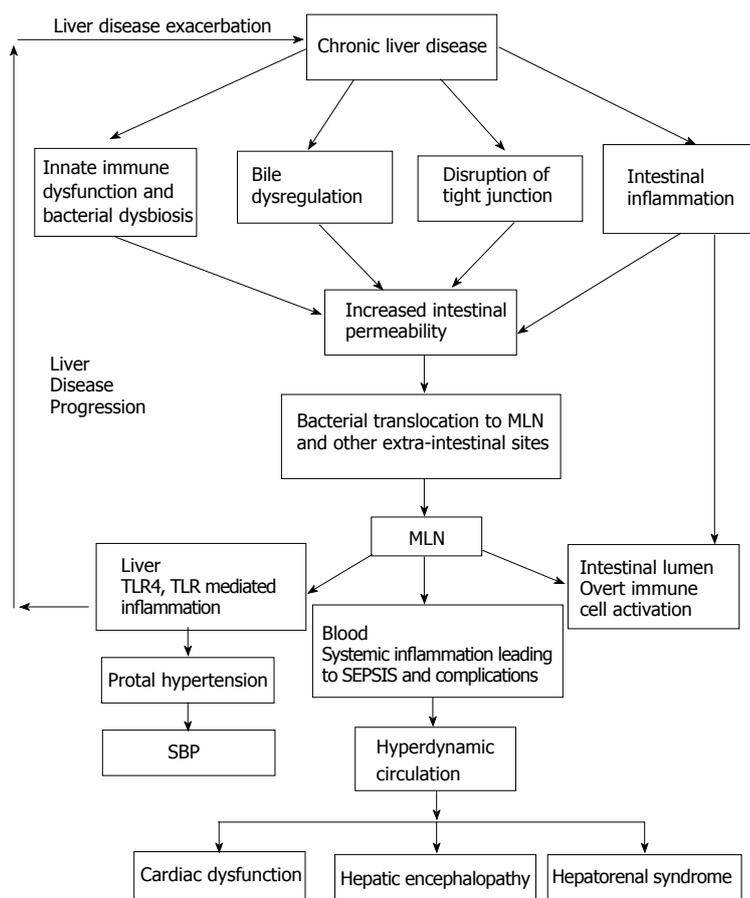


Figure 1 Cyclic cascade of bacterial translocation and associated progression of chronic liver diseases and its complications. The figure enlists the various proposed mechanisms through which chronic liver diseases conditions triggers intestinal permeability and BT. Increased intestinal permeability causes translocation of bacteria across the intestinal epithelium to the MLN and extra-intestinal sites such as liver and blood causing discrete complications in each of the systems. In the intestinal lumen, BT causes overt activation of immune cells and aggravates the pro-inflammatory cytokines in the gut. Translocation of bacteria at pathological levels to MLN is a major risk factor for systemic inflammation which leads to hyperdynamic circulation and is reflected across various organs in the form of complications such as cardiac dysfunction, hepatorenal syndrome and hepatic encephalopathy. Translocation of bacteria to the liver causes TLR4 and TLR9 mediated inflammation which further exacerbates the progression of chronic liver diseases and intestinal permeability. BT to liver also causes portal hypertension, which forms the basis of development of SBP. BT: Bacterial translocation; MLN: Mesenteric lymph node; SBP: Spontaneous bacterial peritonitis.

permeability and BT in CLD remains obscure, and only the probable pathways that might cause the phenomenon have been predicted. The cyclic cascade of events that cause BT and exacerbate CLD have been summarized in Figure 1.

Possible mechanisms of BT in CLD

Compromised Bile in the intestines: Bile flow is altered in liver disease, as the organ is the major producer of bile. Bile acids have been shown to interact with NR’s such as FXR in the GI tract and keep bacteria under check. Hence a compromised bile environment may lead to overgrowth of bacteria in liver disease conditions^[30,44].

Dysbiosis: It is a phenomenon inclusive of any quantitative or qualitative alterations from the symbiosis maintained between the host and microbiota^[41]. Quantitative overgrowth of bacteria also called as small intestinal bacterial overgrowth is usually the first step in the phenomenon of BT. Patients with CLD, especially alcoholic hepatitis have been directly linked

with developing bacterial overgrowth, low motility and increased transit time in the intestine^[41]. The proportion of beneficial and less beneficial (sometimes pathogenic) bacteria is tightly maintained and is termed symbiosis. In CLD, this balance is disrupted leading to increase in pathogenic bacteria and harmful metabolites that damage the epithelium^[41]. Beneficial bacteria such as lactobacillus reduce in numbers and potentially pathogenic bacteria like Enterobacteriaceae increase. Indeed, it was observed that the pathogenic bacteria are more likely to translocate across the intestinal epithelium^[41].

Immune dysfunction: The mucosal immune system prevents the exposure of commensal bacteria to systemic circulation through various secretory mechanisms such as mucus and anti-microbial peptides (AMP). In cirrhotic animal models, Paneth cells, have been observed to have diminished expression of AMPs^[45].

Intestinal inflammation: Both mucosal and sub-mucosal inflammation have been observed in patients

with CLD. A pro-inflammatory cytokine profile is also actively linked with increased intestinal permeability^[43,46-57]. Inflammation and its significance on intestinal permeability are discussed in more detail in the later sections of this review.

Disruption of tight junctions: A single layer of epithelial cells separate 100 trillion gut bacteria from other parts of the body. The epithelial cells are sealed tightly *via* tight junctions (TJ's) that are seen to disassociate in CLD and result in the leaky gut leading to BT^[48,49].

PXR AND ITS ROLE IN COUNTERACTING INFLAMMATION

Inflammation is a response mechanism to meet and overcome the challenges an organism faces from an injury or infection. However, when uncontrolled or unregulated, inflammation is often a pathological driving force in various disease conditions. In the context of this review, inflammation is the most prominent player in the pathophysiology of BT and sets the environment that causes increased intestinal permeability. Studies over the last decade have firmly established the role of PXR as a counter-regulator of inflammation^[7]. Clinical studies have revealed that, in IBD patients a clear pattern of PXR downregulation was observed in inflamed tissues^[50]. In this context, polymorphism of *NR1I2* gene has identified to be associated with more susceptibility to IBD^[51-53]. PXR has been described to interact with various components of the immune response signaling cascade to produce an immune regulating effect.

Nuclear factor kappa B (NF- κ B) is a transcription factor playing a central role in regulating plethora of genes involved in innate and adaptive immunity^[54]. It is well established that drug metabolism is compromised in an inflammatory environment and vice versa^[55]. This phenomenon plays an important role in highlighting the counter-regulation that exists between PXR and NF- κ B. PXR controls drug metabolism by binding to its response elements as heterodimers. PXR forms heterodimers with RXR- α and studies have revealed that this interaction is inhibited by the binding of p-65 subunit of NF- κ B to the RXR unit of PXR heterodimer complex (Figure 2A)^[56]. However, of even more interest are the observations that PXR can reciprocally inhibit NF- κ B and thus making PXR an excellent target to counteract NF- κ B and its associated inflammatory gene kit^[57]. Zhou *et al*^[58] demonstrated an increased NF- κ B activity and inflammatory cytokine profile in PXR null mice when compared to wild type mice that constitutively express PXR. This indicates that in physiological state PXR expression keeps the NF- κ B initiated inflammatory response under check. Additionally, when wild type mice were treated with PCN, a specific PXR agonist, majority of the NF- κ B target genes were downregulated, an

effect that was lost in PXR null mice. This suggests that PCN antagonism of NF- κ B happened in a PXR dependent manner^[58]. Similar findings were observed in DSS-induced colitis mice with and without PCN treatment^[59]. Ultimately, PXR which is responsible for defense against chemicals, and NF- κ B which is responsible for mounting an immune defense, counter-regulate each other to maintain physiological state.

Studies by Wallace *et al*^[7] employed a SJL/J mice model that is characterized by increased monocyte cell infiltration into the hepatic portal tract, revealed that PXR was expressed in infiltrating monocytes. Further in SJL/J-PXR^{+/+} mice, activation of PXR using PCN, downregulated tumor necrosis factor alpha (TNF- α) and Interleukin-1 α (IL-1 α), which are cytokines controlled by NF- κ B. Consistent with other studies, this effect was lost in SJL/J-PXR^{-/-} mice^[7,58]. Thus BT is also associated with considerable recruitment of monocytes cells to the lamina propria, which is accompanied by increased production of TNF- α ^[60,61]. Similarly, Fiorucci *et al*^[60] observed that LPMC's isolated from colitis mice that were treated with Rifaximin, showed complete abrogation of INF γ cytokine production. Unfortunately this study did not consider if activation of PXR by Rifaximin could have produced this effect. However, study by Cheng *et al*^[62] using humanized PXR (hPXR) mice with DSS induced colitis showed that Rifaximin indeed works in a PXR-dependent manner and attenuated NF- κ B mediated cytokines. Interestingly, TNF- α has also been associated with direct downregulation of epithelial TJ proteins^[63]. In this scenario, further studies targeting activation of PXR with an aim to antagonize NF- κ B-induced-TNF- α may pose as an exciting therapeutic outcome especially by controlling mononuclear cell infiltration, with an aim to attenuate intestinal epithelial damage, which is a major prequel to BT.

Even though many studies have established the mutual inhibition between PXR and NF- κ B, the exact mechanism by which PXR represses NF- κ B is not well understood. Indeed, Ye *et al*^[64] showed that PXR activation by a natural PXR ligand Ginkgolide-A (GA) repressed NF- κ B indirectly by enhancing the expression of nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (I κ -B α), an inhibitory protein of NF- κ B activity. When siRNA was used for silencing of PXR, GA did not increase the expression of I κ -B α , showing that the induction happens in a PXR dependent manner^[64] (Figure 2B).

Various plant flavanols like chrysin and isorhamnetin have also been shown to inhibit NF- κ B activity through a PXR dependent manner^[65,66]. Studies using these flavanols in a DSS-induced colitis mouse model showed PXR mediated downregulation of NF- κ B target genes including iNOS, ICAM-1 MCP-1, COX-2, TNF- α IL-2 and IL-6. Intriguingly, both studies showed that PXR activation prevented the degradation of I κ -B α and thus underlining the possibility that PXR might counteract NF- κ B mainly through manipulation of fate of I κ -B α ^[65,66] (Figure 2C).

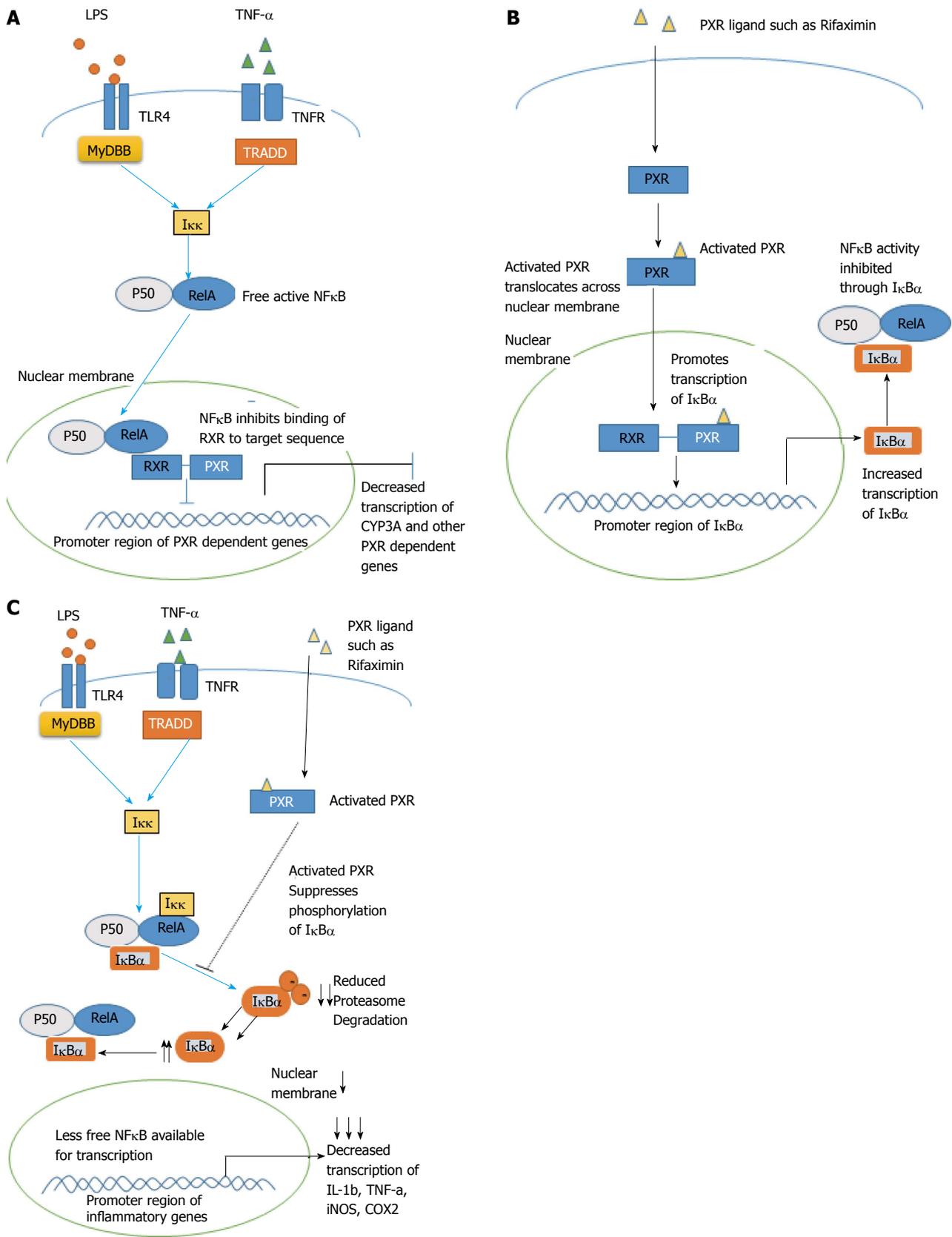


Figure 2 Illustration of counter-regulatory mechanisms existing between nuclear factor kappa B and pregnane X receptor. A: The p-65 subunit of NF- κ B binds to the RXR unit of PXR heterodimer complex and represses its transcriptional activity; B: Activated PXR binds to the promoter region of I κ B α and increases its transcription leading to indirect repression of NF- κ B; C: After activation through its ligands, PXR suppresses the phosphorylation and degradation of I κ B α and thus indirectly represses NF- κ B activity. NF- κ B: Nuclear factor kappa B; PXR: Pregnane X receptor; RXR: Retinoid X receptor; TLR: Toll like receptor.

SUMOYLATION dependent regulation of PXR-NF- κ B axis

SUMOYLATION is a post-translational modification process, in which a small ubiquitin-like modifier (SUMO) protein would be added to the ligand-binding domain of PXR protein^[67]. A SUMO1 binding site was discovered in the ligand-binding domain of PXR^[68]. It was revealed that post SUMOYLATION there is an increase in the transcriptional activity of PXR, marked by increased transcription of PXR target genes (Figure 3A). Also, an increase in interaction between SUMOylated PXR and NR co-repressor (NCOR1) was observed. Thus it is speculated that post-modification, PXR protein might be able to repress NF- κ B indirectly by helping to keep the co-repressors (N-COR)/HDAC3 complex intact by preventing their clearance^[68,69]. Vice versa, endotoxin stimulus such as LPS would signal the clearance of these repressor complex from the promotor region of pro-inflammatory genes and thus enabling NF- κ B to transcribe the inflammatory profile genes^[70]. A similar event has already been established to happen in another NR, PPAR- γ , which post SUMOYLATION transrepresses NF- κ B by preventing recruitment of Ubc5 protein and initiates the clearance of co-repressors^[71]. This mechanism opens attractive opportunities to target the PXR SUMOylation site, particularly in a scenario such as bacterial sepsis, where endotoxin stimulated NF- κ B inflammatory response occurs.

Furthermore, Hu *et al*^[69] revealed that the PXR SUMOylation occurs as a feedback response to inflammatory stimulus such as TNF- α . They specifically identified SUMO3 chains in the post translationally modified (SUMOylated) PXR protein. An extremely interesting aspect of their discovery is that they found through their in vitro assays that SUMOylated form of PXR played a huge role in diminishing inflammation, but was hardly effective in regulating CYP3A expression. Thus, SUMOylation may shift the functional activity of PXR from a ligand-activated transcriptional inducer which upregulates xenobiotic target genes, towards a ligand-activated transcriptional repressor that brings about an immunosuppressive effect (Figure 3B).

PXR and TNF- α

Dysregulation between the intestinal epithelial cells and the innate immune system is often initiated by endotoxins such as LPS and is one of the established pathological mechanism for gut barrier disruption^[73,73]. It has been observed that TNF- α acts as a central mediator of NF- κ B in the initiation of mucosal inflammation^[74]. Goldman *et al*^[74] have reported that an anti-TNF approach which was effective in countering BT. Research conducted by Mencarelli *et al*^[75] revealed that IEC on exposure to TNF- α showed significant dampening of PXR mRNA levels. However, TNF- α was completely antagonized when treated with Rifaximin, which showed significant anti-inflammatory effect through PXR activation. Further, when cells cultured from colon biopsies of IBD patients were induced with

LPS and followed by Rifaximin treatment, an abrogation of the LPS induced NF- κ B target genes such TNF- α , MIP-3 α and IL-8 were observed. These evidence clearly indicate that effectively inducing PXR, which inhibits the effects of LPS induced TNF- α and NF- κ B, may pose as a desired outcome in the treatment of BT.

PXR and MDR1 puzzle

Multi drug resistance gene (MDR) 1 is categorized under the ABC family of transporters and encodes the transmembrane protein P-glycoprotein (P-gp). MDR1 is one of the primary genes regulated by PXR and is widely expressed in intestinal epithelial cells and the liver^[76]. P-gp acts as an ATP-dependent drug efflux system and is responsible for maintaining intestinal homeostasis by pushing out noxious chemicals (from drug or microbial source) from mucosa back into gut lumen^[76,77]. Polymorphism in Mdr1a gene that results in a phenotype with reduced P-gp expression was observed in both Ulcerative colitis and Chron's disease subjects^[78]. Mdr1a^{-/-} knockout mice models confirmed that, in absence of this efflux pump protein, the animals developed spontaneous colitis resembling human IBD. This effect was ameliorated by treatment with oral antibiotics indicating that reducing the bacterial burden is an effective measure to control inflammation. Reducing the toxin accumulation in the gut might be the probable mechanism for ameliorating inflammation and thus underlining the importance of a xenobiotic clearance system in the gut^[77]. Langman *et al*^[79] showed that mRNA levels of both PXR and Mdr1a were reduced in UC patients and also assumed that the dampening of PXR expression might be the probable reason for Mdr1a downregulation.

A recent study performed by Toklu *et al*^[80] hypothesized that PXR stimulation by antibiotics rifampicin and spironolactone may cause an immunosuppressive effect through induction of *Mdr1a* gene (and thus P-gp protein expression). However, these observations were conflicted by Blokzijl *et al*^[81] who showed that PXR protein levels were unchanged between inflamed and uninflamed human colons, in spite of low Mdr1a expression in the same tissues. Thus in such a scenario Mdr1a may be independent of PXR protein concentration. Ros *et al*^[82] reported Mdr1a expression to be unaltered in liver of LPS-treated rats. Conflicting evidence regarding reduced Mdr1a levels in intestine was provided by Kalitsky-Szirtes *et al*^[83]. Further research using PXR null mice may confirm if PXR-induced-P-gp expression is a valid mechanism in controlling BT, most probably by riding the gut of endotoxins, which may otherwise stimulate mucosal immunity.

PXR and LPS

LPS is a major component of the cell walls of gram-negative bacteria and is considered an endotoxin, which potently stimulates host innate immune response^[84,85]. LPS is recognized by its specific receptor toll like receptor 4 (TLR4) and is one of the earliest inflammatory triggers

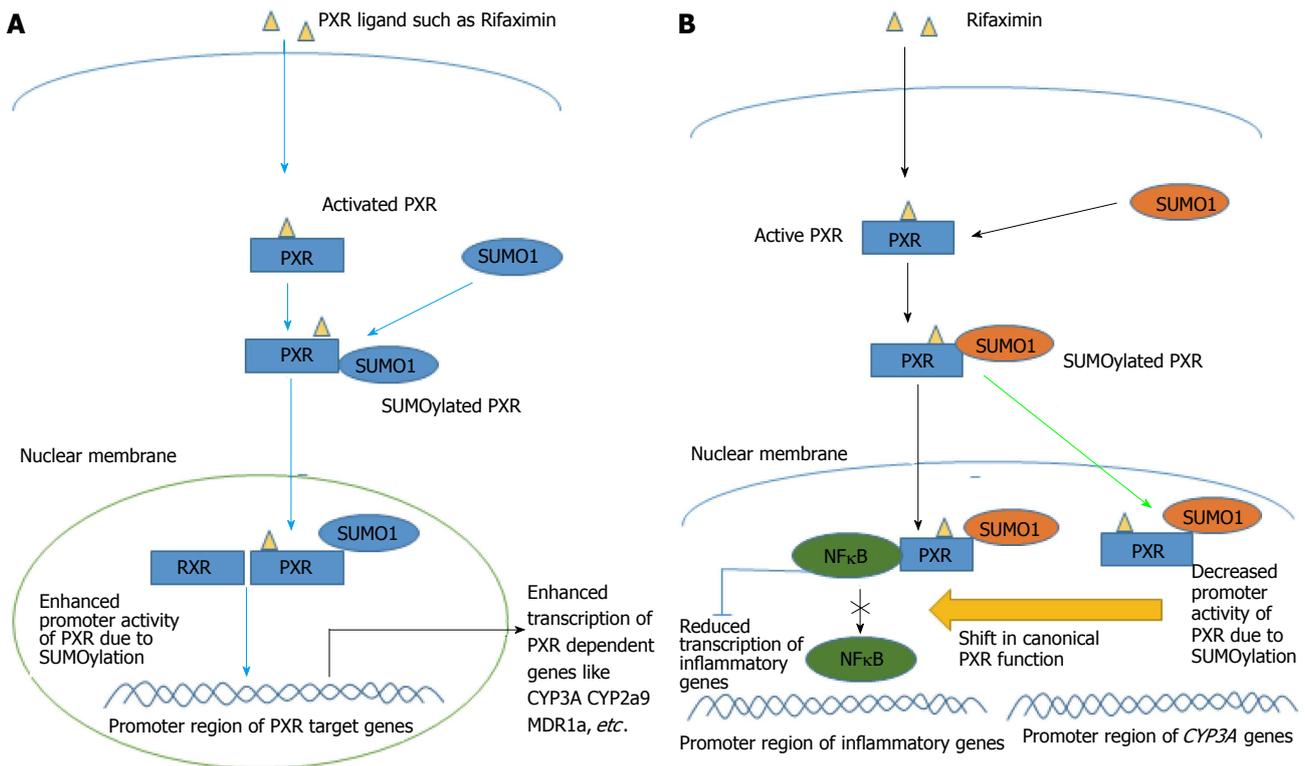


Figure 3 Schematic diagram illustrating the mechanism of modification of pregnane X receptor activity and function after SUMOYLATION. A: SUMO1 binding to PXR has been shown to increase its transcriptional activity; B: Sumo (3) ylation of PXR causes a shift from the canonical transcriptional function of PXR towards the transcriptional repression of NF-κB. NF-κB: Nuclear factor kappa B; PXR: Pregnane X receptor; RXR: Retinoid X receptor.

to induce gut barrier disruption and BT^[86]. The pro-inflammatory effects of LPS are mediated primarily through activation of transcription factors like NF-κB, which is present upstream in the inflammatory cascade^[56,84]. In humans, a physiological BT state has been defined, where 5%-10% of bacteria, translocate across the intestine with minor exposures of LPS, maintaining a tightly regulated tolerance towards the gut microbes and their toxins^[38]. However, when there are alterations in quantity (load) or quality (dysbiosis) of bacteria, the innate immune system is activated overtly. This is often by means of increased exposure to LPS, which stimulate immune cells such as monocytes, neutrophils and lymphocytes. These cells, in turn, produce acute response cytokines including IL-1β, IL-6 and TNF-α, creating an inflammatory profile^[87-89].

LPS-induced inflammation models have highlighted the importance of PXR activity in regulating different phases of the immune response. Diminished expression of *CYP3A* gene was identified during infections and has been replicated in LPS-induced animal models^[90,91]. Interestingly, mRNA levels of PXR were also seen to be downregulated in such models. Indeed, Moriya *et al.*^[92] have indicated that LPS treatment significantly reduced both the gene expression and activity of CYP's, even in mice that were pre-stimulated with PXR activator PCN. LPS caused this effect by inducing cytokines, which bring about the inhibition of PXR in an NF-κB dependent manner. A similar observation was made by Gu *et al.*^[56] where the use of NF-κB suppressor SR1κBα, reversed the

LPS induced downregulation of PXR, proving that "NF-κB stimulated PXR inhibition" is central in bringing out the effects of LPS. While IECs are in constant contact with bacterial products like LPS, immune cells are involved in the response to any dysregulation between IEC and microbial products. It should be noted that PXR is expressed in both these cell types and thus play a major role in regulating both the toxin challenge that is faced by IEC, as well as in response to that challenge that is brought about by immune cells. A recent study identified in the primary culture of hepatocytes (PCH) isolated from WT mice that, PCN pretreatment for 24 h alleviated LPS induced an acute response by decreasing cytokines such as IL-1β, TNF-α and IL-6. However, when PCH from PXR-null mice were treated with LPS, enhanced pro-inflammatory cytokine response was documented^[93]. When PCH isolated from humanized PXR mice were pre-treated with PXR activators, it led to increased production of IL-1Ra, a natural inhibitor of IL-1β^[93]. Thus PXR expression is seen to be important in both dampening endotoxin-stimulated immune response to maintain homeostasis, as well as in resolving the inflammatory state through inducing anti-inflammatory response^[93,94].

Relationship between PXR, LPS and ROS

Xu *et al.*^[95,96] have revealed an interesting pathway in which LPS could suppress the expression of PXR and its associated genes. In their experiment, LPS dose-dependently suppressed PXR mRNA levels in mice and was significantly improved following antioxidants

treatments^[95,96]. Furthermore, inhibition of xanthine oxidase and NADPH oxidase that generates ROS, using specific inhibitors allopurinol and diphenylene iodonium respectively, led to attenuation of LPS induced PXR downregulation^[95]. In this context, the antioxidant melatonin was also observed to produce a similar effect^[97]. Chen *et al.*^[98] revealed that treatment with a free radical trapping agent alpha-phenyl-N-tbutylnitronone prevented LPS from downregulating PXR. Thus, it is well understood that ROS and oxidative stress have an impact in the LPS induced diminishing of PXR expression. PXR ligands such as Danshen, which have an inherent antioxidant property could be used to further understand the LPS-counteracting activity of PXR^[11]. Research performed using PXR ligands or constitutively active PXR (VP-PXR) have revealed that LPS instigated response is regulated by PXR activation^[94,99]. Hence, counteracting LPS induced ROS promises to be a novel opportunity to counter endotoxin-induced inflammatory response, in BT^[94,99].

PXR and TLR4 crosstalk

TLR4 is a transmembrane receptor that recognizes LPS, which is a pathogen associated molecular pattern. LPS can only bind to the TLR4 complex after it has associated itself with LBP (LPS binding protein)^[100]. The recognition also involves additional co-receptors such as CD-14 and MD-2 and adaptor protein MyD88^[101]. Physiologically TLR4 expression and regulation is of significant importance in the intestine. Hence, TLR4 expression is tightly regulated based on the level of LPS in the gut lumen as it directly correlates with the intensity of immune response at any given time. A pathological state such as small intestinal bacterial overgrowth, may challenge this homeostasis leading to overt activation of TLR4 signaling and trigger NF- κ B, which leads to barrier dysfunction and BT^[101,102]. Studies have shown that the crosstalk between TLR4 and PXR could determine the homeostasis in the intestine^[103]. For instance, when TLR4 was activated using its specific agonist KDO2, increased induction of mucosal TNF- α , followed by intestinal permeability was observed in both WT and PXR null mice^[104]. Similarly, when PXR was activated using PCN, a clear reduction in mucosal TNF- α induction was documented. PCN activation did not have any effect in PXR null mice and thus indicating that the TLR4 inhibition was PXR-dependent. This study clearly shows that PXR and TLR4 counter-regulate each other upon their respective activation^[104].

A study by Esposito *et al.*^[105] observed a similar pattern of regulation when Caco2 IECs were induced with Clostridium difficile toxin A (TcdA) to replicate ulceration and inflammation model. They found that the toxin stimulated the expression of TLR4 by 1411% in Caco2 cells. However, this phenomenon was completely reversed by Rifaximin treatment dose dependently, which down-regulated the expression of TLR4, MyD88 and NF- κ B. This study also brings to light

an additional pathway, where by reducing TLR4 levels following PXR activation, the NF- κ B activity might be reciprocally inhibited^[105]. Furthermore, PXR and TLR4 double KO mice models have shed more light into PXR's ability to act as a mediator between the microbes and TLR4. Venkatesh *et al.*^[106] observed that PXR null mice developed leaky gut and showed increased induction of TLR's including TLR4 (1.8 fold increase). However, in TLR4^{-/-} and PXR^{-/-} double KO mice model, the previously observed pathological defects in intestine disappeared. This emphasizes the impact of TLR4 expression, which was particularly high in absence of PXR, in bringing about intestinal inflammation and the gut disruption. In addition, enterocytes isolated from PXR null mice, showed similar results with TLR4 inhibitors. Thus a reciprocal relationship seems to exist between PXR and TLR4 in maintaining homeostasis. They also found that Indole-3 Propionic Acid (IPA), which is an endogenous, microbe derived ligand for PXR, activated PXR and reduced enterocyte TNF- α ^[106]. This is a clear example of the sensing system: PXR, and the symbiotic bacteria working in coherence to repress overt inflammation. Together, these data suggest that PXR regulates the expression of TLR4, and that PXR activation could have a therapeutic effect in BT by counteracting TLR4 mediated gut disruption.

INTESTINAL INTEGRITY AND PXR: TIGHT JUNCTIONS

PXR and maintenance of intestinal integrity

A single layer of epithelial cells serve as physical barrier that prevent the diverse contents of the gut from entering the systemic circulation and other tissues. The integrity of this barrier is governed by junctional complex proteins including TJ and adherent junctions (AJ) and are involved in sealing the gap between two adjacent cells^[107]. The expression of these junctional complexes are tightly controlled and are dynamic in nature, such that allowing passage of only selected molecules across the epithelial barrier^[108]. In disease state, the expression of these junctional complexes are highly compromised leading to a leaky gut, which is the major driving factor for BT and its complications^[109,110].

PXR is extensively expressed by IECs and have been shown to have a direct impact on the signaling molecules that govern intestinal integrity. Accordingly, studies have shown that in PXR null mice, there is a leaky gut like pathology^[106]. Indeed Venkatesh *et al.*^[106] observed reduced mRNA levels of junctional complexes such as Zonula occludens 1 (ZO1) and E-cad in PXR KO mice. However, they also found an increased expression of Claudin-2, which is associated with promoting paracellular transport of microbes and which in high expression state is linked with hyperpermeability in gut. One of the possible mechanisms through which PXR maintains expression of junctional complexes was revealed through the use of PXR-TLR4 double KO mice^[106]. When both PXR and TLR4 were knocked out, the level of TJ expression

was almost relatable to the levels that were found in PXR^{+/+} mice. Hence, PXR may preserve junctional complexes by countering TLR4 and thereby inhibiting the downstream inflammatory cytokines such as TNF- α that are stimulated by TLR4. This shows that PXR knockout state is associated with pattern of upregulation of genes that promote paracellular transport (claudin-2, TNF- α) and downregulation of genes that maintain barrier functions (ZO1) and thereby playing a vital role in maintaining intestinal integrity.

Negative regulation between PXR and MLCK

Several studies that have targeted PXR activation have attributed the preservation of the junctional complexes to PXR's ability to interact with various intercellular signaling mediators. Myosin Light Chain Kinase (MLCK) is associated with regulation of paracellular permeability through its ability to phosphorylate myosin II regulatory light chain (MLC), which underlies the junctional complex arrangement^[111]. Hence through phosphorylation of MLC, MLCK is able to stimulate actomyosin contraction and modulate TJ localization^[111]. In a pathological state such as infection or inflammation TNF- α induced both the expression of MLCK, and its activity and thus influencing intestinal permeability^[112]. He *et al*^[113] observed TNF- α induced MLCK expression was increased through the stimulation of NF- κ B, which acted upstream of MLCK. Hence PXR, which counter-regulates TNF- α mediated NF- κ B could possibly interact with this pathway to preserve TJs. Indeed, study by Garg *et al*^[104] reported that TNF- α exposure induced increased relocalization of ZO1 through the upregulation of MLCK expression. However, PXR activation by Rifaximin, countered the MLCK upregulation in Caco2 IEC cells, through its established function of attenuating NF- κ B. The same results were reproduced in an *in-vivo* DSS mice model, where PCN treatment attenuated MLCK expression and protected against ZO1 mislocalization^[104]. Thus by inhibiting TNF- α induced NF- κ B activation, PXR is able to maintain intestinal integrity through indirectly regulating MLCK^[104].

Preservation of intestinal integrity by PXR through JNK1/2 interference

PXR activation has been reported to influence on the JNK1/2 pathway. C-jun N-terminal kinase (JNKs) are kinases that are activated in response to stress stimuli including various cytokines like TNF- α and are implicated in apoptosis and inflammation^[114-116]. The exact role of JNK1/2 on inflammatory disorders is still unclear. While few studies reported JNK1/2 deletion increased the severity of inflammation in DSS model^[117], others showed that JNK1/2 inhibition is protective^[114,118]. In this context, Mitsuyama *et al*^[119] have reported an increased JNK1/2 expression in IECs of CD patients. Garg *et al*^[104] have reported that TNF- α /INF γ stimulation of Caco-2 cells resulted in increased activation of JNK1/2, an effect that was associated with ZO1 mislocalization. However,

this phenomenon was completely inhibited using JNK inhibitor SP600125. Most importantly, activation of PXR using Rifaximin was seen to attenuate JNK1/2 activity by inducing the transcription of growth arrest and DNA damage inducible 45 β (GADD45 β), a protein that is known to block JNK1/2 activity by preventing its phosphorylation. Thus PXR activation was seen to protect intestinal TJ integrity by preventing the activation/phosphorylation of JNK1/2.

PXR and CDX2: An interaction of two transcription factors

Recently a new mechanism of PXR-related immunosuppression was reported by Dou *et al*^[120] involving the Caudal related Homeobox transcription factor, CDX2. Interestingly, CDX2 has been implicated in intestinal differentiation and in maintaining intestinal integrity^[121,122]. CDX2 is a transcription factor reportedly expressed in the intestine, where it binds to the promoter region of PXR and induces PXR transcription. Further mechanistic studies may establish if CDX2 is a player involved in regulating PXR expression especially in the scenario of mucosal inflammation.

PXR AND BILE ACIDS

Bile acids play a very important physiologic role in the catabolism of cholesterol and are known to regulate bacterial overgrowth owing to their bacteriostatic properties^[123,124]. Bile acids are also ligands to NR's such as FXR and VDR^[125,126]. On activation these NR's regulate the expression of anti-microbial peptides and innate immunity genes, which keep gut microbiome outgrowth in check^[123-126]. However, studies have shown that when bile acids accumulate, they can be potentially toxic, thus highlighting the importance of presence of active bile acid detoxification system to afford protection against their toxicity^[127]. Lithocholic acid (LCA) is a secondary bile acid, which is considered to be toxic at higher concentrations than the basal levels and is a byproduct of gut bacterial biotransformation process^[128]. Makoto Ishit *et al*^[129] have shown that upregulation of PXR and its dependent genes happens as an adaptive response to an increase in LCA, in patients who underwent gastrectomy. They reported that gastrectomy shifted the intestinal PH towards alkaline state due to reduced gastric acid, which led to the increased thriving of LCA-producing bacteria and thus leading to increased accumulation of LCA. This study highlights that PXR is the foremost physiologic and adaptive sensor of LCA especially considering that FXR another important bile acid sensor is unresponsive to LCA^[129]. More importantly, this study using the example of gastrectomy sets the precedence that other pathologic events, such as dysbiosis may also shift the balance of gut microbiome composition and ultimately influence the bile acid metabolism and the genes they control.

Bacterial dysbiosis, an established mechanism to cause BT, has been shown to affect the composition of

bile acid pool^[130]. Disease states such as Non-Alcoholic Fatty Liver Disease (NAFLD) are associated with both increased bile acids and alteration in bacterial gut microbiome communities^[131]. Such states may shift the balance of hydrophobic and hydrophilic secondary bile acids in the overall bile acid pool. In the context of this review, this is of importance, as a linear correlation has been observed between perturbation in gut microbes, disturbance in bile metabolites and disruption of intestinal barrier homeostasis^[132,133]. Increased hydrophobic bile acids such as LCA and Deoxycholic acid (DCA) have been shown to be associated with disruption of gut barrier as shown by Stentman *et al.*^[134] where high fat related concentration of hydrophobic but not hydrophilic bile acids produced barrier disruption. Hughes *et al.*^[135] made an interesting observation that at physiologic levels, LCA increased paracellular permeability in Caco2 intestinal epithelial cells, which was indicated by a decrease in trans-epithelial resistance and increase in mannitol flux. However, at the same physiologic levels, LCA was seen to increase occludin expression. It would be interesting to see if LCA induced occludin expression happens in a PXR dependent manner, as PXR is the major physiologic sensor of LCA. Also, since this study only focused on acute twelve hour effects of LCA at basal levels^[135], future studies at chronic treatment times and higher doses would need to be conducted to illuminate the effect of LCA toxicity on TJ expression.

LCA-feeding has been used a standard *in-vivo* model to induce cholestasis in mice. Fickert *et al.*^[136], used this model to demonstrate that LCA feeding induced disruption of TJ protein ZO1 in both bile duct epithelial cells and between hepatocytes. Vu *et al.*^[137], have found the similar observations that TJ permeability was elevated followed by cholestatic dose of LCA.

Role of PXR in LCA detoxification

The role of PXR in LCA detoxification is paramount, evidenced by studies which showed that PXR KO animals were susceptible to bile toxicity and cholestasis^[138]. In cholestasis, as the disease progresses the bile acid flow is impeded leading to a compromised bile environment. This is exploited by bacterial outgrowth leading to their uncontrolled translocation^[139]. This highlights the fact that, PXR sensing and detoxification may serve as a prophylactic (preventive) setup to maintain homeostasis in bile metabolism and afford protection against cholestasis^[140]. The role of PXR in protection against NAFLD may also be critical, as increased LCA has been associated with high-fat consuming population^[141,142]. Studies by Staudinger *et al.*^[143] and Xie *et al.*^[144] have documented the various mechanisms of PXR-dependent LCA detoxification. PXR has been observed to control the expression of CYP7A1, a rate-limiting enzyme in bile acid production from cholesterol, and is seen to repress CYP7A1 following PXR activation through its ligands^[143]. PXR also directly regulates the expression of Na1-independent organic anion transporter 2, a protein

that is involved in uptake of bile acids by hepatocytes for metabolism^[143,144]. Finally, PXR dependent CYP3A enzymes in the hepatocytes mediate hydroxylation of LCA and prime it for elimination^[145]. Additional mechanisms such as sulfonation of LCA by PXR dependent SULT enzymes have also been described^[146]. Transgenic mice that constitutively expressed activated PXR (VP-PXR) were seen to be resistant to the toxic effects of LCA^[146]. Consequently, PXR plays a very important role prophylactically in affording protection against bile dysregulation and toxicity, as seen in closely related pathologic states such as dysbiosis and NAFLD. The activation of PXR might serve as an attractive option in preventing BT, which might result from direct or indirect effects of bile acid dysregulation.

PXR THE SENSOR OF MICROBIAL METABOLITE CUE

In a physiological state, bacteria have been shown to have an impact on host genes without direct contact^[31]. Gut bacteria communicate with the host mainly through the extensive profile of microbial metabolites that they produce, which interact with a range of physiologic sensors such as NR's in the host cells of intestine and liver^[14]. The enterohepatic circulation gains major importance in this matter, as the liver and intestine interact with each other through bile and metabolite (nutrient and microbial) profile respectively. Compromised bile availability in disease states like cholestasis has been linked with bacterial dysbiosis in the intestine^[147]. Similarly, metabolic disorders such as obesity and Type 1 diabetes have been implicated with alterations in the gut microbiota^[148], which indicate alterations in the proportion of the microbial metabolites the microbiota produce. While some of these microbial metabolites have been characterized as essential nutrients, many other metabolites and the NR's that they interact with are yet to be explored. PXR owing to its flexible binding domain and extensive expression pattern in gut and liver has been identified to interact with wide range of bacterial metabolites through which it is seen to maintain homeostasis along the gut-liver axis.

IPA, a bacterial product of the tryptophan metabolism is being recognized as one of the established ligands of PXR^[149]. Venkatesh *et al.*^[106] highlighted the importance of IPA sensing by PXR, where PXR after activation, maintained gut barrier function by downregulating TLR4 and its downstream effector TNF- α . IPA also increased the mRNA levels of junctional proteins. They demonstrated that either loss or reduced expression of PXR (as seen in inflammatory conditions) or the loss of IPA producing bacteria (as documented through commensal depleted organism models) led to worsening of inflammation and increased intestinal permeability^[106]. It was also revealed that reintroducing *C. Sporogenes* in the presence of their substrate L-Tryptophan in GF mice led to production of IPA and improved cell-cell junctional

Table 1 Table enlisting the documented effect of various natural occurring ligands of pregnane X Receptor

No	Natural ligand and source	Effective response after binding of ligands with PXR	Ref.
1	Baicalein from roots of <i>Scutellaria baicalensis Georgi</i>	Attenuated colonic inflammation in DSS induced colitis mice model through stimulation of CDX2. ↓TNF α and IL-6 mRNA levels in intestinal mucosa	Dou <i>et al</i> ^[122] <i>Plos One</i> 2012
2	Forskolin and 1,9 dideoxyforskolin from roots of <i>Coleus forskohlii</i>	↑CYP3A expression in primary hepatocytes through activation of Protein Kinase A signaling pathway	Ding and Staudinger ^[151] <i>J Pharmacol Exp Ther</i> 2005
3	Z-guggulsterone from <i>Commiphora mukul</i> (Guggul)	↓CYP7A1 gene in HepG2 cells	Owsley and Chiang ^[152] <i>Biochem Biophys Res Commun</i> 2003
4	E-guggulsterone from <i>Commiphora mukul</i> (Guggul)	↑CYP3A11 and CYP3A4 mRNA levels only in cultured hepatocytes from PXR +/+ mice and not in PXR KO mice	Brobst <i>et al</i> ^[153] <i>J Pharmacol Exp Ther</i> 2004
5	Hyperforin from <i>Hypericum perforatum</i> (St. John's wort)	↑CYP3A4 induction in hepatocytes. Induction of CYP2C9 gene expression was also reported in humans	Moore <i>et al</i> ^[154] <i>Proc Natl Acad Sci USA</i> 2000
6	Colupulone from <i>Humulus lupulus</i> (Hop Extract)	↑CYP3A4, CYP2B6 and MDR1 gene expression in primary human hepatocytes dose dependently	Teotico <i>et al</i> ^[155] <i>Mol Pharmacol</i> 2008
7	Kava Kava (<i>Piper methysticum</i>)	↑CYP3A4 mRNA expression in primary human hepatocytes extensively	Raucy ^[156] <i>Drug Metab Dispos</i> 2003
8	Wu Wei Zi [Dibenzocyclooctene lignans: schisandrol B, schisandrin (A and B)]	↑Transcription of CYP3A4, CYP2C9 and MRP2 genes in primary hepatocytes	Mu <i>et al</i> ^[157] <i>J Pharmacol Exp Ther</i> 2006
9	Ginkgolide A from <i>Gingko Biloba</i> extract	Protection against CCL4 induced acute toxicity model in rats, ↑I κ -B α transcription, which in turn inhibited NF- κ B	Ye <i>et al</i> ^[64] <i>Biomol Ther (Seoul)</i> 2016
10	Ginkgolide B from <i>Gingko Biloba</i> extract	↑Nuclear translocation of PXR, and protected HUVEC cells from drug induced apoptosis. Anti-inflammatory role by reducing VCAM-1 and E-selectin induced by TNF α	Zhou <i>et al</i> ^[158] <i>Acta Pharmacol Sin</i> 2016

↑: Upregulation; ↓: Downregulation; PXR: Pregnane X receptor.

complex efficacy. Importantly, the same phenomenon was absent in PXR KO mice. Hence shifting the bacterial composition towards favorable metabolic profile might be an effective method to activate PXR and counteract inflammation^[106]. However, it is also important to consider the level of PXR available to sense IPA, which is often compromised during inflammation.

Gut bacteria also play a vital role in the bio-transformation of various natural herbal products into forms that are beneficial to host. β -glucuronidases produced by symbiotic bacteria have been shown to convert the flavonoid baicalin into baicalein^[122,150]. Interestingly, Dou *et al*^[122] reported that both baicalein and baicalin attenuated gut inflammation induced by DSS *in vivo*. However, baicalin treatment did not have any effect, when the β -glucuronidase inhibitor was used to prevent the bioconversion of baicalin. The study further reported that only baicalein activated PXR to potently produce an anti-inflammatory phenotype in the colitis induced mice. Thus, PXR acts as an important mediator between bacterial derived metabolites and host, and upon activation shapes the immune profile. Table 1 summarizes the various identified natural ligands of PXR along with the effective response they produce after binding with PXR.

CONCLUSION

PXR plays a pivotal role as an endobiotic and xenobiotic sensor scanning for any alterations in the environmental cues and then translates the signals into an epithelial

phenotype that is protective to host. Its role in maintaining homeostasis along the gut liver axis is undisputable. The majority of studies in exploration of PXR pathways, have been conducted only in IBD and associated pathologies. However, PXR is expressed copiously along the gut liver axis and has been proved to have a huge functional impact in both liver and intestine, and poses as an excellent target in CLDs with BT. As summarized above, PXR has important functional implications in each of the major pathophysiological mechanisms attributed to causing BT in CLD states. Targeting PXR with naturally occurring herbs or other polyphenolic compounds may potentially cease BT and attenuate the progression of liver disease or the manifestation of the associated fatal complications of CLD. Moreover, even though Rifaximin is a potent agonist for PXR, it can activate only gut PXR and is associated with adverse hepatotoxic side effects. The plethora of bioactive components from natural herbs are being discovered as effective activators for both human and rodent PXR, promising a fertile research ground for future studies. Using a CCL4 induced mouse cirrhotic model, our lab is currently investigating the effect of a naturally identified PXR ligand Ginkgolide-A, to further comprehend the functional impact of PXR activation on regulating BT.

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Basic Study

Liver atrophy after percutaneous transhepatic portal embolization occurs in two histological phases: Hepatocellular atrophy followed by apoptosis

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Abstract**AIM**

To clarify the histological changes associated with liver atrophy after percutaneous transhepatic portal

embolization (PTPE) in pigs and humans.

METHODS

As a preliminary study, we performed pathological examinations of liver specimens from five pigs that had undergone PTPE in a time-dependent model of liver atrophy. In specimens from embolized lobes (EMB) and nonembolized lobes (controls), we measured the portal vein to central vein distance (PV-CV), the area and number of hepatocytes per lobule, and apoptotic activity using the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay. Immunohistochemical reactivities were evaluated for light chain 3 (LC3) and lysosomal-associated membrane protein 2 (LAMP2) as autophagy markers and for glutamine synthetase and cytochrome P450 2E1 (CYP2E1) as metabolic zonation markers. Samples from ten human livers taken 20-36 d after PTPE were similarly examined.

RESULTS

PV-CVs and lobule areas did not differ between EMB and controls at day 0, but were lower in EMB than in controls at weeks 2, 4, and 6 ($P \leq 0.001$). Hepatocyte numbers were not significantly reduced in EMB at day 0 and week 2 but were reduced at weeks 4 and 6 ($P \leq 0.05$). Apoptotic activity was higher in EMB than in controls at day 0 and week 4. LC3 and LAMP2 staining peaked in EMB at week 2, with no significant difference between EMB and controls at weeks 4 and 6. Glutamine synthetase and CYP2E1 zonation in EMB at weeks 2, 4, and 6 were narrower than those in controls. Human results were consistent with those of porcine specimens.

CONCLUSION

The mechanism of liver atrophy after PTPE has two histological phases: Hepatocellular atrophy is likely caused by autophagy in the first 2 wk and apoptosis thereafter.

Key words: Liver atrophy; Portal vein embolization; Autophagy; Apoptosis; Zonation; Lobule

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Core tip: Liver atrophy after percutaneous transhepatic portal embolization (PTPE) in time-independent human studies is associated with hepatocyte shrinkage and apoptosis. In this preliminary study, we performed pathological examinations of liver specimens from five pigs that had undergone PTPE in a time-dependent model of liver atrophy. Two distinct phases of liver atrophy were identified: A hepatocellular atrophic phase, which may relate to autophagy, and an apoptotic phase. Despite liver atrophy appearing to be mostly resolved 2 wk after embolization, the period after PTPE could beneficially be extended to 4 wk to ensure contralateral hypertrophy and to allow the completion of liver atrophy.

Iwao Y, Ojima H, Kobayashi T, Kishi Y, Nara S, Esaki M, Shimada K, Hiraoka N, Tanabe M, Kanai Y. Liver atrophy

after percutaneous transhepatic portal embolization occurs in two histological phases: Hepatocellular atrophy followed by apoptosis. *World J Hepatol* 2017; 9(32): 1227-1238 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v9/i32/1227.htm> DOI: <http://dx.doi.org/10.4254/wjh.v9.i32.1227>

INTRODUCTION

The interruption of portal blood flow by portal vein embolization or tumor thrombosis, for example, causes liver atrophy^[1]. However, the mechanisms responsible for this effect have not been fully elucidated. Using pig models of percutaneous transhepatic portal vein embolization (PTPE) with absolute ethanol, we previously observed the temporary elevation of serum levels of liver enzymes immediately after ethanol injection. Moreover, in our previous report, macroscopic liver atrophy accompanied by an increased future liver remnant (FLR)/total estimated liver volume ratio was evident 2 wk after PTPE^[2]. These observations suggest that the mechanisms responsible for liver atrophy likely commence soon after the disruption of portal blood flow. Consequently, histopathological changes would likely also be observed soon after PTPE.

In pigs that had undergone PTPE using a combination of coils and polyvinyl alcohol particles, the lobule size in the embolized lobe relative to normal liver reportedly decreased gradually to 23% at 12 d; after 12 d, the size of the embolized lobe remained constant^[3]. Therefore, to clarify the mechanisms responsible for liver atrophy, pathological analysis should be carried out within this time period. However, to the best of our knowledge, such time-course studies have not yet been carried out.

To assess microscopic changes in liver tissues, it is important to study liver lobules, the smallest functional units of the liver. The observation of clear histological changes would be expected when hepatic blood inflow is disturbed and would be dependent on lobule metabolism, which varies in different zones of the lobule. In particular, we focused on the zonation associated with different levels of metabolism, as illuminated by immunohistochemical (IHC) staining for glutamine synthetase (GS)^[4] and cytochrome P450 2E1 (CYP2E1)^[5]. Both markers were observed in the pericentral zone of lobules.

Recently, the relationship between apoptosis and autophagy has been extensively reported^[6]. The molecular mechanism of autophagy was illuminated by the discoveries of the membrane protein autophagy-related gene 5 in yeast and the microtubule-associated protein 1 light chain 3 (LC3) in mammals^[7]. Consequently, IHC staining for these proteins can be used to evaluate levels of autophagy^[8,9]. Recent studies have used lysosomal-associated membrane protein 2 (LAMP2) to evaluate autophagy because it is related to autolysosomes for some kinds of autophagy^[10]. Autophagy in the liver is reportedly

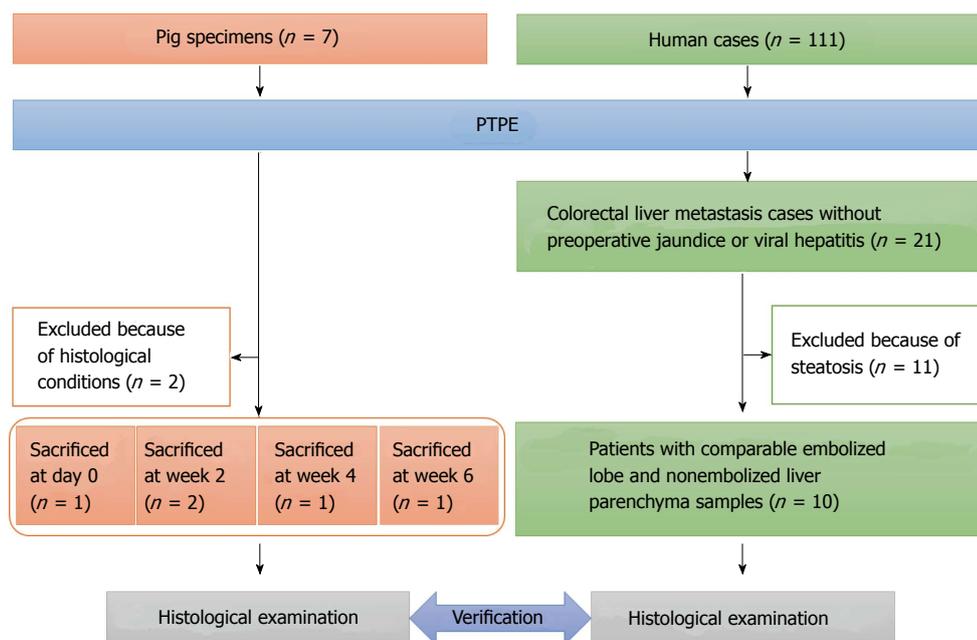


Figure 1 Flow chart of the pig specimens and human cases examined in this study. PTPE: Percutaneous transhepatic portal vein embolization.

caused by starvation and is related to hepatocellular atrophy^[11]. The interruption of portal blood flow, which contains a wealth of nutrients^[12], is considered a form of starvation. Therefore, autophagy may be related to both cellular shrinking and apoptosis. However, the relationship between portal venous obstruction and autophagy has not been reported.

The aim of this study was to investigate, using specimens from a previously reported porcine PTPE model^[2], the microscopic changes associated with apoptosis and autophagy in the days and weeks following portal venous obstruction and to clarify the mechanism by which interrupted portal blood flow causes liver atrophy. Furthermore, we sought to verify the integrity of our pig results by performing the same histopathological investigations in specimens resected from human patients who had undergone PTPE^[13-16].

MATERIALS AND METHODS

Animal specimens

Liver specimens obtained from seven female domestic pigs (Saitama Experimental Animal Supply, Saitama, Japan) weighing 30.0-35.0 kg were used in this study. All pigs underwent segmental PTPE under fluoroscopic guidance with injection of 10 mL absolute ethanol, as we described previously^[2]. Specimens from two pigs were excluded from this study because their quality was unsuitable for pathological analysis. Finally, specimens from five pigs were selected for analysis; one pig was sacrificed on day 0, two pigs at week 2, one pig at week 4, and one pig at week 6 (Figure 1).

The removed pig livers were observed macroscopically (Figure 2A and B). No pig livers exhibited bleeding, degeneration, or necrosis. To evaluate the pure

histological changes of the embolized area compared with those of the nonembolized area without histological regenerative reactions, formalin-fixed paraffin-embedded specimens were produced from samples resected from the embolized segment and a nonembolized lobe (control) far from the lobe containing the embolized segment.

Patients

Formalin-fixed paraffin-embedded specimens obtained from 111 patients who underwent major hepatectomy with preoperative PTPE between 2004 and 2010 were collected at the Hepatobiliary Pancreatic Surgery Division of the National Cancer Center Hospital, Tokyo, Japan. Of these 111 patients, 21 had colorectal liver metastases without preoperative jaundice or viral hepatitis. To facilitate the histological evaluation of liver lobules, 11 patients were excluded because of steatosis. In total, 10 patients with comparable embolized lobe and nonembolized liver parenchyma samples (*e.g.*, the caudate lobe or partial hepatectomy from contralateral lobe of PTPE) were selected (Figure 1). All patients (male-to-female ratio: 4:6, median age: 59 years, range: 43-76 years) underwent hepatectomy with PTPE based on their individual clinical status (Table 1), and samples were collected between 20 and 36 d later (median: 22 d). All patients underwent PTPE *via* the ipsilateral approach using a 21-G needle (Top, Tokyo, Japan) under ultrasonographic guidance. A 5-Fr sheath (introducer set, Medikit, Tokyo, Japan) was introduced into a branch of the portal vein under fluoroscopic guidance and a 5-Fr balloon catheter (Selection Balloon Catheter, Terumo Clinical Supply, Gifu, Japan) was used for the injection of absolute ethanol (99.5% ethanol, Fuso Pharmaceutical Industries, Osaka, Japan). The study was approved by the Ethics Committee of our

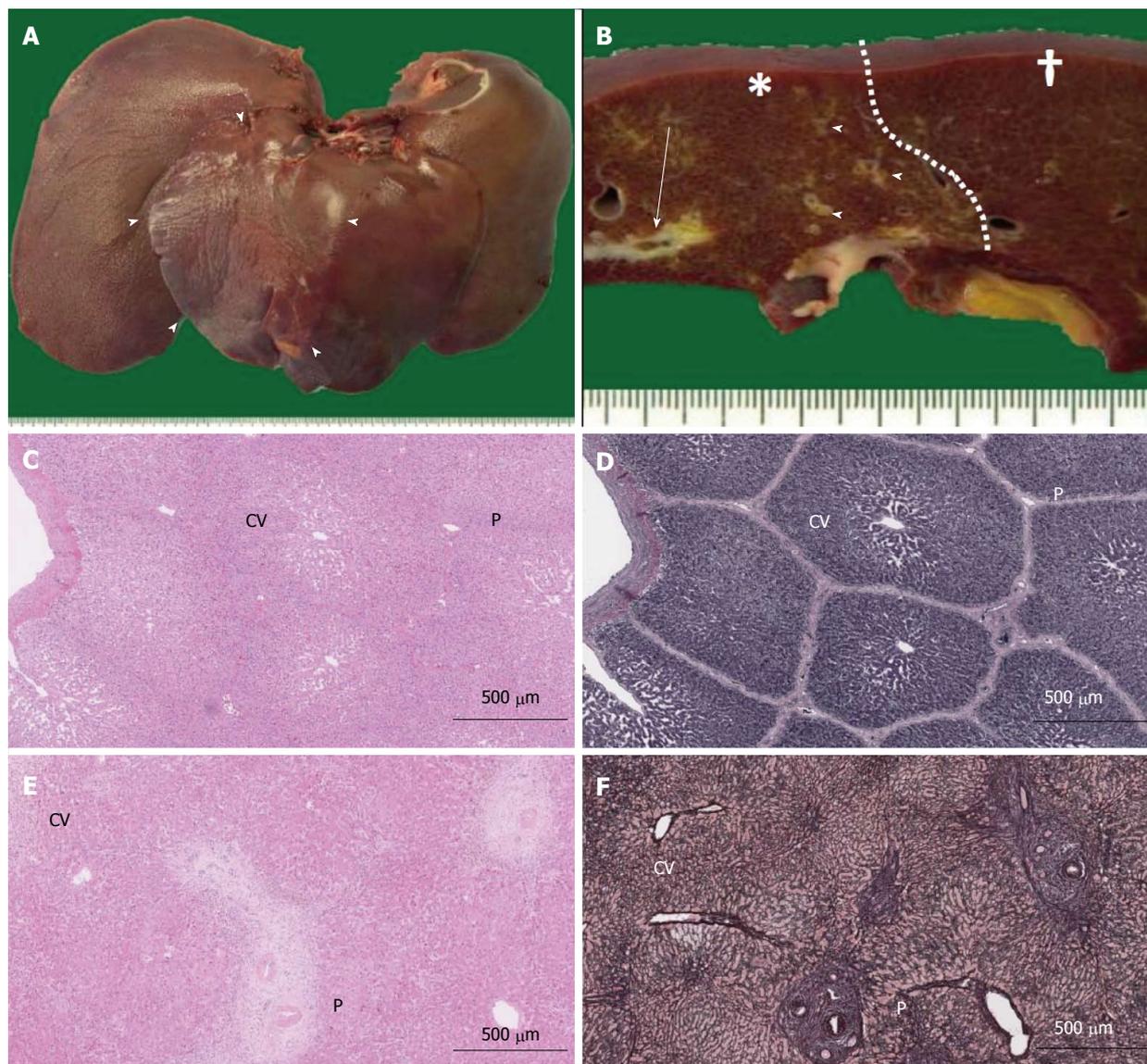


Figure 2 Macroscopic images of pig livers following interruption of portal blood flow and structural comparison of pig and human liver lobules. A: Liver removed from a pig at week 2 after PTPE. Segmental macroscopic atrophy could be seen (arrowheads); B: Portal thrombosis was observed at the cut surface (arrow) in the embolized area (*), and fibrous thickening of the portal areas was observed (arrowheads) compared with nonembolized area (†). A clear border (dotted line) was observed between these areas; C-F: Microscopic views (C, D: Pig; E, F: Human) show central veins (CV) and portal areas (P) in HE-stained sections (C, E), but these features are more clearly observed in silver-stained sections (D, F). Lobule structure was less well defined in human specimens (F).

institution. All patients gave written informed consent for inclusion in this study (ID: 2007-022).

Histological examination

All histological examinations were carried out using digital images scanned by Nanozoomer Digital Pathology (NDP, Hamamatsu Photonics, Hamamatsu, Japan) evaluated by two experienced pathologists (Yasuhito Iwao and Hidenori Ojima) who were blinded to all experimental and clinical data. The pathologists conferred if the original evaluations differed.

Morphological study of the lobule

Sections were stained with hematoxylin and eosin (HE), and the morphological changes in embolized and nonembolized lobules were evaluated at 50 random locations on NDP images. The distance between the

endothelium of the portal vein in the portal triad and the associated central vein in the same lobule (PV-CV) and the cross sectional area of the lobule (which has a convex shape around a single central vein) were recorded. After the median lobule size (median, ± 0.100 mm²) of each group was determined, the number of hepatocytes in each lobule was counted for 20 randomly selected lobules (Figure 2C and D). Hepatocyte density was calculated by dividing the number of hepatocytes by the area of the counted lobule for pig specimens. For human specimens, the hepatocyte density was counted within 20 randomly selected 1-mm-diameter circles.

Evaluation of apoptotic activity

Apoptosis of hepatocytes was quantified by terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick-end labeling (TUNEL) assay (In situ cell

Table 1 Clinical background of the patients who provided the human specimens

Patient	Gender	Age (yr)	Primary tumor site	Surgery
1	F	74	Rectum	Ex Rt
2	F	57	Rectum	Ex Rt
3	M	76	Cecum	Ex Rt
4	M	43	S/C	Ex Rt
5	M	56	Rectum	Ex Rt
6	M	53	Rectum	Ex Rt + nonAnat S3
7	F	47	S/C	Ex Rt
8	F	61	Rectum	Ex Rt
9	F	64	S/C	Ex Rt
10	F	63	Rectum	Ex Rt + nonAnat S3

All patients underwent right-sided percutaneous transhepatic portal embolization. F: Female; M: Male; S/C: Sigmoid colon; Ex Rt: Extended right hemilobectomy; nonAnat S3: Non-anatomical liver resection of segment 3.

death detection kit, POD, Roche Diagnostic, Mannheim, Germany). The proportion of TUNEL-positive hepatocytes was counted five times in ten random high-power fields.

Immunohistochemical staining

Sections (4- μ m thick) were deparaffinized and incubated in an autoclave for 10 min at 121 °C and 1.5 bar. IHC staining was performed using a polymer system (Dako, Glostrup, Denmark) with 3,3'-diaminobenzidine (DAB/Tris tablets, Muto Pure Chemicals, Tokyo, Japan) as the chromogen. A mouse monoclonal antibody (1:50, sc-271625, clone G-2, Santa Cruz Biotechnology, Santa Cruz, CA, United States) was used for LC3, a rabbit polyclonal antibody (1:100, bs-2379R, Bioss, Beijing, China) was used for LAMP2, a mouse monoclonal antibody (1:2000, MAB302, clone GS-6, Millipore, Billerica, United States) was used for GS, and a rabbit polyclonal antibody (1:100, bs-4562R, Bioss, Beijing, China) was used for CYP2E1. The sections were incubated for 2 h at room temperature.

After sections stained for LC3 were scanned and captured by NDP, the digital images were analyzed using ImageJ version 1.48 (National Institutes of Health, Bethesda, Maryland, United States). To facilitate comparisons between pig specimens, the IHC intensity of LC3 was evaluated for each lobule and then divided by the IHC intensity of nerve in the same portal area as the positive control.

Electron microscopy

Formalin-fixed pig liver specimens were analyzed using a Hitachi H-7650 (Hitachi, Tokyo, Japan) transmission electron microscope. Magnification at 80 kV achieved a clear depiction of the hepatocyte organelles.

Statistical analysis

Statistical analysis was performed with the Statistical Package for Social Sciences version 22 (SPSS Inc., Chicago, IL, United States). The Mann-Whitney *U* test

was used to assess differences between embolized and nonembolized samples at each time point. For nonparametric multiple comparisons, the Kruskal-Wallis test was applied. Differences were considered significant at $P < 0.05$. Data are expressed as medians unless otherwise indicated.

Animal care and use statement

The animal experiment protocols were described in our previous report^[2]. All protocols were approved by the Committee for Ethics in Animal Experimentation and were conducted in accordance with the Guidelines for Animal Experiments of our institution (ID: K03-004).

RESULTS

Changes in PV-CV distance, lobule area, and number of hepatocytes per lobule

The PV-CV distance in embolized and control specimens did not differ significantly at day 0 (0.571 mm vs 0.485 mm, respectively). However, at weeks 2, 4, and 6, the PV-CV distance was significantly reduced in embolized specimens (week 2: 0.364 mm, week 4: 0.335 mm, and week 6: 0.372 mm, $P < 0.001$, $P = 0.001$, $P = 0.001$, respectively) compared with control specimens. Moreover, at weeks 2, 4, and 6, the PV-CV distance was significantly reduced in embolized specimens compared with embolized specimens at day 0 ($P < 0.001$, $P = 0.001$, $P = 0.001$, respectively) (Figure 3A). The lobule cross sections of embolized specimens at weeks 2, 4, and 6 (week 2: 0.368 mm², week 4: 0.532 mm², and week 6: 0.462 mm²) were significantly smaller than those of control specimens and were also smaller than embolized specimens at day 0 (1.096 mm²) ($P < 0.001$ for all) (Figure 3B). The PV-CV distances and lobule areas in embolized specimens at weeks 2, 4, and 6 did not differ significantly.

The number of hepatocytes in lobules of median size did not differ significantly between embolized and control specimens until 4 wk after PTPE (week 4: 1459 and 2055, respectively, $P = 0.025$; week 6: 1494 and 2642, $P < 0.001$). At weeks 4 and 6, the number of hepatocytes per median-sized lobule in embolized specimens was significantly smaller than those in embolized and in control specimens at day 0 and week 2 ($P < 0.001$ for all) (Figure 3C). Therefore, the hepatocyte density in embolized specimens peaked at week 2 (5878/mm²) (Figure 3D).

Evaluation of apoptotic activity

The fraction of TUNEL-positive hepatocytes was higher in embolized than in control specimens at day 0 and week 4 (11.1% vs 2.37% on day 0 and 5.51% vs 0.493% at week 4, $P = 0.018$, $P = 0.009$, respectively; Figure 3E).

Transition of LC3/LAMP2 IHC intensity in the lobule and GS/CYP2E1 zonation

The IHC intensity, as measured by Image J, for LC3

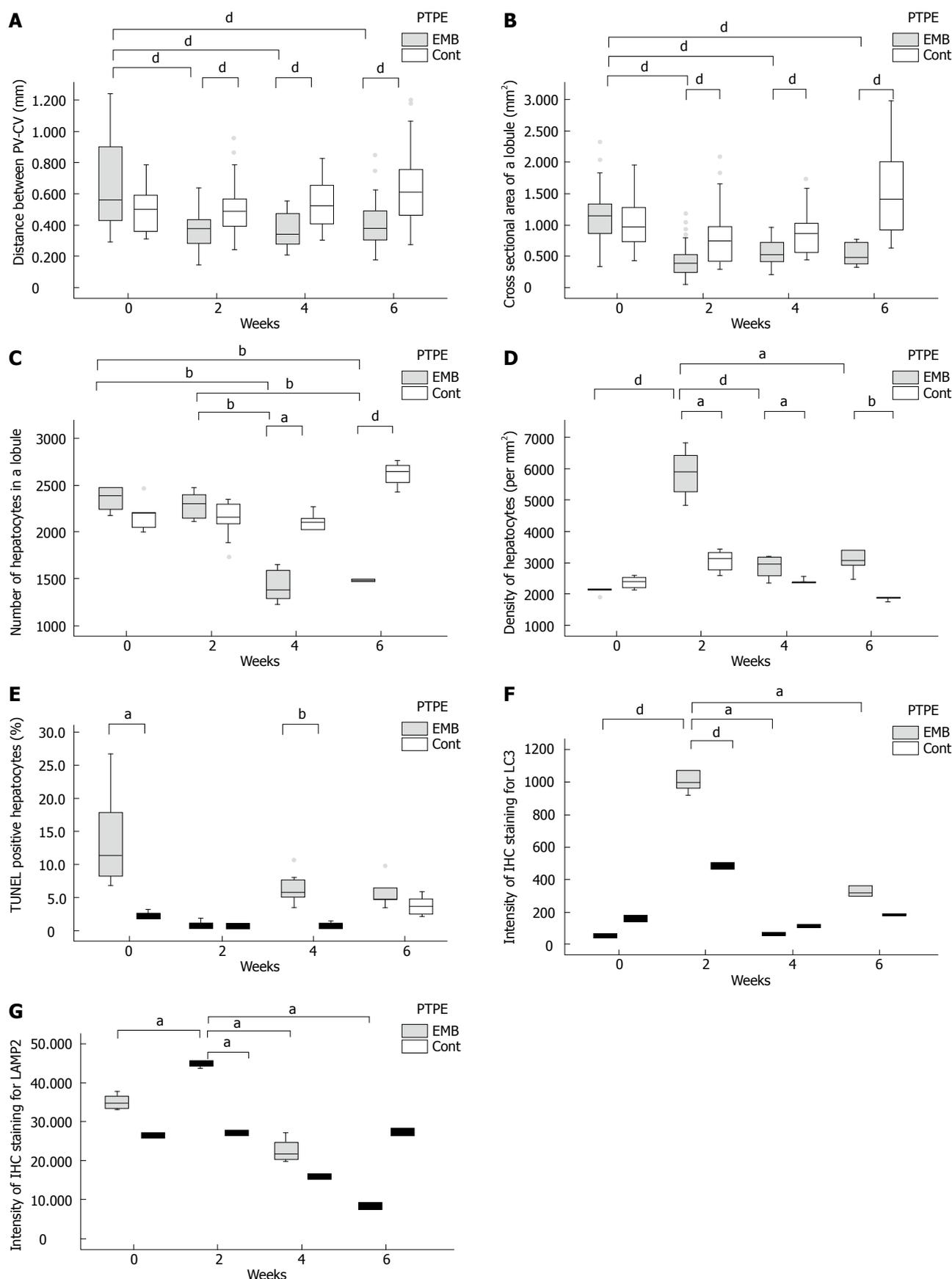


Figure 3 Histological changes in pig livers following the interruption of portal blood flow. A and B: The distance between the portal vein and central vein (A) and the cross sectional area of the lobule (B) of the embolized segment at week 2 after PTPE differed significantly from those of controls; C and D: The number of hepatocytes per lobule (C) of the embolized segment was significantly lower than in control lobes at week 4, and the density of hepatocytes (D) in the embolized area at week 2 was highest; E-G: The fraction of TUNEL-positive hepatocytes (E) in the embolized segment was significantly higher than in control segments at week 4, and the LC3 (F) and LAMP2 (G) intensity was highest in embolized lobes at week 2. ^a*P* ≤ 0.05, ^b*P* ≤ 0.01, ^d*P* ≤ 0.001. EMB: Embolized area; Cont: Control lobe area.

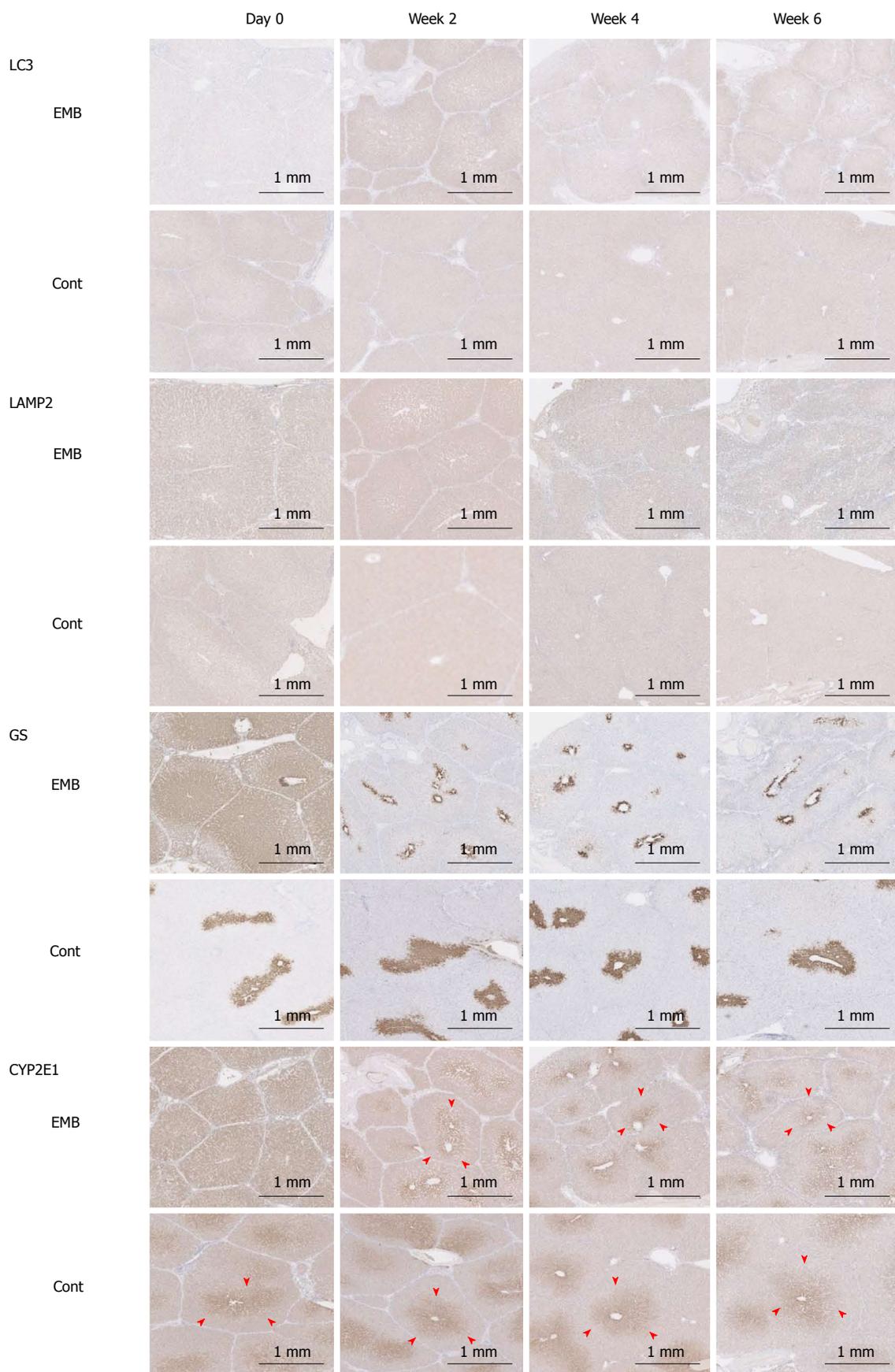


Figure 4 Light chain 3, lysosomal-associated membrane protein 2, glutamine synthetase, and cytochrome P450 2E1 immunohistochemical staining intensities. Expression of LC3 and LAMP2 was highest in the embolized segment of porcine specimens at 2 wk after PTPE. Zonation of GS and CYP2E1 (arrowheads) was expanded in the embolized area immediately after interruption of portal blood flow, but was reduced in the embolized lobe at 2 wk. EMB: Embolized area; Cont: Control lobe area; LC3: Light chain 3; LAMP2: Lysosomal-associated membrane protein 2; GS: Glutamine synthetase; CYP2E1: Cytochrome P450 2E1; IHC: Immunohistochemical.

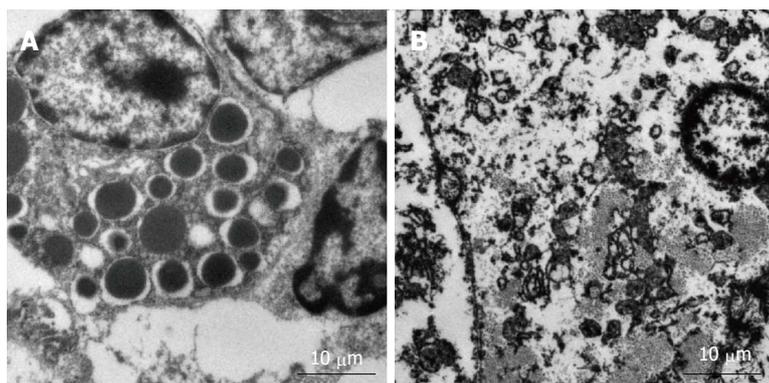


Figure 5 Electron microscopic findings at week 2. A and B: Many more autophagic vacuoles were found in embolized samples (A) compared with control samples (B) at week 2.

and LAMP2 in embolized specimens at week 2 (0.994 and 45.4, respectively) was significantly higher than that in control specimens (0.486, $P = 0.046$ and 27.0, $P = 0.014$, respectively). Moreover, the LC3 and LAMP2 intensities of embolized specimens at week 2 were significantly higher than those in all other specimens ($P \leq 0.025$ and $P \leq 0.014$ for all; Figure 3F, G and Figure 4). GS and CYP2E1 staining intensities in embolized specimens were not closely associated with those of control specimens at day 0. The extent of the stained zones decreased after 2 wk (Figure 4).

Electron microscopy

Clear findings were hard to establish because of the poor condition of pig liver specimens that had been fixed in formalin some time previously. There was the suggestion of a peak of autophagic vacuoles in embolized samples at week 2 (Figure 5), which was consistent with the IHC staining intensity of LC3.

PV-CV distance and hepatocyte density in human specimens

We sought to validate our findings in porcine samples by repeating the analytical procedures in human liver specimens from patients following PTPE. Because human lobule structures are not as well defined as those in porcine specimens, the PV-CV distance and hepatocyte density were assessed in a morphological study (Figure 2E and F). PV-CV was significantly shorter in embolized specimens than in nonembolized specimens (0.455 mm vs 0.563 mm, $P < 0.001$) (Figure 6A), as was also observed in porcine specimens 4 wk after PTPE. The hepatocyte density in embolized specimens was significantly higher than that in nonembolized specimens (2111/mm² vs 1772/mm², $P = 0.038$) (Figure 6B).

Evaluation of apoptotic activity, LC3 intensity, and GS and CYP2E1 zonation in human specimens

A significantly greater fraction of hepatocytes was TUNEL-positive in embolized specimens than in nonembolized specimens (2.804% vs 0.559%, $P <$

0.001) (Figure 6C). However, the LC3 intensity did not differ significantly between embolized and nonembolized specimens (Figure 6D, E and H). The extents of GS and CYP2E1 zonation were reduced in embolized specimens compared with nonembolized specimens (Figure 6F, G, I and J); similar results were observed in porcine specimens collected 4 wk after PTPE.

DISCUSSION

Interruption of the portal blood flow causes shrinkage of the embolized lobe and compensatory enlargement of the nonembolized lobes. The effects of portal venous obstruction on hepatocyte volume and apoptosis have been previously reported^[13-16]. However, these studies used only human specimens in which the atrophy process was complete. As a result, the process of liver atrophy could not be studied in detail. Our morphological study focused on changes in the lobules over time in porcine samples. We observed two distinct phases of liver atrophy following portal blood flow disruption. The first phase was characterized by lobule shrinkage without a fall in the number of hepatocytes and was accompanied by strong expressions of LC3 and LAMP2 in the first 2 wk after portal venous obstruction. The second phase, which occurred between 2 and 4 wk after portal venous obstruction, was characterized by a reduction in the number of hepatocytes without changes in lobular size. This reduction was accompanied by decreased LC3 and LAMP2 intensity and an increased fraction of TUNEL-positive cells (Figure 7).

Soon after the injection of ethanol, the zonation of GS and CYP2E1 in embolized specimens expanded markedly. Increased GS zonation could represent accelerated ammonia metabolism resulting from the degradation of denatured proteins^[17]. Moreover, it has been reported that CYP2E1 is directly associated with ethanol metabolism^[18]. Furthermore, in this study, the fraction of TUNEL-positive hepatocytes was observed to increase in embolized specimens at day 0; this finding may reflect damage caused by ethanol.

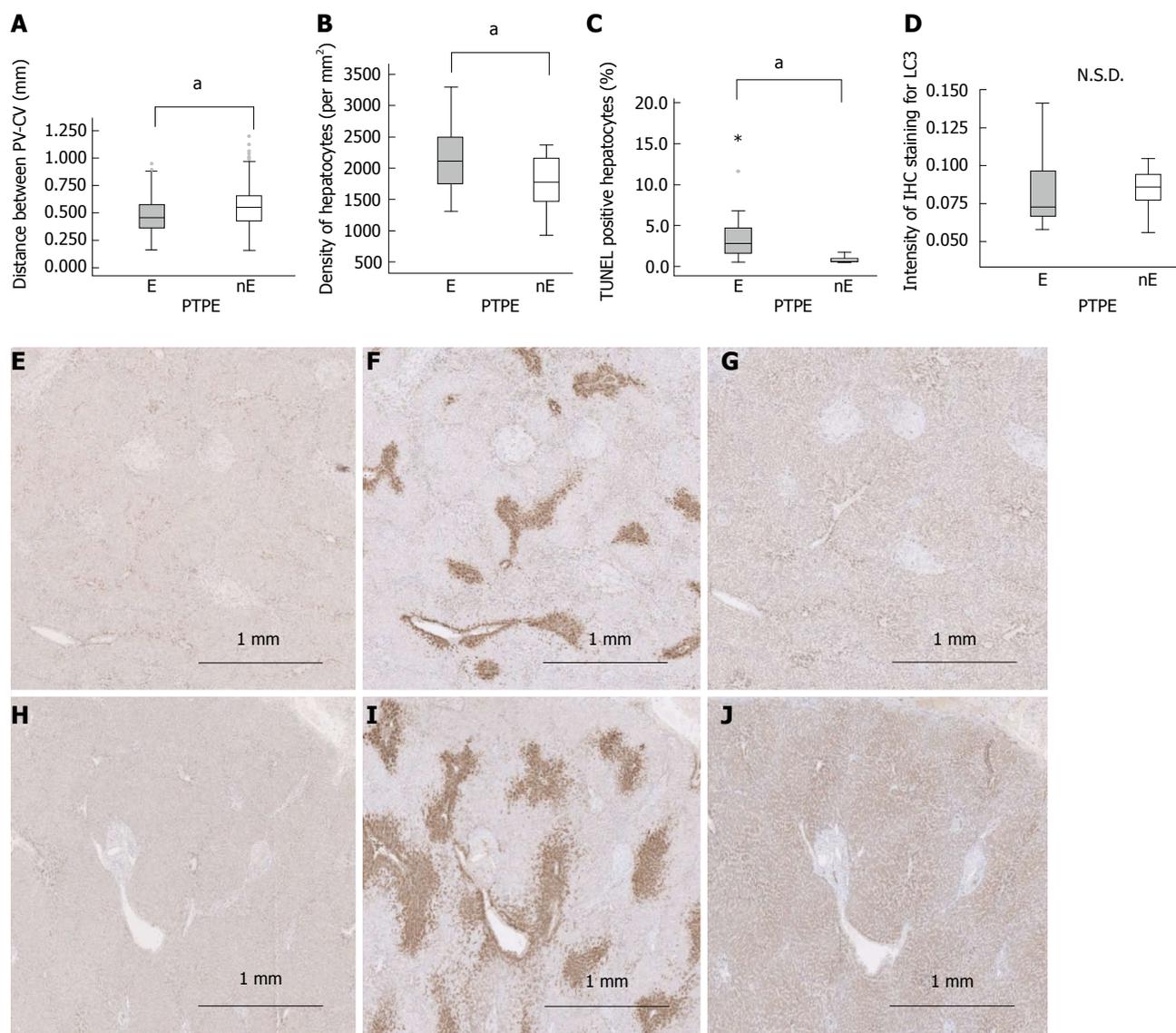


Figure 6 Human specimens. A: The distance between the portal vein and central vein; B: Hepatocyte density; C: Fraction of TUNEL-positive hepatocytes differed significantly between embolized and nonembolized lobes; D: Intensity of IHC staining for LC3; E-J: IHC for LC3 (E, H), GS (F, I), and CYP2E1 (G, J). LC3 expression did not differ significantly (D) between the embolized (E) and the nonembolized area (H), but the zonation of GS and CYP2E1 was narrower in the embolized lobe (F, G) than the nonembolized lobe (I, J). $^aP \leq 0.001$. N.S.D: No significant difference; E: Embolized area; nE: Nonembolized area.

Hepatocytes in the embolized lobule may degenerate soon after ethanol injection. These changes are consistent with the clinical observation that circulating levels of transaminases are transiently elevated after ethanol injection to patients undergoing portal vein embolization^[19]. In addition, we found that the proportion of TUNEL-positive hepatocytes decreased in the first 2 wk and did not differ between embolized and control specimens at week 2. However, hepatocyte numbers were reported to be restored in 3-4 d after partial hepatectomy^[20], and hepatocyte replication in the embolized lobe was reported to be slightly increased approximately 7 d after PTPE with coils and particles^[3]. Perhaps the cellular damage observed at day 0 in our study was repaired *via* regeneration within the first few days.

The PV-CV distance and lobule size were reduced

without the loss of hepatocytes in embolized specimens at week 2. This first phase could be considered a hepatocellular atrophic phase. Interruption of the portal blood flow (which is rich in nutrients from the gastrointestinal tract) may starve hepatocytes after embolization. Starvation reportedly causes autophagy and hepatocyte atrophy^[11]. Interestingly, in our study, LC3 and LAMP2 expression was significantly increased in embolized specimens at week 2. Simultaneously, GS and CYP2E1 zonation were reduced at week 2 as starvation caused a reduction in metabolism. Moreover, we found an increase in the number of autophagic vacuoles in embolized specimens at week 2. Thus, we speculated that disruption of the portal blood flow caused hepatocyte shrinkage by activating autophagy.

Between weeks 2 and 4, the number of hepatocytes in embolized specimens decreased without

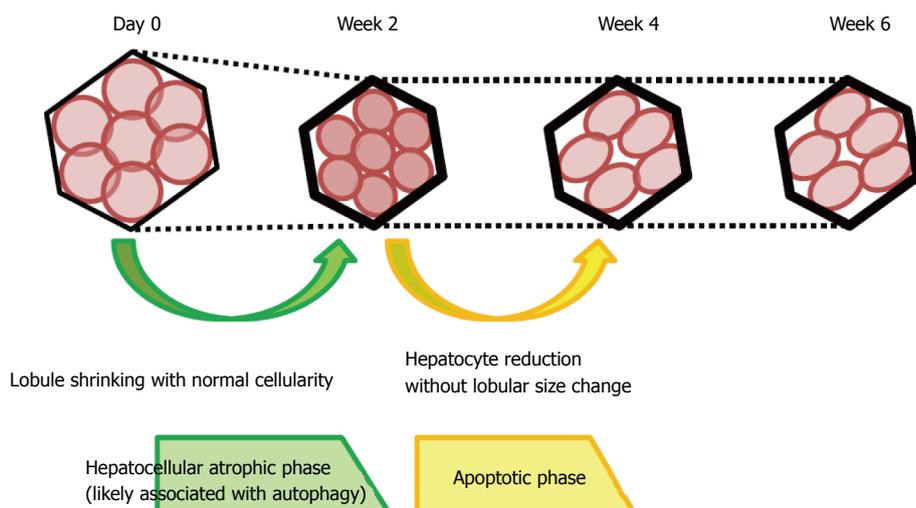


Figure 7 Schema of the histological changes occurring following interruption of portal blood flow. At 2 wk after obstruction of the portal vein, lobular shrinkage was observed without reduction in hepatocyte number, but with strong LC3 and LAMP2 expression. These changes may be associated with autophagy, and this process is termed the hepatocellular atrophic phase. At week 4, hepatocyte numbers fell, without a reduction in lobule size, but with an elevation of TUNEL staining. These secondary changes may be attributed to apoptosis occurring after autophagy, characterizing the hepatocellular atrophic phase. No significant histological changes were observed at week 6 compared with week 4.

significant changes in the lobule size. During the same period, LC3 and LAMP2 expressions fell and a larger proportion of hepatocytes became TUNEL-positive. Consequently, this phase may be regarded as encompassing the deactivation of autophagy and the activation of apoptosis. Recently, autophagy was reported to induce cell death^[21]. Therefore, the TUNEL-positive cell death we observed might represent caspase-independent apoptosis, rather than caspase-dependent apoptosis^[22]. Because hepatocyte numbers decreased while TUNEL-positive staining increased after the activation of autophagy, we characterized the phase occurring 2-4 wk after PTPE as the "apoptotic phase". During this phase, the zonation of GS and CYP2E1 did not differ significantly from that observed in embolized specimens at week 2. Between weeks 4 and 6, no morphological or IHC changes were observed at the lobular level and no significant difference was observed in the proportion of TUNEL-positive cells between embolized and control specimens at week 6. Therefore, the liver atrophy process likely terminates between weeks 4 and 6. Our results corroborate that FLR hypertrophy usually takes 4 wk to complete after PTPE because the liver atrophy process is not complete until week 4, although it appears at the macro level to have resolved by week 2.

Hepatectomy is usually performed around 4 wk after PTPE. Consequently, we sought to validate our porcine model observations in human specimens. The observations we made concerning the PV-CV distance, hepatocyte density, TUNEL staining, LC3 and LAMP2 expression, and GS and CYP2E1 zonation in embolized and nonembolized specimens at 4 wk after PTPE in porcine samples also applied to human specimens taken between 20 and 36 d after PTPE. Moreover, the TUNEL results supported those already

reported for clinical samples^[14-17]. Furthermore, the pigs we used underwent the same PTPE protocol that humans undergo clinically. Because the histological observations made using this porcine model did not contradict the human results (Table 2), the mechanism by which the interruption of portal blood flow causes liver atrophy may be similar in pigs and in humans.

The limitations of this preliminary study were that the number of pigs was insufficient for a detailed histopathological study to provide unequivocal evidence of the relationship between hepatocellular atrophy and autophagy. Further we attempted Western blotting for LC3-II, but it was not successful. However, we believe that our results and speculations provide a basis for understanding the mechanism of liver atrophy after interruption of the portal blood flow and will facilitate further study. Future research will hopefully provide a sound theoretical basis for planning treatment strategies for acute portal obstruction-related liver dysfunction or disease and chronic ischemic-related liver diseases with liver atrophy.

In conclusion, to investigate the mechanism by which portal vein obstruction causes liver atrophy, we investigated the histological changes in pig livers following PTPE and observed two distinct phases. The first phase, termed the hepatocellular atrophic phase, is characterized by lobular shrinkage without hepatocyte loss and with high levels of LC3 and LAMP2 expression. This phase lasted for the first 2 wk following PTPE. The second phase, which occurs between weeks 2 and 4, is termed the apoptotic phase and is characterized by a reduction in hepatocyte numbers without a reduction in lobular size. This is accompanied by reduced LC3 and LAMP2 expression and increased TUNEL staining. Human liver specimens resected after PTPE had many similar characteristics

Table 2 Comparison of the results for pigs and humans

	Pig liver specimens at week 4	Human liver specimens resected around week 4
PV-CV distance	EMB < Cont	EMB < nonEMB
Hepatocyte density	EMB > Cont	EMB > nonEMB
TUNEL-positive cells	EMB > Cont	EMB > nonEMB
LC3 Intensity	N.S.D.	N.S.D.
GS zonation	EMB narrower than Cont	EMB narrower than nonEMB
CYP2E1 zonation	EMB narrower than Cont	EMB narrower than nonEMB

PV-CV: Portal vein to central vein; EMB: Embolized lobe; Cont: Control lobe; nonEMB: Nonembolized lobe; N.S.D: No significant difference.

to specimens collected from pigs at week 4. Therefore, our findings suggest that the mechanism by which the interruption of portal blood flow causes liver atrophy may be similar in pigs and in humans.

ARTICLE HIGHLIGHTS

Research background

The interruption of portal blood flow by portal vein embolization or tumor thrombosis, for example, causes liver atrophy. However, the mechanisms responsible for this effect have not been fully elucidated.

Research motivation

The previous study suggested that the mechanisms responsible for liver atrophy likely commence soon after the disruption of portal blood flow. Consequently, histopathological changes would likely also be observed soon after percutaneous transhepatic portal embolization (PTPE). Recently, the relationship between apoptosis and autophagy has been extensively reported. Autophagy in the liver is reportedly caused by starvation and is related to hepatocellular atrophy, and, moreover, interruption of the portal blood flow, which contains a wealth of nutrients, is considered a form of starvation. Therefore, autophagy may be related to both cellular shrinking and apoptosis. However, the relationship between portal venous obstruction and autophagy has not been reported. To clarify the mechanisms responsible for liver atrophy, histopathological analysis should be carried out repeatedly within the first few weeks after PTPE. However, to the best of our knowledge, such time-course studies have not yet been carried out. The results and hypotheses will provide a basis for understanding the mechanism of liver atrophy after interruption of the portal blood flow and will facilitate further study.

Research objectives

The aim of this study was to investigate, using specimens from a previously reported porcine PTPE model, the microscopic changes associated with apoptosis and autophagy in the days and weeks following portal venous obstruction and to clarify the mechanism by which interrupted portal blood flow causes liver atrophy. Furthermore, to understand the mechanism of liver atrophy in humans after PTPE, the authors sought to verify the integrity of the pig results by performing the same histopathological investigations in specimens resected from human patients who had undergone PTPE.

Research methods

The authors performed histopathological examinations of liver specimens from five pigs that had undergone PTPE in a time-dependent model of liver atrophy. In specimens from embolized lobes (EMB) and nonembolized lobes (controls), the authors measured the portal vein to central vein distance (PV-CV), the area and number of hepatocytes per lobule, and apoptotic activity using the terminal deoxynucleotidyl transferase dUTP nick-end labeling assay. Immunohistochemical reactivities were evaluated for light chain 3 (LC3) and lysosomal-associated membrane protein 2 (LAMP2) as autophagy markers and for glutamine synthetase and cytochrome P450 2E1 (CYP2E1) as metabolic zonation markers. Samples from ten human livers taken 20–36 d after PTPE were similarly examined.

Research results

PV-CVs and lobule areas did not differ between EMB and controls at day 0, but were lower in EMB than in controls at weeks 2, 4, and 6. Hepatocyte numbers were not significantly reduced in EMB at day 0 and week 2 but were reduced at weeks 4 and 6. Apoptotic activity was higher in EMB than in controls at day 0 and week 4. LC3 and LAMP2 staining peaked in EMB at week 2, with no significant difference between EMB and controls at weeks 4 and 6. Glutamine synthetase and CYP2E1 zonation in EMB at weeks 2, 4, and 6 were narrower than those in controls. Human results were consistent with those of porcine specimens. However the number of pigs was insufficient for a detailed histopathological study to provide unequivocal evidence of the relationship between hepatocellular atrophy and autophagy.

Research conclusions

To investigate the mechanism by which portal vein obstruction causes liver atrophy, the authors examined the histological changes in pig livers following PTPE and observed two distinct phases. The first phase, termed the hepatocellular atrophic phase, is characterized by lobular shrinkage without hepatocyte loss and with high levels of LC3 and LAMP2 expression. This phase lasted for the first 2 wk following PTPE. The second phase, which occurs between weeks 2 and 4, is termed the apoptotic phase and is characterized by a reduction in hepatocyte numbers without a reduction in lobular size. This is accompanied by reduced LC3 and LAMP2 expression and increased TUNEL staining. Human liver specimens resected after PTPE had many similar characteristics to specimens collected from pigs at week 4. Despite liver atrophy appearing to be mostly resolved 2 wk after embolization, the period after PTPE could beneficially be extended to 4 wk to ensure contralateral hypertrophy and to allow the completion of liver atrophy.

Research perspectives

Histopathological analysis is the best way to clarify the mechanisms responsible for liver atrophy. To assess microscopic changes in liver tissues, it is important to study liver lobules, the smallest functional units of the liver. The observation of clear histological changes would be expected. To clarify the more detailed mechanism of liver atrophy after interruption of the portal blood flow, the authors have to study the histopathological changes using not only the pig model but also small animal models, *e.g.*, mouse models, because such animals are easy to handle. After such detailed studies, future research will hopefully provide a basis for understanding the mechanism of liver atrophy after interruption of the portal blood flow and also give a sound theoretical basis for planning treatment strategies for acute portal obstruction-related liver dysfunction or disease and chronic ischemic-related liver diseases with liver atrophy.

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