described by a single exponential ($y = ae^{-0034\ min}$, r = 0.99). Thus, MLCs and MSCs display functionally similar ATP degradation pathways.

Discussion

The present studies extend the observations regarding the specialized function of cholangiocytes by identifying and characterizing the elements of the purinergic signaling axis in cholangiocytes derived from distinct functional areas along the intrahepatic bile ducts. Using molecular, pharmacological, and functional biophysical approaches the principal findings in these studies of mouse cholangiocytes are: (1) both small and large cholangiocytes express a repertoire of both P2X and P2Y receptors; (2) both small and large cholangiocytes develop polarized epithelial monolayers with a high transepithelial resistance and demonstrate rapid increases in $[Ca^{2+}]_i$ and transepithelial secretion (I_{sc}) upon exposure to extracellular nucleotides; (3) nucleotide-stimulated secretion is dependent on IP3 receptor-mediated increases in $[Ca^{2+}]_i$ and Ca^{2+}-activated Cl^- channel activation; (4) both small and large cholangiocytes demonstrate mechanosensitive ATP release which is dependent on intact vesicular trafficking pathways; and (5) the magnitude of mechanosensitive ATP release is significantly greater in small versus large cholangiocytes. Thus, these studies demonstrate that both small and large cholangiocytes express all components of the purinergic signaling axis and collectively, provide a working model for mechanosensitive ATP-stimulated secretion along intrahepatic bile ducts. Additionally, the ATP-mediated secretory pathway identified in the mouse small cholangiocytes, which do not exhibit secretin-stimulated secretion,[3,17] represent the first identification of a secretory pathway in these specialized cells. The existence of a gradient along the biliary axis, wherein ATP released from small cholangiocytes "upstream" may represent an important paracrine signal to the "downstream" P2 receptor-expressing large cholangiocytes, has important implications for bile formation (Fig. 8).

Although regulated ATP release has been identified in all liver cells studied, including both human and rat hepatic parenchymal cells and biliary epithelial cells,[20,22] these are the first studies to characterize ATP release in mouse cholangiocytes, and several observations deserve highlighting. First, the magnitude of ATP release from small cholangiocytes was significantly greater than that from large cholangiocytes. Because the mechanism of cholangiocyte ATP release has not been identified, the cellular basis for this difference in

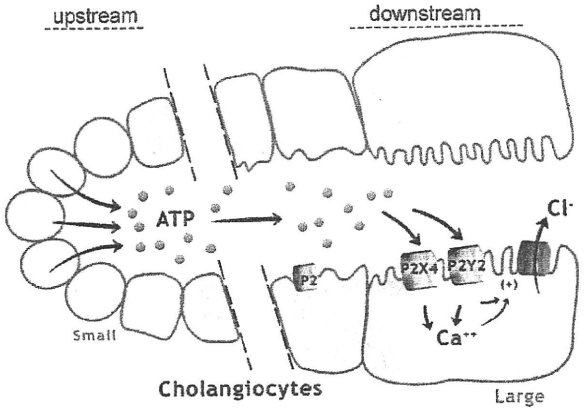

Fig. 8. Proposed model of the purinergic signaling axis along the intrahepatic bile duct. ATP released from small cholangiocytes lining the "upstream" small intrahepatic bile ducts may contribute importantly to local purinergic signaling, serve as a source for ATP in bile, and represent an important paracrine signal to the large cholangiocytes lining the larger "downstream" bile ducts. Both small and large cholangiocytes express a full array of P2 receptors and respond to extracellular nucleotides with increases in $[Ca^{2+}]_i$ and Cl^- secretion.

ATP release cannot be determined. Although CFTR has been proposed as a regulator of ATP release,[12,24,25] MSC do not express CFTR,[17] suggesting alternate ATP release pathways in these cells. One proposed alternate mechanism involves exocytosis of ATP-enriched vesicles. In fact, biliary cells possess a dense population of vesicles ~140 nm in diameter in the subapical space,[26] and increases in cell volume increase the rate of exocytosis to values sufficient to replace ~30% of plasma membrane surface area within minutes. In the current studies, stimuli associated with ATP release were also associated with parallel increases in the rate of exocytosis, and disruption of vesicular trafficking significantly decreased ATP release. Notably, overall rates of exocytosis in response to mechanosensitive stimuli did not vary significantly between MLCs and MSCs, despite a significantly greater release of ATP from MSCs, given the same stimulus. This may suggest the existence of distinct vesicle populations contributing to regulated ATP release. In fact, recent findings in rat liver cells suggest that a distinct population of ATP-enriched vesicles may contribute to regulated ATP release.[27] In some cell types, the concentration of ATP within secretory vesicles may approach 50 mM[28] and, therefore, only several vesicles per cell may account for substantial differences in the concentration of ATP released into the extracellular space. Differences observed in the magnitude of ATP release between MSCs and MLCs may be related to variation in the regulation and/or trafficking of specific vesicles involved in ATP transport (either ATP-containing

vesicles and/or vesicles transporting an ATP transporter to the membrane). This regulation may occur at the level of vesicle "priming", trafficking, or membrane fusion/release, though clearly further work is required. Nonetheless, if these observations apply to *in vivo* conditions, greater ATP release from small cholangiocytes would translate into a significant increase in the concentration of ATP in bile in the "upstream" intrahepatic ducts, given their smaller cross-sectional area and relative volume.[29]

Second, it is notable that extracellular nucleotides elicit secretory responses when applied at both apical and basolateral membranes. The apical membrane specifically represents an anatomic orientation that is well suited for hepatocyte-to-cholangiocyte or cholangiocyte-to-cholangiocyte signaling by release of ATP into bile. This is notably distinct from secretin and other hormones that are delivered to the basolateral membrane through the bloodstream.[1] ATP release from the hepatocyte canalicular membrane may signal to downstream small and large cholangiocytes through apical P2 receptor stimulation in a process known as hepatobiliary coupling. Hepatobiliary coupling has also been described for bile acids, which are released from the hepatocyte canalicular membrane and may be transported into "downstream" cholangiocytes via the apical Na^+-dependent bile acid transporter located on large, but not small, cholangiocytes.[30] Interestingly, Ursodeoxycholic acid is associated with cholangiocyte ATP release and Cl^- secretion.[24] Thus, the ductal concentration of ATP appears to be an important determinant of bile formation and may represent a final common pathway in coupling hepatocyte transport to cholangiocyte secretion.

Lastly, the relative importance of secretin- versus P2 receptor-mediated secretion, in bile formation is unknown. The molecular identity of the Cl^- channel(s) activated in response to ATP remains undefined in biliary epithelium, though it appears to be unrelated to CFTR.[10] Furthermore, although we have previously identified the Ca^{2+}-activated K^+ channels, SK2 and IK-1, in rat and human biliary epithelial cells,[7,8] the expression and contribution of these channels to secretion in mouse cholangiocytes has not been defined.

In conclusion, the present studies represent a functional characterization of the purinergic signaling axis in mouse cholangiocytes from distinct areas of the intrahepatic biliary tree. The findings support a model wherein ATP released from small cholangiocytes lining the "upstream" small intrahepatic bile ducts may contribute importantly to local purinergic signaling, serve as a source for ATP in bile, and represent an important paracrine signal to the large cholangiocytes lining the larger "downstream" bile ducts. Targeting P2 receptor-mediated signaling pathways in intrahepatic biliary epithelial cells may provide new and innovative strategies for stimulating bile formation in the treatment of cholestatic liver diseases.

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Inhibitory effect on hepatitis B virus in vitro by a peroxisome proliferator-activated receptor-γ ligand, rosiglitazone

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ABSTRACT

Although chronic infection of hepatitis B virus (HBV) is currently managed with nucleot(s)ide analogues or interferon-α, the control of HBV infection still remains a clinical challenge. Peroxisome proliferator-activated receptor (PPAR) is a ligand-activated transcription factor, that plays a role in glucose and lipid metabolism, immune reactions, and inflammation. In this study, the suppressive effect of PPAR ligands on HBV replication was examined in vitro using a PPARα ligand, bezafibrate, and a PPARγ ligand, rosiglitazone. The effects were examined in HepG2 cells transfected with a plasmid containing 1.3-fold HBV genome. Whereas bezafibrate showed no effect against HBV replication, rosiglitazone reduced the amount of HBV DNA, hepatitis B surface antigen, and hepatitis B e antigen in the culture supernatant. Southern blot analysis showed that the replicative intermediates of HBV in the cells were also inhibited. It was confirmed that GW9662, an antagonist of PPARγ, reduced the suppressive effect of rosiglitazone on HBV. Moreover, rosiglitazone showed a synergistic effect on HBV replication with lamivudine or interferon-α-2b. In conclusion, this study showed that rosiglitazone inhibited the replication of HBV in vitro, and suggested that the combination therapy of rosiglitazone and nucleot(s)ide analogues or interferon could be a therapeutic option for chronic HBV infection.

1. Introduction

Hepatitis B virus (HBV) is a major health problem over the world, and about 350 million people have continuous infection of HBV. Fifteen to 40% of HBV-infected patients develop cirrhosis, liver failure, or hepatocellular carcinoma (HCC) [1]. Currently, there is no therapy that eliminates completely the infection in most chronically infected patients. Few patients achieve hepatitis B surface antigen (HBsAg) loss with existing drugs, and the loss of hepatitis B e antigen (HBeAg) is thought to be one of the signs that indicate a good response to therapy. Chronic hepatitis B patients are clinically treated with interferon (IFN)-α and nucleot(s)ide analogues such as lamivudine, adefovir, and entecavir. Nucleot(s)ide analogues are potent but have some problems including drug resistance with continuous treatment [2], and IFN-α has the disadvantages of limited effectiveness and side effects. Therefore, new and improved antiviral therapies with novel targets and mechanisms are still needed.

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors consisting of three isotypes: PPARα, PPARβ, and PPARγ. They exist in many kinds of cells including hepatocytes [3–5] and regulate the transcription of distinct genes through heterodimerization with the retinoid X receptors (RXRs). Among these transcription factors, PPARα and PPARγ together with their obligate partner RXR are the three main nuclear receptors expressed in the liver [6–8]. They were first identified as regulators of lipid and glucose metabolism [9]. In recent reports, PPARs may also be involved in the complex regulation of immune and inflammatory processes [10]. The activation of PPARα and PPARγ correlated with the inhibition of inflammatory cell responses in a variety of cell types. PPARγ ligands inhibit the expression of inflammatory genes such as interleukin (IL)-1β and tumor necrosis factor (TNF)-α [11]. Therefore, there is a possibility that ligands of PPARs may affect the replication of viruses. It was reported that the replication of respiratory syncytial virus (RSV) and human immunodeficiency virus type 1 (HIV-1) was suppressed by PPARγ ligands [12,13]. The involvement of nuclear hormone receptors, such as RXRα and PPARα, in the transcription and replication of HBV has been suggested [14]. It was also reported that HBV X protein induced hepatic steatosis via the activation of PPARγ [15]. However, the direct effect of the ligands of PPARs on the replication of HBV remains unknown.

In this study, the effect of the ligands of PPARs on the replication of HBV was examined in vitro using a PPARα ligand, bezafibrate, and a PPARγ ligand, rosiglitazone. The therapeutic

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application as a combination therapy with existing drugs for chronic hepatitis B, lamivudine and IFN-α-2b, was also investigated in the same system.

2. Materials and methods

2.1. Drugs

Rosiglitazone was purchased from Cayman Chemical Company (Ann Arbor, MI), and bezafibrate was provided by Kissei Pharmaceutical Co., Ltd. (Nagano, Japan). Lamivudine was purchased from Toronto Research Chemicals, Inc. (Ontario, Canada), and IFN-α-2b was provided by Schering-Plough K.K. (Tokyo, Japan). An antagonist of PPARγ, GW9662, was purchased from Calbiochem (Darmstadt, Germany).

2.2. Plasmid

Plasmid containing 1.3 times whole HBV DNA was constructed using a genotype B2 HBV strain obtained from a fulminant hepatitis patient [16]. The strain had mutations in the core promoter region (A1762T/G1764A) and in the precore region (G1896A). These mutations were converted into wild-type nucleotides using site-directed mutagenesis, and the plasmid was used as a wild-type HBV clone. A clone with only A1762T/G1764A or G1896A was constructed also in the same way.

2.3. Cell culture

Human hepatoma HepG2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C and 5% CO_2.

2.4. Cytotoxicity assay

The drug-induced cytotoxicity of bezafibrate and rosiglitazone was examined using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2*H*-tetrazolium (MTS) assay. HepG2 cells were seeded in 96-well tissue culture plates at the concentration of 1.0×10^4/well. At 24 h after seeding of the cells, the medium was exchanged to media containing several different concentrations of rosiglitazone or bezafibrate with 0.1% DMSO. At 24 h after addition of the drugs, CellTiter 96® AQ$_{UEOUS}$ One Solution Reagent (Promega, Madison, WI) was added, followed by incubation for an hour. Absorbance was measured at 490 nm, and the 50% cytotoxicity concentration (CC_{50}) for each drug was calculated.

2.5. Transfection and addition of drugs

HepG2 cells were seeded in 24-well tissue culture plates at the concentration of 1.25×10^5/well for real-time polymerase chain reaction (PCR), or 6-well tissue culture plates at the concentration of 7.5×10^5/well for Southern blotting analysis. Twenty-four hours later, the plasmids containing 1.3-fold HBV genome were transfected to the cells using TransIT LT-1 Mirus Transfection Reagent (Mirus, Madison, WI). At 24 h post-transfection, medium containing rosiglitazone, bezafibrate, lamivudine, or IFN-α-2b, was added to the cells with 0.1% DMSO. Alternatively, these drugs were added in combination. At day 3 after the addition of the drugs, the culture supernatant and cells were collected separately.

2.6. Quantification of HBV DNA, HBsAg, and HBeAg in the culture supernatant

To digest the input plasmid DNA in the culture supernatant, 5 μl of the supernatant were treated with 5 U of DNase I (TaKaRa Bio, Inc., Shiga, Japan) at 37 °C for 2 h, and the reaction was stopped with EDTA. Total DNA in the culture supernatant was extracted using SMITEST EX-R&D (Genome Science Co. Ltd., Tokyo, Japan), and 10 μl of 200 μl DNA solution was subjected to real-time PCR using a LightCycler system (Roche Diagnostics, Mannheim, Germany) as described previously [16]. Dose–response curves were plotted to determine the 50% effective concentration (EC_{50}) for each drug. HBsAg and HBeAg in 50 μl of the culture supernatant were assayed by enzyme-linked immunosorbent assay (ELISA), using and HBsAg ELISA kit (Hope Laboratories, Belmont, CA) and ELISA kit for HBeAg (BioChain Institute, Inc., Hayward, CA), respectively.

2.7. Detection of replicative intermediates of HBV in the cells

The core particle-associated HBV DNA in the cells was isolated as described previously [16] with slight modifications. The cells were washed with phosphate-buffered saline (PBS) and lysed in 400 μl lysis buffer (50 mM Tris–HCl, 1 mM EDTA, 1% Nonidet P-40) per well. The lysed cells were centrifuged and the supernatant was collected. The samples were reacted with DNase I to remove unprotected DNA, and the reaction was stopped by EDTA. Total DNA was extracted using EX-R&D, and analyzed with Southern blotting analysis using a full-length HBV DNA probe labeled with PCR DIG Probe Synthesis kit (Roche Diagnostics). The signal was analyzed with LAS-1000 Image Analyzer (Fuji Photo Film, Tokyo, Japan) and quantified by densitometry with ImageJ 1.39u (The National Institute of Health, Bethesda, MD).

2.8. Analysis of drug combination

The doses in the experiment for combined drugs were determined as described previously [17] with modification: of the five concentrations tested for each drug, the middle dose was approximately equal to the EC_{50}, and two higher doses were three and nine times the EC_{50}, and the two lower doses were 0.11 and 0.33 times the EC_{50}. The effect on HBV replication of the combination of rosiglitazone and lamivudine, or rosiglitazone and IFN-α-2b, was analyzed in HBV-transfected HepG2 cells as described above. Evaluation of the drug interactions in the combination treatments was conducted against the corresponding monotherapies using the CalcuSyn® for Windows computer program (Biosoft, Inc., Cambridge, United Kingdom), and synergism was evaluated.

2.9. Statistical analysis

Statistical analyses were performed using Fisher's exact probability test for comparison of proportions between two groups. Differences were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. Cell toxicity assay

The cytotoxicity of rosiglitazone, bezafibrate, lamivudine, and IFN-α-2b to HepG2 cells was evaluated using the MTS assay. Rosiglitzone and bezafibrate showed no cytotoxicity at concentrations lower than 100 μM (Fig. 1A). The CC_{50} of rosiglitazone and bezafibrate was calculated to be 220 and 255 μM, respectively. Lamivudine and IFN-α-2b had no cytotoxicity at concentrations lower than 1 μM and 900 U/ml, respectively (data not shown). The effect of each drug on HBV was analyzed at the concentration without toxicity to HepG2 cells in the following experiments.

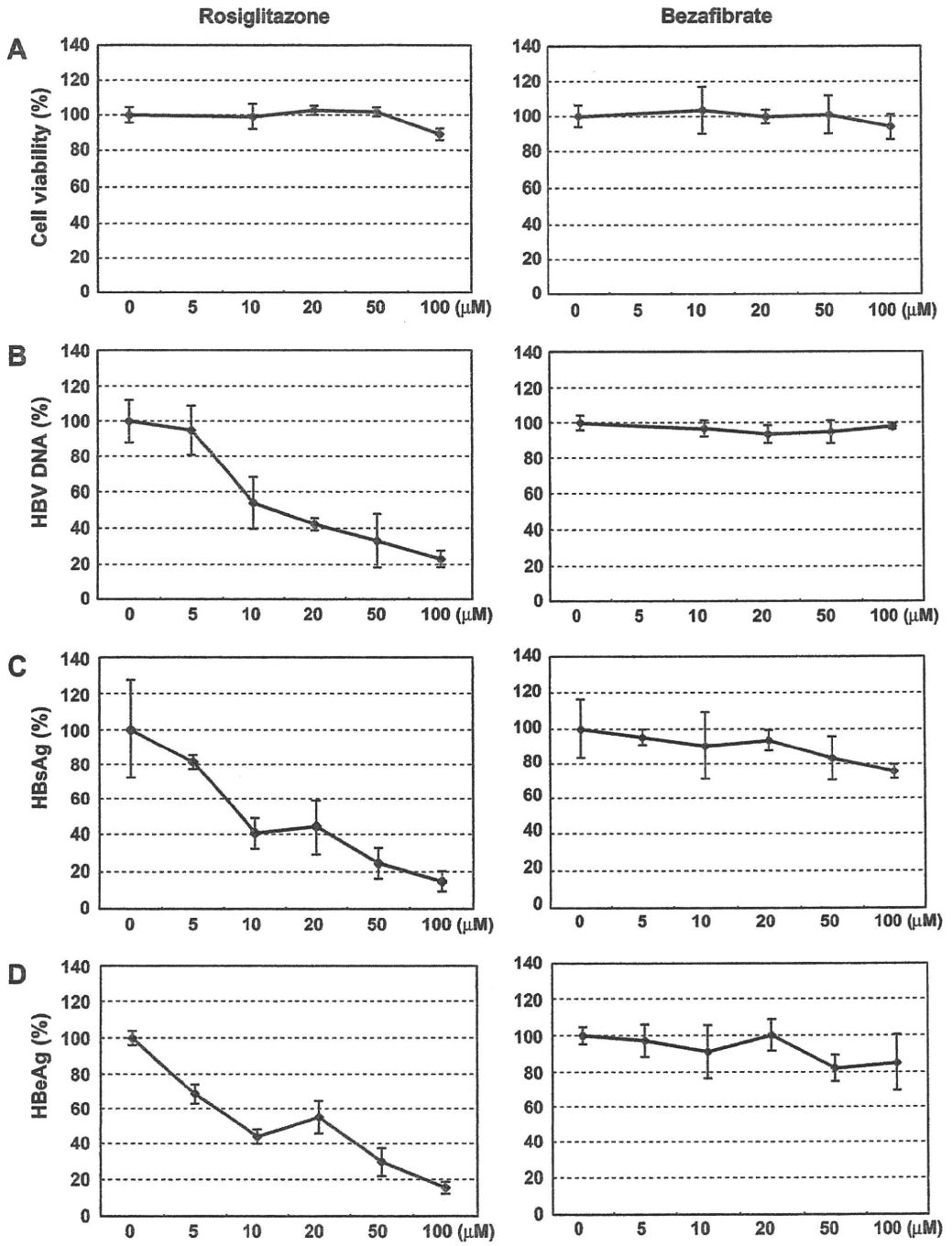

Fig. 1. Cytotoxicity (A) and effect on hepatitis B virus (HBV) replication [HBV DNA (B), hepatitis B surface antigen (HBsAg) (C), and hepatitis B e antigen (HBeAg) (D) in the culture supernatant] of a peroxisome proliferator-activated receptor (PPAR) γ ligand, rosiglitazone, and a PPARα ligand, bezafibrate. All values are expressed as percentages relative to drug-free control.

3.2. Effect of drugs to HBV replication: extracellular HBsAg, HBeAg, and HBV DNA

It was investigated whether each drug could suppress the replication of HBV. The amount of HBV DNA in the culture supernatant was dose-dependently decreased by the addition of rosiglitazone (Fig. 1B). The EC_{50} for rosiglitazone on the extracellular HBV DNA was calculated to be 24.3 μM. Both HBsAg and HBeAg in the culture supernatant were also dose-dependently decreased by rosiglitazone (Fig. 1C and D). In contrast, bezafibrate demonstrated no suppressive effect at concentration without cytotoxicity. Lamivudine or IFN-α-2b decreased the extracellular HBV DNA dose-dependently without cytotoxicity (data not shown) as previously reported [18–20].

3.3. Effect of rosiglitazone to intracellular intermediates of HBV

The suppressive effect on the HBV replicative intermediates in the HepG2 cells was assessed with Southern blotting analysis. The amount of the intracellular replicative intermediates was also dose-dependently suppressed by rosiglitazone (Fig. 2), and the EC_{50} for rosiglitazone was calculated to be 3.7 μM. This result

showed a greater effect against intracellular HBV replicative intermediates than extracellular HBV DNA.

3.4. Suppressive effect of rosiglitazone against HBV mutants

The effect of rosiglitazone was confirmed using mutant HBV strains with common mutations in the core promoter region (A1762T/G1764A) and/or in the precore region (G1896A). These mutations were reported to affect the HBV replication [21,22], and to be associated with HBeAg/Ab seroconversion [23,24] and fulminant hepatitis [25,26]. In this study, HBV with A1762T/G1764A showed greater replication capacity in comparison with the wild-type strains as described previously [21]. Rosiglitazone also suppressed the mutant HBV strain with enhanced replication capacity (Fig. 3A). The replication of the clone with G1896A, which is the main cause of HBeAg loss in the natural course of HBV carriers [23], was also suppressed by 20 μM of rosiglitazone. The clone with both mutations was similarly suppressed.

3.5. Addition of PPARγ antagonist with rosiglitazone

We examined whether an irreversible PPARγ antagonist, GW9662, reduces the suppressive effect of rosiglitazone on the replication of HBV. When both rosiglitazone and GW9662 were added, the amount of HBV DNA in the medium was significantly larger than that suppressed by rosiglitazone monotherapy (Fig. 3B). Therefore, it was confirmed that the suppressive effect of rosiglitazone on HBV was specifically via PPARγ.

3.6. Combination treatment with rosiglitazone and lamivudine or IFN-α-2b

To evaluate the antiviral effect of drug combinations of rosiglitazone and lamivudine or IFN-α-2b, HBV-transfected HepG2 cells were cultured with various concentrations of these drugs in combination. Fig. 4A shows the dose–effect curves for the decreased HBV DNA in the medium at various concentrations of rosiglitazone and/or lamivudine. The concentration ratio of lamivudine/rosiglitazone in combination treatment was 1/100. The amount of HBV DNA was suppressed more greatly with the combination treatment than with the monotherapies. Using these dose–effect curves, a normalized isobologram was constructed (Fig. 4C). The single points, which show the effects of the combination treatment, were located below lines indicating the effects of combined drug concentration deduced from the concentration of monotreatment. The result indicated that these drugs exerted a synergistic effect. Also, the combination index (CI) calculated for the drug combination showed that the interactions of these drugs were synergistic, as all CI values were less than 1 (Fig. 4E). The effect of the combination of rosiglitazone and IFN-α-2b was examined similarly (Fig. 4B), and both the isobologram and CI analyses showed a synergistic effect (Fig. 4D and F).

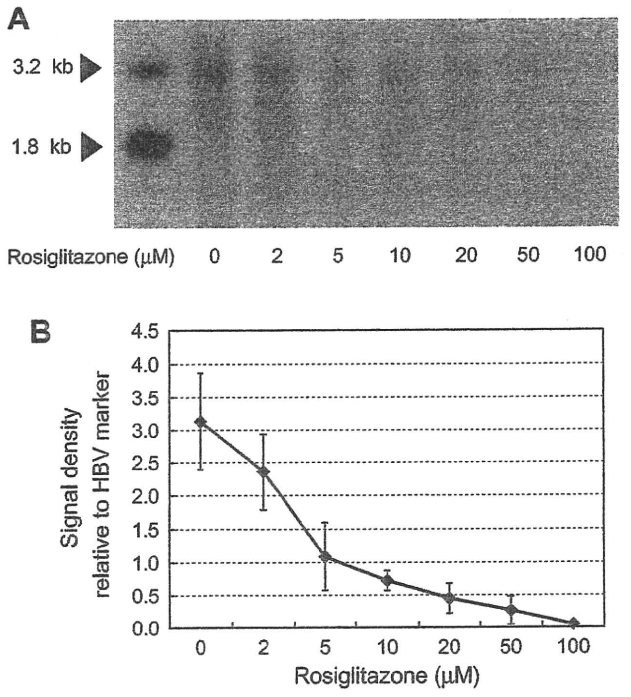

Fig. 2. Effect of rosiglitazone on intracellular HBV replicative intermediates assayed with Southern blotting analysis. (A) A representative result of Southern blotting analysis. (B) Amount of HBV replicative intermediates quantified with densitometry.

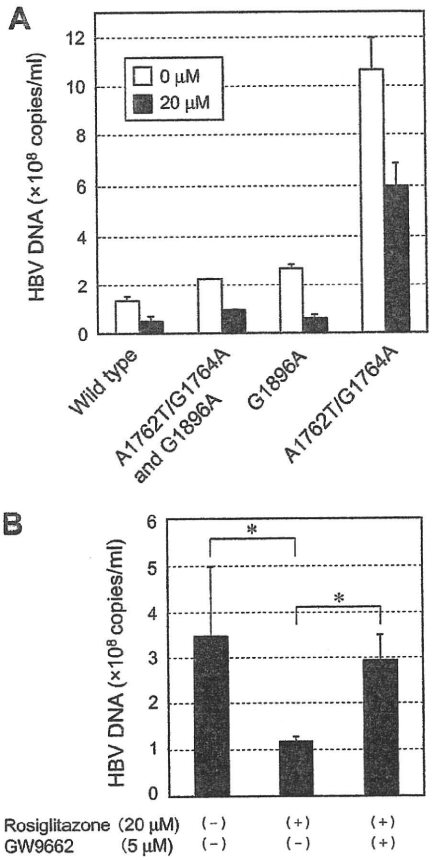

Fig. 3. (A) Effect of rosiglitazone on the replication of HBV with common mutations in the core promoter region and/or precore region. (B) Inhibition of rosiglitazone by a PPARγ antagonist, GW9662. *$P < 0.05$.

4. Discussion

In this study, it was demonstrated that a PPARγ ligand, rosiglitazone, suppressed the replication of HBV in HepG2 cells. In contrast, a PPARα ligand, bezafibrate, did not have such an effect. Because both HBV DNA in the culture supernatant and replicative intermediates in the cells were dose-dependently decreased by rosiglitazone, it appeared that rosiglitazone prevented the synthesis of pregenome RNA or reverse transcription of HBV DNA. The effect was greater on the intracellular replicative intermediates of HBV

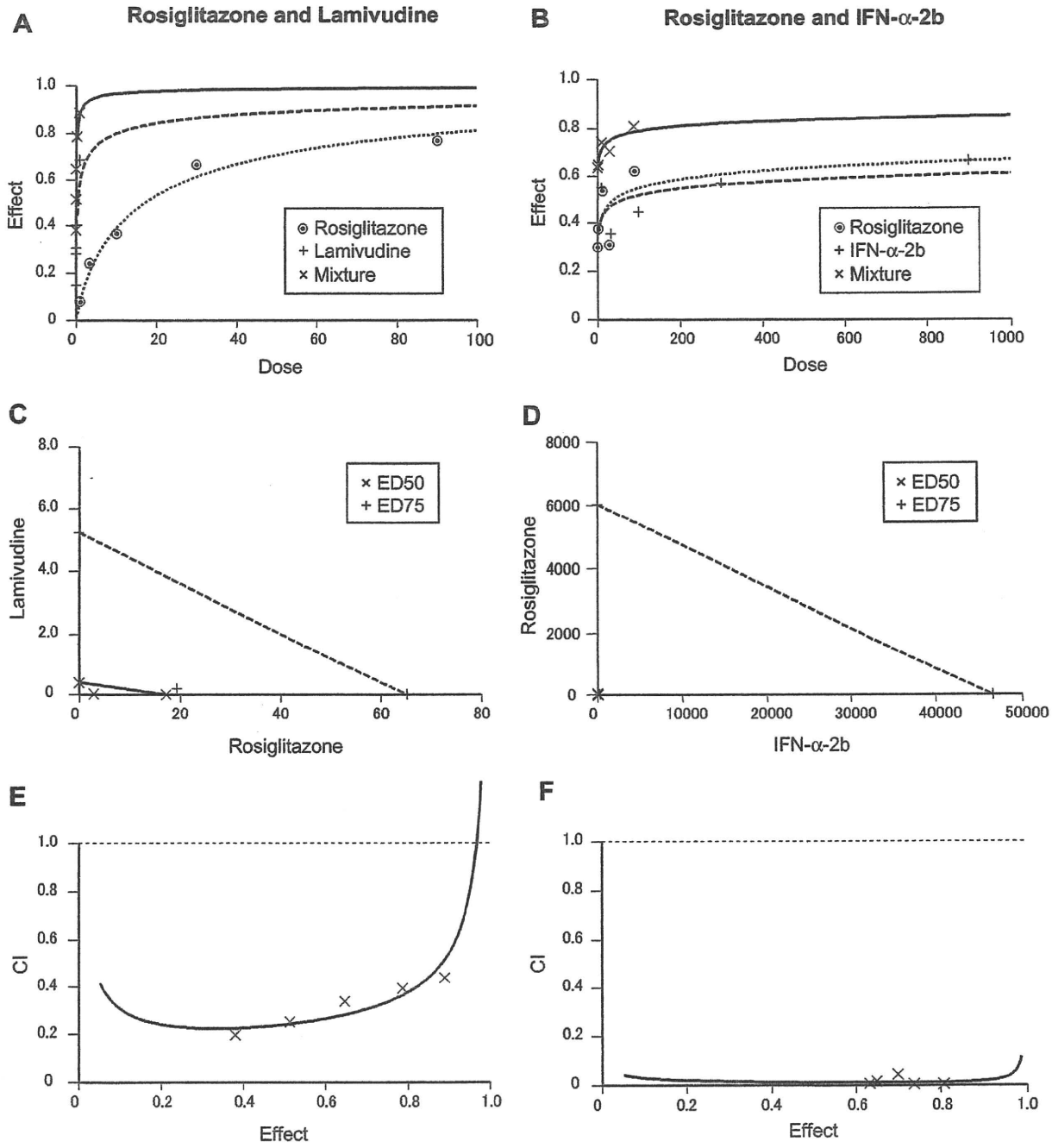

Fig. 4. Analysis of interactions between rosiglitazone and lamivudine or interferon (IFN)-α-2b using CalcuSyn software (Biosoft, Inc., Cambridge, United Kingdom). (A and B) The dose–effect curves for the decrease of HBV DNA in the medium with rosiglitazone and/or lamivudine (A), and rosiglitazone and/or IFN-α-2b (B). The concentration ratio in combination treatment was 1/100 and 1 mol/10^6 U, respectively. (C and D) Isobolograms for the combination treatments of rosiglitazone and lamivudine (C), and rosiglitazone and IFN-α-2b (D). EC_{50} and EC_{70} values for the combination treatments are shown as single points. The lines connecting the axes indicate the expected EC_{50} and EC_{70} values for drug combinations as calculated from the monotherapies. (E and F) Combination index (CI)-fraction plots for the combination treatments of rosiglitazone and lamivudine (E), and rosiglitazone and IFN-α-2b (F). CI less than 1.0 indicates synergism, and CI greater than 1.0 indicates antagonism.

than on the extracellular HBV DNA. This difference might be due to a time-lag in the effect: extracellular HBV DNA might decrease more slowly. The concentration of rosiglitazone in this examination was higher than that in physiological plasma after oral administration [27]. On the other hand, the concentration of lamivudine or IFN-α-2b in the examination was lower than that in plasma [28]. Therefore, it was thought that the suppressive effect of rosiglitazone on HBV might be weaker than that of lamivudine or IFN-α-2b in the clinical situation for the treatment of chronic HBV infection. However, rosiglitazone showed synergistic interactions with lamivudine and IFN-α-2b to suppress the replication of HBV. This result may suggest the possibility of novel combination treatments for chronic HBV infection: the administration of rosiglitazone in combination with current standard therapy such as nucleot(s)ide analogues or IFN. It is necessary to examine the effect of the combination therapies with these drugs in vivo.

Generally, the natural course of patients with chronic HBV infection includes an immunotolerant phase, a hepatitis phase, and an inactive phase. In the hepatitis phase, seroconversion of HBeAg/Ab occurs frequently along with the emergence of the pre-core mutation [23] or core promoter mutations [24]. The present study showed that rosiglitazone could suppress HBV strains with these mutations. Therefore, rosiglitazone therapy may be useful at any phases in the natural course of HBV infection.

Although the results from the experiment using the PPARγ antagonist confirmed that the suppressive effect of rosiglitazone on HBV was specifically via PPARγ, the mechanism in detail was not clarified in this study. Previous studies showed that several liver-enriched transcription factors, including CCAAT/enhancer-binding protein (C/EBP), hepatocyte nuclear factors (HNF) 3 and 4, RXRα, PPARα, and ubiquitous transcription factors including Sp1 and regulatory factor X 1 (RFX1) modulates the core promoter activity in vitro [29–33]. It was reported that PPARα and RXRα together activated replication of HBV in HBV-transfected non-hepatoma cells [14,34]. Using an HBV transgenic mouse model, Guidotti et al. demonstrated that the activation of PPARα increased the transcription and replication of HBV and suggested that even a modest alteration in transcription could have a big impact on virus replication [35]. However, PPARγ had not been reported to be involved in HBV replication. PPARγ plays important roles in glucose, lipid, and steroid metabolism in the liver [9]. It was reported that PPARγ ligands inhibited 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD-1), which is predominantly expressed in liver, adipose, gonadal, and central nervous system tissue [36]. 11β-HSD-1 serves as a reductase that converts inactive cortisone into the active glucocorticoid cortisol. The HBV genome has a glucocorticoid-responsive element with which corticosteroid directly combines, and glucocorticoid enhances directly the replication of HBV [37]. It was hypothesized that PPARγ ligands might reduce corticosteroid in the liver cells, and suppress the replication of HBV.

PPAR ligands can activate kinases that lead to phosphorylation cascades. The activation can trigger transcriptional changes leading to several levels of downstream regulation [38]. Ligand binding, corepressor/coactivator recruitment, or heterodimerization with RXRα can be altered influencing PPAR-mediated transcription. There is a possibility that these interactions play some role in the suppression of HBV.

Recently, the presence of androgen responsive element in the HBV genome was reported [39]. This finding suggests an alternative mechanism for rosiglitazone. It was reported that PPARγ ligands had an anti-androgen effect in a prostate cancer cell line [40]. Rosiglitzone may also have such an effect, and there is a possibility that the effect leads to the inhibition of HBV replication. The detailed mechanism will be elucidated in future studies.

Interestingly, it was reported previously that PPARγ ligands inhibited HIV-1 replication in vitro [41] and in vivo [13]. The suppressive mechanism of PPARγ ligands was supposed to involve the transrepression of nuclear factor-kappa B (NF-κB) and HIV-1 long terminal repeat promoter transcription [13]. HIV-1 and HBV have a similar replication strategy such as reverse transcription, and several kinds of drugs can be effective for both of viruses. Whether PPARγ ligands suppress these viruses via a similar pathway needs to be elucidated.

In conclusion, this study showed that a PPARγ ligand, rosiglitazone, had a suppressive effect on HBV replication in vitro. As the effect was synergistic with lamivudine or IFN-α-2b, there is a possibility that combination therapies of these drugs could be an option for the treatment at patients with chronic HBV infection. The effectiveness of the therapy should be confirmed in vivo.

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Murine models of autoimmune cholangitis

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Purpose of review

Primary biliary cirrhosis (PBC) is a human autoimmune liver disease whose molecular pathogenesis is poorly understood because of the difficulty in accessing human tissue and the absence of appropriate animal models. Recently, several unique murine models of human PBC have been discovered. These models have great potential for illustrating the cause and the cellular events that lead to biliary-specific damage. The purpose of this review is to summarize recent progress in these models.

Recent findings

The murine models of autoimmune cholangitis include the transforming growth factor beta receptor II (TGF-βRII) dominant-negative (dnTGF-βRII), IL-2 receptor α deleted (IL-2Rα$^{-/-}$), scurfy, nonobese diabetic (NOD) c3c4, and Ae2 gene-disrupted (Ae2$_{a,b}^{-/-}$) mice. Recently, we have also established a successful murine model following the immunization with a chemical mimicry of the lipoyl-lysine residue of the E2 component of PDC-E2.

Summary

These emerging murine models have greatly enabled researchers to address the pathogenesis of human PBC and to elucidate pathogenic factors. These models will ultimately lead to new therapeutic options for human PBC.

Keywords

$CD8^+$ T cells, cytokines, primary biliary cirrhosis, regulatory B cells

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Introduction

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by progressive bile duct destruction leading to cirrhosis and liver failure [1]. PBC patients are clinically relatively homogeneous, and over 95% develop antimitochondrial autoantibodies (AMA), the most highly disease-specific and directed autoantibody in human autoimmune disease [1,2]. PBC is characterized by destruction of small bile ducts, a female predominance, a clustering within other autoimmune disease (such as scleroderma, Sjögren's syndrome, and systemic lupus), and a genetic basis for susceptibility [3]. On the basis of rigorous studies with PBC patients and human cell lines, we have developed murine models of autoimmune cholangitis that are parallel to human PBC. We should note that the study of these models is still in its infancy. To put the models in perspective, lupus mice have been studied as an autoimmune murine model since 1959 [4–7] and the immunological studies with New Zealand and MRL/lpr mice [8,9] have greatly advanced our understanding of autoimmunity [10–13], although the complete basis of immunopathology of these models is still unclear.

dnTGF-βRII mice

Mice transgenic for directed expression of dnTGF-βRII, under the $CD4^+$ promoter lacking a $CD8^+$ cells silencer [14] demonstrate features characteristic of human PBC [14,15]. Transforming growth factor (TGF)-β mediates pleiotropic functions on various cells and plays a central negative regulatory role in autoimmunity [16]. TGF-β receptor II is essential for signal transduction of TGF-β that regulates activation of lymphocytes. dnTGF-βRII mice demonstrate 100% AMA positivity, directed specifically toward PDC-E2, BDOADC-E2, and OGDC-E2, the major autoantigens in human PBC. Liver histology in dnTGF-βRII mice shows lymphoid cell infiltration of portal tracts accompanied by bile duct injury, as seen in human PBC. This model is very similar to human PBC from both a serologic and a histological perspective.

Role of lymphoid cells in dnTGF-βRII primary biliary cirrhosis

To assess the pathogenic contribution of T cells in dnTGF-βRII autoimmune cholangitis, we performed a series of adoptive transfer studies with splenic $CD4^+$ and/or $CD8^+$ T cells derived from dnTGF-βRII mice into

$Rag1^{-/-}$ recipients [17]. $Rag1^{-/-}$ recipients of dnTGF-βRII unfractionated splenocytes develop features of liver disease similar to human PBC, suggesting that splenic T and B cells loss of tolerance, not a specific abnormality in biliary targets, can cause autoimmune cholangitis. More importantly, adoptive transfer of $CD8^+$ but not $CD4^+$ T cells into $Rag1^{-/-}$ mice led to a liver histopathology remarkably similar to PBC, suggesting a prominent role of $CD8^+$ T-cell mediation for the pathogenesis in PBC. In contrast, $Rag\text{-}1^{-/-}$ recipients of $CD4^+$ T cells of dnTGF-βRII mice predominantly developed inflammatory bowel disease associated with higher levels of serum IFN-γ and TNF-α. These data suggest that $CD8^+$ T cells are the primary contributor for bile duct destruction in this model [17].

B-cell contribution in dnTGF-βRII mice

Despite the relatively constant occurrence of serum AMA and the accumulation of B cells among liver infiltrates in some cases [18], the contribution of B cells to the pathogenesis of PBC remains unclear [19]. To assess whether B-cell deficiency would ameliorate liver disease, dnTGF-βRII mice were crossed with B-cell-deficient mice ($Ig\mu^{-/-}$) and evaluated for the development of liver inflammation, as well as the severity of accompanying colitis [20]. Contrary to expectations, genetic B-cell deprivation exacerbated both PBC-like liver disease and colitis [20]. Also, B-cell deprivation expanded the $CD8^+$ T-cell population relative to $CD4^+$ T cells and diminished hepatic regulatory T (Treg) cells frequency in the $CD4^+$ T-cell population. In $Ig\mu^{-/-}$ dnTGF-βII mice, regulatory B cells (Breg) produced anti-inflammatory cytokines, especially IL-10 [21]. B-cell adoptive transfer study from two major B cell pools, the peritoneal cavity (PerC) and the spleen (Spl), demonstrated that B cells from the PerC, but not the Spl of dnTGF-βRII mice, regulate PBC.

To examine the effect of therapeutic B-cell depletion, we treated dnTGF-βRII mice in young (4–6 weeks) and old (20–22 weeks) mice by intraperitoneal injection of anti-mouse CD20 antibody (mAb) every 2 weeks, and compared the disease phenotype with control Ab treatment [22]. Treatment of young mice fully depleted serum AMA and decreased liver inflammation and activated hepatic $CD8^+$ T cells, but significantly exacerbated colon inflammation [22]. In contrast, anti-CD20 treatment of animals with established disease was ineffective.

Clearly knocking out B cells had a different effect than CD-20 treatment. One explanation of these contradictory results is that the $Ig\mu^{-/-}$ mice suffered deletion of all B-cell subsets, including subsets that could drive the development of T regulatory cells or that could act as B regulatory cells. In contrast, anti-CD20 treatment does not deplete all B cells and could preferentially remove cells contributing to pathology, thus enhancing cells contributing to regulation in young mice. We are actively exploring the mechanisms of B-cell depletion versus knockout.

Natural killer T-cell contribution in dnTGF-βRII mice

Natural killer T (NKT) cells bridge innate and adoptive immunity and demonstrate both immunoregulatory and effector functions [23,24]. NKT cells are primed for pro-inflammatory and anti-inflammatory phenotypes under a DC-derived cytokine environment such as IL-12 or IL-10 [25]. We generated and investigated $CD1d^{-/-}$-dnTGF-βRII mice [26] and such mice exhibited decreased lymphoid cell infiltrates, milder damage of bile ducts compared with those of control mice ($CD1d^{+/-}$-dnTGF-βRII mice), suggesting CD1d-restricted NKT cells are primarily pro-inflammatory phenotypes in a T helper 1 (Th1) cytokine bias and promote deprivation of TGF-β signaling [26]. The contribution of NKT cells to biliary disorder is consistent with our results in an infection-mediated model of PBC [27]. We have shown that human PBC patients develop seroreactivity to *Novosphingobium* bacterial species [28]. When we infected mice with *Novosphingobium*, they suffered an acute infectious phase followed by a chronic phase of liver disorder similar to PBC [27]. In the chronic phase, T cells could transfer the disease to naive mice. NKT cells respond to *Novosphingobium* and were essential to promoting the development of autoreactive T cells. This model provides additional support for a pathological effector role for NKT cells in PBC.

Cytokine/chemokine contribution in dnTGF-βRII mice

IL-12p40 depletion led to a marked diminution in the levels of pro-inflammatory Th1 cytokines in the livers of dnTGF-βRII mice with accompanying reductions in cellular infiltrates in portal tracts associated with diminished bile duct damage [29]. In contrast, IFN-γ deprivation demonstrated no significant effect on the immunopathology of autoimmune cholangitis. The lack of a necessary pathological role for IFN-γ is consistent with results of other autoimmune diseases including EAE and type I diabetes (REFS) and may reflect a more critical role for T helper 17 (Th17) cells. These experiments suggest that IL-12p40 is a major determinant of dnTGF-βRII autoimmune cholangitis, and that IL-12p40 suppression may be a therapeutic option for human PBC [30].

Immunomodulation by β-glucosylceramide

β-Glucosylceramide (Alabaster, Alabama, USA) administration age in dnTGF-βRII mice beginning at 6 weeks of age ameliorates cholangitis accompanied by a significant reduction of hepatic $CD8^+$ memory T cells [31]. β-Glucosylceramide is a naturally occurring glycosphingolipid and has been shown to function as a 'fine-tuning factor' in several murine models of immune-mediated

disorders [32–35]. Interestingly, there were no changes in antimitochondrial antibodies, $CD4^+$ T cells, $CD19^+$ B cells or NKT cell populations, indicating that the beneficial effects of β-glucosylceramide were targeted specific to liver infiltrating $CD8^+$ T cells.

IL-2Rα$^{-/-}$ mice

IL-2Rα$^{-/-}$ mice demonstrate a low frequency of Treg cells among peripheral blood mononuclear cells (PBMCs) as seen in human PBC, which is reflective of the critical role of the IL-2 receptor in maintaining Treg cell survival [36,37]. IL-2Rα$^{-/-}$ mice also demonstrate 100% serum positivity against PDC-E2, 80% antinuclear antibody (ANA) positivity and lymphocyte infiltration around portal tracts, especially intraepithelial $CD8^+$ T cells in the interlobular bile ducts, accompanied by cholangiocyte damage [36]. IL-2Rα$^{-/-}$ mice also demonstrate an increased number and frequency of $CD44^+$ memory $CD4^+$ and $CD8^+$ T cells and decreased CD4/CD8 ratio in liver infiltrates [36,38]. Mild B-cell enrichment is also observed in this model [39]. The role of IL-2Rα (CD25) and its relationship with Treg cells has been well demonstrated in murine and human autoimmune diseases such as inflammatory bowel disease, and more recently, in murine autoimmune cholangitis models and human PBC [37,40–43]. IL-2Rα$^{-/-}$ mice demonstrate autoimmune cholangitis concomitant with intestinal inflammation. However, colitis rarely co-exists in human PBC [3]; therefore, we hypothesized that there are distinct effector mechanisms in selective targeting of autoimmune response against bile duct and colon [39]. IL-2Rα$^{-/-}$-CD4$^{-/-}$ mice demonstrated exacerbated intrahepatic biliary ductular destruction but diminished colitis. In contrast, IL-2Rα$^{-/-}$-CD8$^{-/-}$ mice lacked biliary ductular destruction with an exacerbation of colitis [39]. These results are similar to those from adoptive transfer studies in dnTGF-βRII mice in which $CD8^+$ but not $CD4^+$ T cells are the major contributors for autoimmune cholangitis [17]. Most importantly, the predominant role of T cells in IL-2Rα$^{-/-}$ mice was demonstrated by the lack of pathological conditions in IL-2Rα$^{-/-}$ TCR-β$^{-/-}$ mice, similar to absence of inflammation in Rag-1$^{-/-}$-dnTGF-βRII mice [15]. Taken together, these data further support a key role of $CD8^+$ T cells in the pathogenesis of autoimmune cholangitis.

In addition to severe cellular infiltration in the portal tracts of IL-2Rα$^{-/-}$-CD4$^{-/-}$ mice, pro-inflammatory and Th1 cytokines, such as TNF-α, IFN-γ, IL-2, IL-12p40, and IL-17, were elevated. IL-2Rα$^{-/-}$-CD8$^{-/-}$ mice, lacking biliary ductular destruction, also had elevated serum levels of Th1 and inflammatory cytokines but significantly higher levels of interleukin-17 (IL-17) compared with IL-2Rα$^{-/-}$ mice [39]. Splenic CD4 T cells cocultured with liver nonparenchymal cells increased IL-17 production approximately 10-fold compared with T-cell culture alone, suggesting a role of the liver microenvironment in Th17 induction [44]. In a recent human PBC study, Th17-related cytokines such as IL-23p19 and IL-17 were significantly elevated in the sera of PBC patients than in healthy and chronic hepatitis B affected patients [45]. IL-17 receptor signaling in cholangiocytes also induces production of IL-6, IL-1β, IL-23p19, and chemokines (CXCL1, 2, 3, 6, 8, and CCL2, 20) [46], suggesting cholangiocytes support Th17 cell development. Tight junctions between mouse cholangiocytes are disrupted by Th1 and pro-inflammatory cytokines such as IFN-γ and TNF-α, suggesting a possible role for these cytokines in advancing tissue damage [47].

NOD.c3c4 mice

NOD.c3c4 mice spontaneously develop AMA and liver disorder similar to some features of human PBC [48,49]. These mice demonstrate seropositivity to PDC-E2 up to 50–60%, ANA positivity of 80–90%, and lymphocyte infiltration around portal tracts with chronic nonsuppurative destructive cholangitis and epithelioid granuloma formation as seen in human PBC liver [48–50]. Liver disorder can be transferred with whole splenocytes or splenic $CD4^+$ T cells [48,49]. However, it is important to note that extrahepatic bile duct involvement of NOD.c3c4 mice is not seen in human PBC [48].

Scurfy mice

As a decreased frequency of Treg cells have been reported in human PBC, we also studied Scurfy mice, which have a forkhead box 3 (Foxp3) gene mutation that results in a deficiency of functional Treg cells [51,52]. Scurfy mice demonstrate serological, histological, and cytokine features characteristic of autoimmune cholangitis, including an expanded $CD8^+$ T-cell population, similar to human PBC. Severe bile duct destruction has been shown in more than 90% of mice [53]. Naturally occurring Treg cells specifically express the transcription factor known as Foxp3, which is essential for the development, maintenance, and function of Treg cells [54,55]. Importantly, Foxp3 expression confers suppressive activity to conventional non-Treg cells and it is also critical for T-cell receptor-positive T cells to differentiate to Treg cells in the thymus [56]. Scurfy mice as an autoimmune cholangitis model suggests that the lack of Foxp3 protein, which results in abnormal Treg function, is responsible for the loss of tolerance in the liver, leading to autoreactive CD8 T cell-mediated bile duct injury [53]. These findings reflect the importance of Treg cells in autoimmune cholangitis.

Ae2$_{a,b}^{-/-}$ mice

Ae2$_{a,b}^{-/-}$ mice demonstrate immunologic and hepatobiliary features similar to those found in human PBC:

enhanced production of IL-12p70 and interferon-γ, expanded $CD8^+$ T-cell population, and reduced Treg cells [57]. Although PBC is classified as an autoimmune disease, the fact that ursodeoxycholic acid, a bile acid that induces bicarbonate-rich choleresis, improves the clinical course of PBC led to the hypothesis that defective Cl^-/$HCO3^-$ exchange plays a role in the pathogenesis of PBC [58,59]. *AE2* gene expression is reduced in liver biopsy specimens and blood mononuclear cells from patients with PBC [60,61]. Although the mechanisms leading to the AE2 deficiency in the liver and lymphocytes in human PBC remain unclear, study of $Ae2_{a,b}^{-/-}$ mice suggests that AE2 dysfunction is involved in the pathogenesis of PBC [57].

Cholangiocytes in $Ae2_{a,b}^{-/-}$ mice have increased expression of genes involved in antigen presentation by major histocompatibility complex (MHC) class I molecule (*Usp2* and *H2-D1*) [57]. This suggests there are enhanced presentations of breakdown products of cellular proteins to CD8 T cells by cholangiocytes. Therefore, Ae2 deficiency might cause oxidative stress in cholangiocytes with increased degradation of cell proteins and presentation of autoepitopes to cytotoxic T cells [62,63]. These alterations in the characteristics of cholangiocytes, in combination with defective Treg cell function and CD8 T-cell expansion, may contribute to the selective damage of bile ducts in *Ae2$_{a,b}$*-targeted mice [57]. The $Ae2_{a,b}^{-/-}$ mouse model provides clues to the pathogenesis of PBC and postulates a role of Ae2 in cholangiocyte function and in the homeostasis of the immune system [57]. Moreover, the findings in $Ae2_{a,b}^{-/-}$ mice point to Ae2 as a pharmacologic target to modulate T-cell responses [57].

2-Octynoic acid–bovine serum albumin immunized mice

Like most autoimmune diseases, PBC pathogenesis is believed to be multifactorial, with genetic and environmental factors interacting to determine disease onset and progression [64]. We have identified several potential environmental initiators, including bacteria [65–67] and chemical xenobiotics [68–70]. Following the discovery that AMA reacts against 2-octynoic acid-modified PDC-E2 peptide, 2-octynoic acid conjugated bovine serum albumin (BSA) was used as an immunogen into B6 mice and NOD.1101 mice [71,72]. In these studies, 2-octynoic acid conjugated with BSA was a potent immunodominant epitope that led to loss of tolerance against PDC-E2 and induced a PBC-like liver disease in both strains.

Conclusion

The immunological characteristics of spontaneous and induced murine autoimmune cholangitis models are noted in Table 1 and comparison of these features to human PBC in Table 2. The models demonstrate several serological, biochemical, and histological features of human PBC, especially within the hepatic $CD8^+$ T-cell

Table 1 Significant observations in the murine models of autoimmune cholangitis

dnTGF-βRII mice	IL-2Rα$^{-/-}$ mice	NOD.c3c4 mice	Scurfy mice	Ae2$_{a,b}^{-/-}$ mice	Xenobiotic-induced mice
Adoptive transfer of dnTGF-βRII CD4 T cells causes IBD to Rag1$^{-/-}$ recipients	IL-2Rα$^{-/-}$-CD4$^{-/-}$ mice develop autoimmune cholangitis with Th1 cytokines	OD.c3c4 CD4 T cells transfer autoimmune cholangitis to NOD.c3c4-scid recipients	Scurfy mice develop Treg dysfunction then lead to autoreactive CD8 T cell-mediated bile duct injury	Cholangiocytes in Ae2$_{a,b}^{-/-}$ mice express MHC class I as a possible antigen presenting cells to CD8 T cell	2-Octynoic acid-BSA immunized mice develop autoimmune cholangitis with AMA
Adoptive transfer of dnTGF-βRII CD8 T cells causes autoimmune cholangitis to Rag1$^{-/-}$ recipients	IL-2Rα$^{-/-}$CD8$^{-/-}$ mice develop autoimmune colitis with Th1 and Th17 cytokines				
Genetic B cell depletion exacerbates autoimmune cholangitis and colitis	IL-2Rα$^{-/-}$TCRβ$^{-/-}$ mice develop neither autoimmune cholangitis nor colitis				
Therapeutic depletion of B cells exacerbates autoimmune cholangitis in young mice					
Genetic NKT cell depletion inhibits the pathogenesis of autoimmune cholangitis					
Genetic IL12 depletion inhibits the pathogenesis of autoimmune cholangitis					
Genetic INFγ depletion does not affect the pathogenesis for autoimmune cholangitis					

Table 2 Comparison between human PBC and the murine autoimmune cholangitis models

Index	Human PBC	dnTGF-βRII mice	IL-2Ra$^{-/-}$ mice	NOD.c3c4 mice	Ae2a,b$^{-/-}$ mice	Scurfy mice	2-OA$_{hp}$ mice
Classification	N/A	Spontaneous	Spontaneous	Spontaneous	Spontaneous	Spontaneous	Induced
Background strain	N/A	C57BL/6	C57BL/6	NOD	FVB/N, 129/Sv, Balb/c, SJL	C57BL/6	C57BL/6, NOD 1101
Clinical features							
Female : male ratio	9 : 1	1 : 1	1 : 1	1 : 1	1 : 1	0 : 1 (X/X females fatal at 4 weeks)	1 : 1
Age of onset	40–60 years	4 weeks	4 weeks	8–20 weeks	? (6–15 months for AMA production)	3–4 weeks	4–12 weeks
B-cell immunity							
AMA	90–95%	100%	100%	50–60%	40–80%	100%	100%
Dominant AMA target protein	PDC-E2	PDC-E2	PDC-E2	PDC-E2	PDC-E2	PDC-E2	PDC-E2
Dominant epitope	Lipoyl domain	Lipoyl domain	Lipoyl domain	Lipoyl domain	Lipoyl domain	Lipoyl domain	Lipoyl domain
Liver histology							
Portal lymphoid infiltrates	+++	+++	+++	+++	+–+++	++–+++	+
CD4 cell	+	+	++	++	+	+	+++
CD8 cell	++	++	++	+	++	++	++
B cell	+	+	+	–	+	+	+
Bile duct destruction	+–+++	++	+–++	+	+–+++	+–++	+++
Granuloma	+–++	–	–	+	?	–	+
Eosinophilia	+	–	–	+	+	(+)	–
Pro-inflammatory cytokines	+	+	+	+	+	+	+
References	[1,3,15]	[14,17,20,24,25]	[34–37]	[46–48]	[55]	[51]	[68,69]

2-OA, 2-octynoic acid; AE, anion exchanger; AMA, antimitochondrial antibody; ANA, antinuclear antibody.

expansion. Despite limitations, these models have greatly enabled us to address issues in the pathogenesis of human PBC and hopefully to elucidate etiopathogenic and effector mechanisms.

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Research Article

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Possible involvement and the mechanisms of excess *trans*-fatty acid consumption in severe NAFLD in mice

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Background & Aims: Excessive *trans*-fatty acids (TFA) consumption has been thought to be a risk factor mainly for coronary artery diseases while less attention has been paid to liver disease. We aimed to clarify the impact of TFA-rich oil consumption on the hepatic pathophysiology compared to natural oil.

Methods: Mice were fed either a low-fat (LF) or high-fat (HF) diet made of either natural oil as control (LF-C or HF-C) or partially hydrogenated oil, TFA-rich oil (LF-T or HF-T) for 24 weeks. We evaluated the liver and body weight, serological features, liver lipid content and composition, liver histology and hepatic lipid metabolism-related gene expression profile. In addition, primary cultures of mice Kupffer cells (KCs) were evaluated for cytokine secretion and phagocytotic ability after incubation in *cis*- or *trans*-fatty acid-containing medium.

Results: The HF-T-fed mice showed significant increases of the liver and body weights, plasma alanine-aminotransferase, free fatty acid and hepatic triglyceride content compared to the HF-C group, whereas the LF-T group did not differ from the LF-C group. HF-T-fed mice developed severe steatosis, along with increased lipogenic gene expression and hepatic TFA accumulation. KCs showed increased tumor necrosis factor secretion and attenuated phagocytotic ability in the TFA-containing medium compared to its *cis*-isomer.

Conclusions: Excessive consumption of the TFA-rich oil up-regulated the lipogenic gene expression along with marked hepatic lipid accumulation. TFA might be pathogenic through causing severe steatosis and modulating the function of KCs. The quantity and composition of dietary lipids could be responsible for the pathogenesis of non-alcoholic steatohepatitis.

Keywords: *trans*-Fatty acid; NASH; NAFLD; Metabolic syndrome; Kupffer cell.
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Abbreviations: NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; FFA, free fatty acid; LPS, lipopolysaccharide; TFA, *trans*-fatty acid; ALT, alanine-aminotransferase; LF(-C or -T), low-fat (control or TFA-rich) diet; HF(-C or -T), high-fat (control or TFA-rich) diet; KCs, Kupffer cells (KCs); AST, aspartate-aminotransferase; TG, triglyceride; ELISA, Enzyme-Linked ImmunoSorbent Assay; HDL, high density lipoprotein; (V)LDL, (very) low density lipoprotein; NAS, NAFLD activity score; TBARS, thiobarbituric acid reactive substances; TNFα, tumor necrosis factor α; IL-6, interleukin-6; SD, standard deviation; iNOS, inducible nitric oxide synthase; TGF-β, transforming growth factor-β; SREBP-1, sterol regulatory element-binding protein-1; FAS, fatty acid synthase; ACC, acetyl CoA carboxylase; PPAR, peroxisome proliferator activated receptor; PGC-1β, PPARγ coactivator-1β; PUFA, polyunsaturated fatty acid; MUFA, monounsaturated fatty acid; SFA, saturated fatty acid.

Introduction

In concordance with the prevalence of obesity, the incidence of non-alcoholic fatty liver disease (NAFLD) has increased and is nowadays recognized as the most common liver disease [2]. It is known that a part of NAFLD can progress to non-alcoholic steatohepatitis (NASH), liver fibrosis, cirrhosis and hepatocellular carcinoma [9]. Nevertheless, the mechanisms of NAFLD-to-NASH transition remain to be clarified; NAFLD appears to originate from the dysregulation of hepatic lipid metabolism as a part of the metabolic syndrome accompanied by visceral obesity, dyslipidemia, atherosclerosis, and insulin resistance [25]. According to the hypothetical theory named the 2-hit theory [5], the secondary hit to NAFLD that can be due to free fatty acid (FFA)s, oxidative stress, lipopolysaccharide (LPS) and inflammatory cytokines, causes NASH as a consequence.

In terms of the "first hit", the lipid accumulation in the liver is induced by high-fat diets [6,23] that include various lipid species. Such dietary lipid species uniquely affect the obesity phenotype, liver histology and gene expression pattern in the rat liver [3]. In this context, lipid species could play a potential role in the pathogenesis of NAFLD and/or NASH.

trans-Fatty acid (TFA) is produced through the industrial hardening of the vegetable oils to make the products more stable and robust, and thus easier to handle or store. Excess consumption of TFA is known as a risk factor for coronary artery diseases, insulin resistance and obesity accompanied by systemic inflammation, the features of metabolic syndrome [20,29]. Nevertheless, little is known about the effects on the liver induced by lipids.

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Fast-foods, containing large amount of TFA in the form of margarine, spreads or frying oils, cause body-weight gain and abnormal serum alanine-aminotransferase (ALT) elevations in healthy subjects [15]. In addition, TFA-rich chow leads to hepatic steatosis [30], ALT elevations and insulin resistance in mice [17]; although the mechanisms have not been completely clarified. Therefore, we aimed to investigate the impact of the dietary lipid species and their quantities on the pathogenicity of hepatic inflammation and steatosis in mice comparing in particular natural oil and industrially produced partially hydrogenated TFA-rich oil of the same origin.

Materials and methods

Animal treatment

All the animal experiments were conducted under the approval of the Institutional Animal Care and Use Committees of Tohoku University. Female C57BL/6Njcl mice (8–10 weeks) were randomly assigned to four groups ($n = 6$ per group) and fed the designated chows (ORIENTAL YEAST Co. Ltd., Tokyo, Japan) *ad libitum* for 24 weeks, respectively. Low-fat diet (LF) and high-fat diet (HF) were made of either natural canola oil as control oil (LF-C and HF-C) or industry produced partially hydrogenated canola oil as TFA-rich oil (28.5% TFA/total fat, LF-T and HF-T), respectively (Table 1). After 12 h of fasting, the mice were sacrificed under diethylether anesthesia and the livers were removed and weighed. The divided livers were either stored at −80 °C for lipid, protein and gene expression analysis, or fixed in 4% paraformaldehyde and embedded in paraffin for histological evaluation. Standard chow-fed female C57BL/6Njcl mice (6–10 weeks) were used as a source of primary Kupffer cells (KCs).

Chemistry

Plasma aspartate-aminotransferase (AST), ALT, triglyceride (TG) and total cholesterol were measured with FUJI DRI-CHEM 7000 (FUJIFILM, Tokyo, Japan) at Biomedical Research Core of Tohoku University Graduate School of Medicine. Plasma adiponectin (AdipoGen, Seoul, Korea) and leptin (RayBio, GA, USA) were measured by Enzyme-Linked ImmunoSorbent Assay (ELISA). Plasma FFA, high density lipoprotein (HDL)-cholesterol and (very) low density lipoprotein ((V)LDL)-cholesterol were measured by enzymatic assay kits (BioVision, CA, USA).

Histology and immunohistochemistry

The thin-sliced specimens were stained with hematoxylin and eosin to evaluate steatosis and inflammation or Sirius red to evaluate fibrosis of the liver. The histology was scored by the NAFLD activity score (NAS) [16]. KCs were stained with anti-F4/80 monoclonal antibody (Abcam, Cambridge, UK) and neutrophils were detected by myeroperoxidase immunostaining (Abcam). Apoptosis was evaluated by TUNEL method using an ApopTag kit (Chemicon, CA, USA).

Table 1. Diet compositions.

	Low-fat diet		High-fat diet	
	Control oil (LF-C) kcal%	TEA-rich oil (LF-T) kcal%	Control (HF-C) kcal%	TEA-rich (HF-T) kcal%
Diet compositions				
Protein	13.8	13.8	18.8	18.8
Carbohydrate	74.4	74.4	17.6	17.6
Over all fat	11.8	11.8	63.6	63.6
Fat composition (g/100 g)				
Saturated	7.8	21.7	7.8	21.7
(*cis*-)Monounsaturated	62.5	45.3	62.5	45.3
Polyunsaturated	29.7	4.5	29.7	4.5
trans- (%)		28.5		28.5

Immunoblot analysis and real-time RT-PCR

Liver protein extracts were evaluated by immunoblot analysis with the following primary antibodies: phosphor-AKT (Thr308 and Ser473), total AKT (Cell Signaling Technology, Danvers, MA) and β-actin (Sigma, MO, USA). RNA extracted from the livers was subjected to real-time RT-PCR analysis using the specifically designed primer sets purchased form TAKARA BIO Perfect Real Time Support System (TAKARA BIO INC., Tokyo, Japan) and One Step SYBR Prime Script RT-PCR Kit II (TAKARA BIO INC.), and only PGC-1β was analyzed using the specifically designed TaqMan primer set and 1-step kit (Applied Biosystems, CA, USA). All results were normalized by GAPDH as the internal control.

Lipidomic analysis of the liver

Hepatic TG and FFA content were measured by enzymatic assay kit (BioVision) and were normalized by the liver weight. Hepatic lipid peroxide was evaluated by measuring TBARS (thiobarbituric acid reactive substances, Cayman Chemical Company, USA) in the liver and was normalized by the protein level [18]. Total lipids from the liver were extracted by Folch's procedure [10]. The lipids were methylated and evaluated by gas chromatography as previously reported [31].

Isolation and culture of primary Kupffer cells

KCs were isolated as reported previously [28]. Briefly, the mice livers were digested by two-step collagenase perfusion. The minced livers were subjected to the gradient centrifugation of Percoll (Sigma) and succeeding counterflow centrifugal elutriation. The viabilities of the obtained cells evaluated by trypan blue staining were more than 85%, and the purity was more than 90% determined by the population of CD11b positive cells counted by FACS Calibur (Becton Dickinson, Tokyo, Japan). KCs were suspended in RPMI1640 medium with 10% fetal bovine serum and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin sulfate) and incubated overnight at 37 °C in 5% CO_2 incubator for the succeeding examinations.

Fatty acid treatment

Fatty acids (Larodan Fine Chemicals, Malmo, Sweden) were dissolved in RPMI1640 medium with 1% fatty acid-free bovine serum albumin (Calbiochem, Darmstadt, Germany) and adjusted to a final concentration of 200 μM with 1% bovine serum albumin, 1% ITS-A supplement (GIBCO, CA, USA) and antibiotics same as above. After overnight incubation, KCs were washed and the medium was changed to fatty acid-containing medium or fatty acid-free medium as the control, and incubated for another 24 h.

Cytokine production by KCs stimulated with lipopolysaccharide

After 24 h incubation, KCs were stimulated by LPS (100 ng/ml, SIGMA) combined with LPS-binding protein (200 pg/ml, ALEXIS BIOCHEMICALS, Lausanne, Switzerland) for 6 h, and the cell viability was determined by MTS assay (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt and phenazine ethosulfate, Promega, Tokyo, Japan). The supernatants were subjected to ELISA (Thermo Fisher Scientific Inc., IL, USA) for the evaluation of the tumor necrosis factor-alfa (TNFα) and interleukin-6 (IL-6) production.

Phagocytotic ability of KCs

After 24 h incubation, KCs were incubated at 37 °C for 1 h with 1 μm latex beads (75 ng/ml, SIGMA) or at 4 °C in the fatty acid-free medium as control. After incubation, the cells were washed 3 times, detached with trypsin/EDTA and analyzed by FACS calibur [1].

Statistical analysis

The results are shown as the mean ± standard deviation (SD), and were analyzed by SPSS software (SPSS INC., Tokyo, Japan).

The differences between the groups were tested by ANOVA, followed by Tukey post hoc test. A p values less than 0.05 were considered statistically significant.

Results

Physiological and biochemical characteristics

Body weight was similar between LF-fed mice, increased in HF-fed mice compared to LF-fed mice, and strikingly HF-T-fed mice weighed 1.3-fold more than HF-C-fed mice (Table 2). Liver weight was significantly increased in only HF-T-fed mice by approximately 2-fold compared to the other groups. The liver–body weight ratio was significantly increased by 1.2- and 1.6-fold in LF-T-fed and HF-T-fed mice, respectively, compared to the corresponding control groups with the same dietary composition, and decreased by approximately 20% in the HF-C-fed mice compared to the LF-C-fed mice.

Plasma AST, ALT, TG, FFA and leptin were similar between the LF groups irrespective of the dietary lipid source, but in the LF-T group, total cholesterol, HDL-cholesterol, (V)LDL-cholesterol and adiponectin were significantly decreased compared to the LF-C group (Table 2). In contrast, some serum markers were elevated in the HF-T group compared to the HF-C group, particularly AST, ALT, TG, total cholesterol, (V)LDL-cholesterol, FFA and leptin were significantly increased. As for the control oil-fed mice, total cholesterol, HDL-cholesterol, (V)LDL-cholesterol and adiponectin were lower, whereas plasma leptin was higher in HF-C-fed than in LF-C-fed mice. Between TFA-rich oil-fed mice, all serum markers except adiponectin were also significantly higher in HF-T-fed than in LF-T-fed mice.

Liver histology

There were few lipid droplets in LF-C-fed mice liver. Mild microvesicular and macrovesicular steatosis was present around zone 1 in LF-T-fed mice livers and abundant large lipid droplets around zones 1 and 2 in HF-C-fed mice livers. Inflammation and ballooning degeneration were minimal in these groups (Fig. 1A). However, the HF-T-fed mice livers were characterized by foamy, prominent microvesicular steatosis throughout the lobe and some macrovesicular lipid droplets in zones 1 and 2. Most of the hepatocytes were expanded with marked small lipid droplets that surrounded the nuclei, and the severely expanded hepatocytes presented the phenotype of ballooning degeneration (Fig. 1A); moreover, some of the fatty hepatocytes were surrounded by infiltrated neutrophils confirmed by immunostaining for myeloperoxidase, forming lipogranuloma (Fig. 1B) accompanied by ballooning hepatocytes (Fig. 1C). The number of neutrophils was increased in HF-T-fed mice livers (Fig. 1D). However, when evaluated by NAS, the HF-T group did not show significant differences (Table 2).

To investigate the involvement of KCs in the pathological difference between the HF-C group and HF-T group, we performed immunohistochemical staining for F4/80, a macrophage-restricted surface glycoprotein. F4/80-positive cells were more prevalent in the HF-T group (Fig. 1E). Although fibrosis was not identified visually by Sirius red staining in any of the groups (not shown), collagen type1, α1 mRNA expression in the liver, as an early fibrosis marker, increased only in HF-T-fed mice by 3.6-fold compared to LF-C-fed mice (Fig. 1F). TUNEL assay did not reveal conspicuous apoptotic hepatocytes in each group, however some non-parenchymal cells were TUNEL positive (Supplementary Fig. 1).

Lipid and lipid peroxide content and fatty acid composition of liver

The hepatic total lipid (Fig. 2A), TG (Fig. 2B), FFA (Fig. 2C) and lipid peroxide contents (Fig. 2D) did not differ between the LF-C and LF-T groups. On the other hand, reflecting the marked liver weight gain and histological steatotic changes, hepatic total lipid, TG and lipid peroxide content were significantly increased in the HF-T group compared to the HF-C group, while FFA content did not differ. All of these markers had a tendency to be elevated in the HF groups compared to the LF groups and when compared between the corresponding dietary oil-fed groups, although the TG increase in HF-C-fed mice was not statistically significant.

Table 2. Influence of *trans*-fatty acid-rich oil intake for the physiological and biochemical characteristics.

	Low-fat diet		High-fat diet	
	Control oil (LF-C)	TFA-rich oil (LF-T)	Control oil (HF-C)	TFA-rich oil (HF-T)
Body weight (g)	24.4 ± 2.1	23.1 ± 1.3	31.8 ± 3.6‖	40.9 ± 7.0*‡†
Liver weight (g)	1.08 ± 0.16	1.22 ± 0.08	1.11 ± 0.11	2.40 ± 1.01*‡†
Liver–body weight ratio (%)	4.5 ± 0.4	5.4 ± 0.2*	3.5 ± 0.3‖	5.6 ± 1.6*†
Plasma characteristics				
Aspartate-aminotransferase (IU/L)	95.2 ± 12.4	82.5 ± 20.8	136.8 ± 47.0	262.2 ± 72.0*‡†
Alanine-aminotransferase (IU/L)	48.8 ± 15.0	37.0 ± 7.3	50.4 ± 10.9	244.0 ± 105.7*‡†
Triglyceride (mg/dl)	60.3 ± 19.2	51.0 ± 12.8	62.4 ± 14.8	124.8 ± 45.0*‡†
Total cholesterol (mg/dl)	77.0 ± 8.9	47.5 ± 6.1*	55.2 ± 5.0	87.8 ± 10.1*‖
HDL-cholesterol (mg/dl)	51.6 ± 8.3	26.2 ± 3.9*	33.3 ± 7.2‖	38.6 ± 5.0‖†
(V)LDL-cholesterol (mg/dl)	16.8 ± 2.1	12.0 ± 1.5*	11.9 ± 1.0‖	17.4 ± 1.7*‖
Free fatty acids (nmol/ml)	1.77 ± 0.38	1.43 ± 0.31	1.99 ± 0.58	3.64 ± 0.42*‡†
Adiponectin (μg/ml)	25.5 ± 1.4	18.2 ± 1.4*	20.0 ± 1.5‖	20.0 ± 1.4†
Leptin (ng/L)	5.6 ± 0.7	5.3 ± 0.6	13.8 ± 2.0‖	23.7 ± 2.3*‡†
Total: HDL-cholesterol ratio	1.54 ± 0.06	2.33 ± 0.5*	1.71 ± 0.37	2.25 ± 0.87†
NAFLD activity score				
Steatosis	0.33 ± 0.52	0.17 ± 0.41	1.67 ± 0.82‖	1.17 ± 0.41‖
Inflammation	0.33 ± 0.52	0.33 ± 0.52	0.83 ± 0.75	1.00 ± 0.63
Ballooning	0.00 ± 0.00	1.00 ± 0.63*	1.00 ± 0.00‖	1.67 ± 0.82†

All values are means ± SD ($n = 6$ per each group).

* Significantly different from the corresponding control group with the same dietary composition; $p < 0.05$.

‖ Significantly different from the low-fat diet with the same dietary lipid as a source; $p < 0.05$.

† Significantly different from low-fat control diet group; $p < 0.05$.

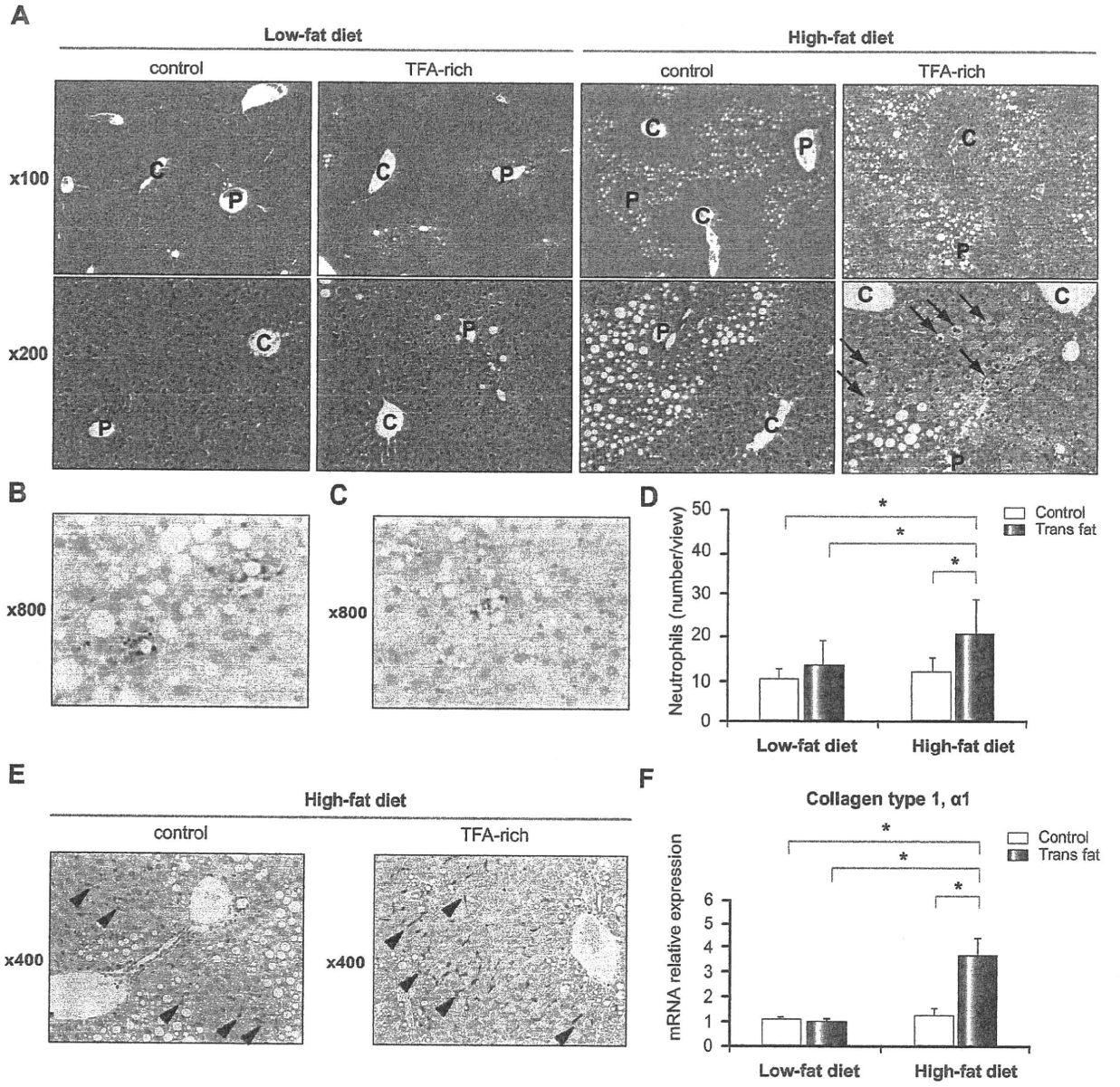

Fig. 1. Distinct steatotic features of the liver. (A) Representative liver histology stained with H&E. Remarkably expanded hepatocytes with extensive small lipid droplets make a feature of ballooning degeneration (arrows). Neutrophils confirmed by myeloperoxidase staining were (B) forming lipogranulomas and (C) surrounding the ballooning degenerated hepatocytes. (D) The number of neutrophils is increased in HF-T-fed mice liver. (E) KCs were detected by anti-F4/80 immunohistochemical staining (arrow heads). (F) Quantitative RT-PCR revealed elevation of collagen type 1, α1 mRNA expression in liver of HF-T-fed mice. P, portal tract; C, central vein. *p <0.05.

We evaluated the lipid composition of the liver to examine the pathological condition in the model. Compared to the LF-C group, the sum of total polyunsaturated fatty acid (PUFA), n-6 PUFA and n-3 PUFA was decreased in the LF-T group, but did not differ significantly in the other groups (Fig. 2E.). In the HF-C group, the sum of saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) was decreased, and total PUFA, n-6 PUFA and n-3 PUFA were increased compared to the LF-C group. However, in the HF-T group, total PUFA and n-6 PUFA decreased significantly compared to the LF-C group, and their proportions were similar to those of the LF-T group. The potentially beneficial lipid n-3 PUFA that is thought to prevent insulin resistance and hepatic steatosis [11], was increased even in the HF-T group compared to the LF-T group, the level of which was similar to that of the LF-C group.

The content of individual fatty acids in the liver coordinated nearly synergistically with the sum of the content of the fatty acids in the same unsaturation grade (Fig. 2E and Table 3). The unique accumulation of elaidic acid (18:1(9-*trans*)), chief component of dietary TFA, was noteworthy in the LF-T and HF-T groups. The content of arachidonic acid (20:4n-6) alone decreased to 70% only in the HF-T group, which was similar to the LF-T group in