Table 5. The reasons of 30 non-participants for preferring surgery or local ablation therapies

Statements[a]	Number of respondents (%)	
	Pt with surgery ($n = 4$)	Pt with local ablation therapies ($n = 25$)
I thought the probability of recurrences would be lower	4 (100)	1 (4)
I thought the survival period would be longer	0	0
I thought the treatment was less burdensome	0	20 (80)
I thought the hospitalization period was shorter	0	12 (48)
I thought the medical cost was fewer	0	1 (4)
Other	0	
I heard that the prognosis were the same		1 (4)
I did not want to increase wound any more		1 (4)

[a]More than one response was allowed.

Table 6. What non-participants referred to when they made a decision

	Number of respondents (%)	
	Pt with surgery ($n = 4$)	Pt with local ablation therapies ($n = 25$)
What non-participants referred to[a]		
Informed consent form	0	13 (52)
Consultation with surgeon in charge	3 (75)	2 (8)
Consultation with physician in charge	1 (25)	21 (84)
Consultation with general practitioner	0	9 (36)
Opinion of other patients	0	2 (8)
Opinion of my family	1 (25)	3 (12)
Other		
My close friend who was clinician suggested	1 (25)	
My friend suggested		1 (4)
The explanation about the prognosis		1 (4)
The information from internet		1 (4)
The information from newspaper		2 (8)
When they made a decision		
Before invitation to the study	1 (25)	13 (52)
After invitation to the study	1 (25)	8 (32)
Do not know or no answer	2 (50)	4 (16)

[a]More than one response was allowed.

referred them to a specialist hospital. Non-participants who received surgery believed in the survival benefits from surgery and relied on surgeon recommendations. On the other hand, patients without strong preference participated in the trial largely because of altruistic motivations. In summary, we found that patients tended to choose less invasive treatment methods even if there is a lack of superiority evidence or an inferiority possibility compared with the standard treatment. Many studies have reported a number of complex barriers in appropriately conducting RCTs (13–18), and we found a couple of these factors that contributed to the incompletion of this trial.

One barrier is that LAT, which had been performed in patients with unrespectable hepatic malignancies, has become popular in treating patients with small HCC due to its superiority in local tumor control and minimal invasiveness. It has become so popular that even without appropriate evidence that LAT has equivalent survival benefits compared with surgery, many general practitioners have recommended it to their patients as an alternative therapy.

Another barrier was patient fear towards a possible allocation into a treatment group that they did not prefer. Although some studies reported that a barrier to trial entry was patient difficulty in understanding the randomization concept and associated patient uneasiness (19–21), our study did not find this as an issue. Only one in 12 respondents that disliked randomization could not understand the randomization concept. Consequently, unbiased and objective explanations by clinicians are crucial in the consent process. However, in our study, we found that the more we stressed the clinical equipoise, the more the patients preferred LAT.

Although the lack of participation was based on these simple reasons, the solution is not simple. In order to increase the number of participants, there are a few possible study designs. One is a randomized consent design, where patients are randomly allocated into a specific treatment group before they provide consent (22,23). If patients decline the allocated treatment, they are then possibly allocated to the other treatment. Even if we apply this design, apart from its ethical problems, the effort will likely fail because most patients allocated to the surgery group will decline. Another possible solution is a randomized trial with a non-randomized part. Specifically, consenting patients are randomized into the two treatment groups, and those that refuse their allocated treatment are enrolled into a non-randomized study. At the conclusion of such a study, the endpoints of the randomized group and the non-randomized group are compared. In such a design, the results may include biases. Moreover, if there is an imbalance in the number of patients between the treatment groups in the non-randomized study, it is difficult to obtain appropriate results.

Furthermore, when there is a discrepancy in results between the randomized and non-randomized study groups, there is difficulty in the interpretation of the results.

In conclusion, when innovative and less burdensome treatments become widespread, they are difficult to compare with standard therapy utilizing a RCT. In light of the increasing number of organ preserving therapies, investigators should evaluate the efficacy and safety of innovative treatments with RCTs as early as possible (24).

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Conflict of interest statement

None declared.

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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.

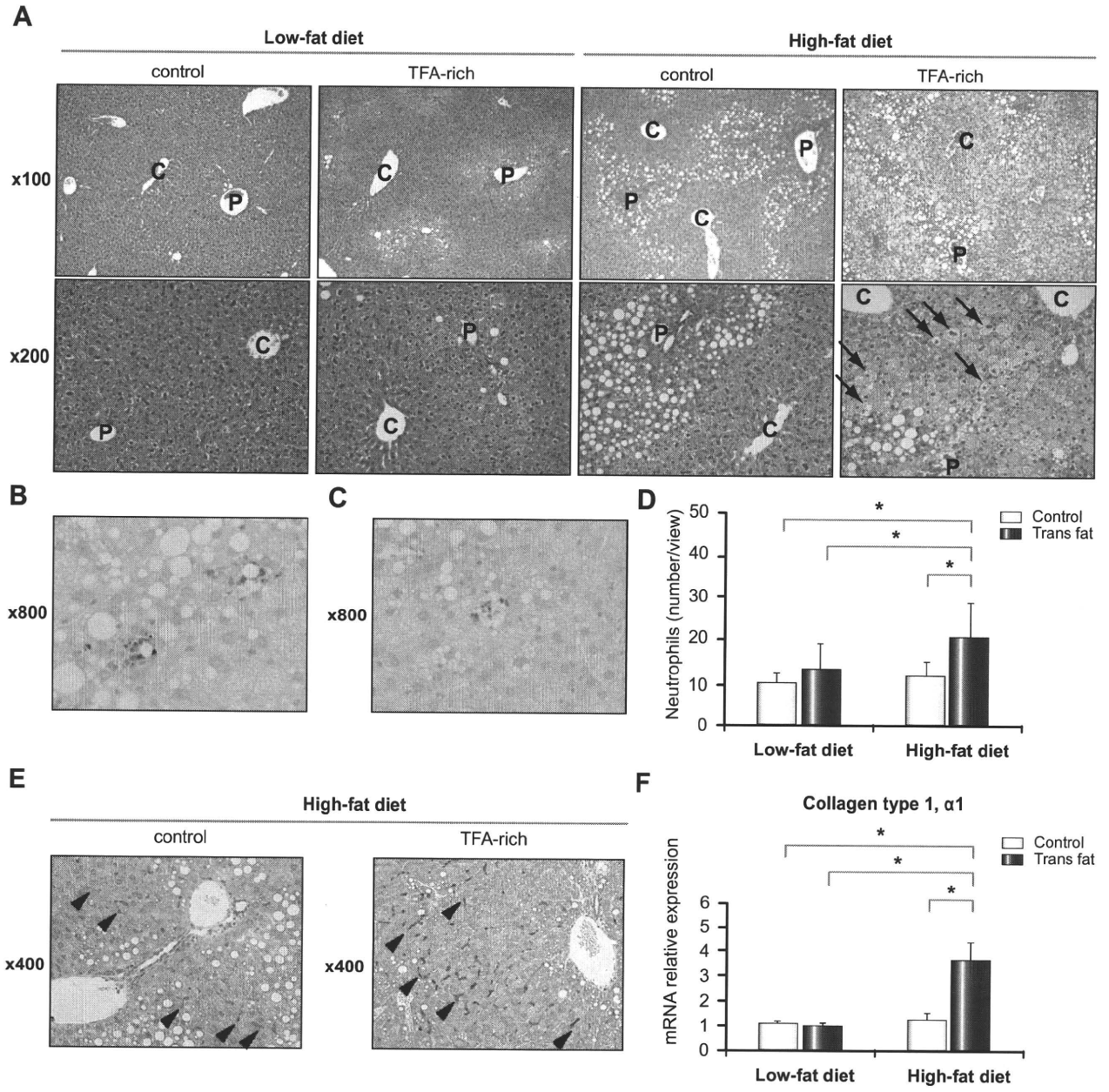

Fig. 1. Distinct steatotic features of the liver. (A) Representative liver histology stained with H&E. Remarkably expanded hepatocytes with extensive small lipid droplets make a feature of ballooning degeneration (arrows). Neutrophils confirmed by myeloperoxidase staining were (B) forming lipogranulomas 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 (20:4*n*-6) alone decreased to 70% only in the HF-T group, which was similar to the LF-T group in

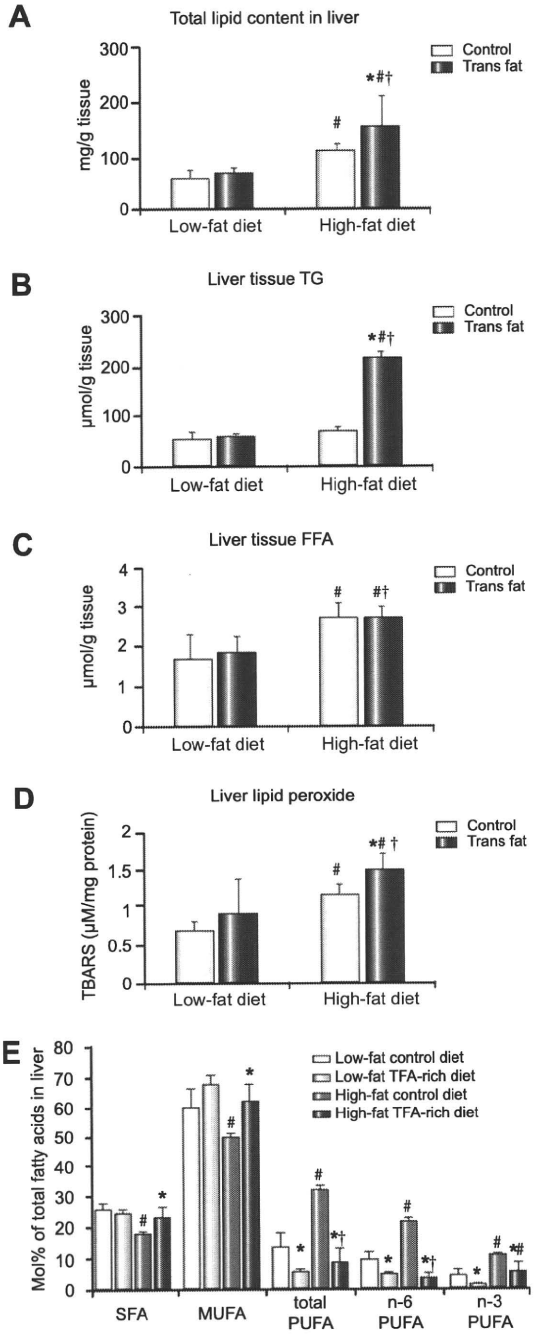

Fig. 2. Lipid accumulation in the liver. (A) Total lipid, (B) TG, and (C) FFA in the liver were measured and normalized to the tissue weight. (D) Lipid peroxide in the liver was measured and normalized by each amount of protein. (E) The hepatic fatty acid composition analyzed by gas chromatography was organized as follows: SFA, saturated fatty acid; MUFA, *cis*-monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; *n*-6, *n*-6 PUFA; and *n*-3, *n*-3 PUFA ($n = 6$ for each group). *Significantly different from the control group with the same dietary composition; ‡significantly different from the low-fat diet with same dietary oil; †significantly different from LF-C-fed and HF-T-fed group.

terms of linoleic acid (18:2*n*-6), the precursor of arachidonic acid. Except for elaidic acid and arachidonic acid, there were no specific alterations for the HF-T group.

Cytokine-, adipokine- and lipid metabolism-related gene expression in liver

Real-time RCR showed that TNFα and inducible nitric oxide synthase (iNOS) mRNA expression increased in the HF groups compared to the LF groups by approximately 2-fold when evaluated for each C or T group (Table 4), while no difference was seen in IL-6 and transforming growth factor-β (TGF-β) mRNA expression in liver among all groups. In addition, adiponectin receptor 1 and 2 gene expression was measured as adipokine related genes, but they did not differ among all groups.

To examine the potential mechanisms of hepatic steatosis by the TFA-rich diet, we determined the expression of known mediators of lipogenesis, fatty acid oxidation and TG excretion in liver, the imbalance of which is thought to lead to steatosis. Sterol regulatory element-binding protein-1 (SREBP-1) induces fatty acid synthase (FAS) and acetyl CoA carboxylase (ACC), and is implicated in steatosis [27]. In relation to hepatic fatty acid synthesis, the mRNA expression of SREBP-1 was significantly elevated in the LF-T, HF-C and HF-T groups when compared to the LF-C group, whereas FAS and ACC were elevated significantly only in the HF-T group (Table 4). Peroxisome proliferator activated receptor γ (PPARγ) is also implicated in steatosis [33]. The expression of PPARγ1 did not differ among the groups, while PPARγ2 was significantly elevated 13-fold in the HF-C group and, remarkably, 50-fold in the HF-T group. Although, the expression of PPARγ coactivator-1β (PGC-1β), known to coactivate the SREBP-1 and stimulate lipogenic gene expression [19], was decreased in HF-fed mouse livers. The fatty acid oxidation-related genes of PPARα and carnitine palmitoyl transferase-1, and the TG excretion-related genes of microsomal triglyceride transfer protein and apolipoprotein B did not differ among the groups; fatty acid oxidation-related genes showed a tendency to be decreased in the LF-T group, but without statistical significance.

Concerning cholesterol metabolism-related gene expression in liver, SREBP-2 was increased in the HF groups, but was not affected by the dietary lipid sources. Hydroxymethylglutaryl-CoA synthase-1 and reductase were significantly increased and apolipoprotein A-1, a component of HDL, was decreased only in the HF-T group, while they did not change in the LF-T group, which showed an alteration of plasma cholesterol fraction (Table 4).

Phosphorylation status of AKT in high-fat diet-fed mice livers

As Koppe et al. suggested that TFA feeding increased insulin resistance in mice [17], and to determine if the exacerbating effects of TFA intake on liver were associated with increased insulin resistance, we evaluated the hepatic phosphorylation status of AKT (Fig. 3A). The phospho-AKT(Thr308) level was significantly decreased (Fig. 3B) and the phospho-AKT(Ser473) level was also decreased, but without statistical significance (Fig. 3C) as determined by densitometrical analysis.

TFA increases TNFα production and alters phagocytotic ability of KCs

The *cis*- or *trans*-fatty acid-containing medium showed no cytotoxicity towards KCs when compared to the fatty acid-free control medium (Fig. 4A). TNFα production of KCs induced by LPS was increased in both the C18:1 and C18:2 TFA-containing medium compared to that of *cis*-structural isomer-containing

Table 3. Individual fatty acid composition of the liver.

		Low-fat diet		High-fat diet	
		Control oil (LF-C)	TFA-rich oil (LF-T)	Control oil (HF-C)	TFA-rich oil (HF-T)
SFA					
Myristic	14:0	0.6 ± 0.1	0.5 ± 0.0	0.3 ± 0.1[‡]	0.4 ± 0.1[*†]
Palmitic	16:0	21.0 ± 1.6	19.2 ± 1.1	12.1 ± 0.6[‡]	18.7 ± 3.6[*]
Stearic	18:0	3.7 ± 0.8	3.6 ± 0.6	4.6 ± 0.6	3.3 ± 1.2
Arachidic	20:0	0.6 ± 0.3	1.0 ± 0.1[*]	0.6 ± 0.3	0.9 ± 0.3
MUFA					
Palmitoleic	16:1 *n*-7	6.9 ± 4.9	7.9 ± 4.8	2.5 ± 0.2	4.9 ± 1.0
Oleic	18:1	53.4 ± 6.7	59.8 ± 2.3	47.4 ± 1.3	57.2 ± 4.8[*‡†]
Elaidic	18:1 (9-*trans*)	0.0 ± 0.0	2.2 ± 0.5[*]	0.3 ± 0.2	6.2 ± 2.4[*‡†]
PUFA					
Linoleic	18:2 *n*-6	6.5 ± 2.1	2.1 ± 0.4[*]	18.1 ± 1.6[‡]	2.6 ± 1.4[*†]
α-Linolenic	18:3 *n*-3	0.6 ± 0.4	0.0 ± 0.0[*]	3.2 ± 0.5[‡]	0.2 ± 0.2[*]
Dihomo-γ-linolenic	20:3 *n*-6	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.2[‡]	0.2 ± 0.2
Arachidonic	20:4 *n*-6	2.5 ± 0.9	2.2 ± 0.5	2.6 ± 0.6	0.6 ± 0.3[*‡†]
Eicosapentaenoic	20:5 *n*-3	0.4 ± 0.2	0.1 ± 0.0	1.4 ± 0.1[‡]	1.2 ± 0.9[‡†]
Docosapentaenoic	22:5 *n*-3	0.2 ± 0.1	0.0 ± 0.0	0.8 ± 0.0[‡]	0.4 ± 0.3[*‡†]
Docosahexaenoic	22:6 *n*-3	3.0 ± 1.1	1.0 ± 0.3	5.2 ± 0.4	3.2 ± 2.4

The relative percentage (mean ± SD) of each fatty acid to the total fatty acids is presented ($n = 6$ 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; $p < 0.05$.

medium (Fig. 4B). However, IL-6 production by KCs did not differ between *cis*- and *trans*-fatty acid-containing medium for both C18:1 and C18:2, respectively (Fig. 4C). The phagocytotic ability of KCs incubated in *trans*-C18:1-containing medium was lower than that in *cis*-C18:1-containing medium (Fig. 4D), while the influence of the structural difference of C18:2 fatty acid was small (Fig. 4E).

Discussion

Both of the dietary lipid species [3] and their amounts [6,23] are known to affect hepatic steatosis and inflammation. TFAs have been mainly linked with coronary heart disease, possibly by decreasing HDL-cholesterol and increasing LDL-cholesterol [20,29]; while little attention has been paid to liver disease, even

Table 4. Cytokine-, adipokine- and lipid metabolism-related gene expression in liver.

		Low-fat TFA-rich (LF-T)	High-fat control (HF-C)	High-fat TFA-rich (HF-T)
Cytokine and adipokine				
Tumor necrosis factor	TNF	0.98 ± 0.20	2.11 ± 0.73[‡]	1.94 ± 0.77[‡†]
Interleukin-6	IL-6	1.30 ± 0.28	1.16 ± 0.14	1.10 ± 0.25
Transforming growth factor-β	TGB-β	0.86 ± 0.17	1.35 ± 0.50	0.98 ± 0.22
Nitric oxide synthase 2, inducible	iNOS	1.29 ± 0.34	2.23 ± 0.75[‡]	2.69 ± 0.74[‡†]
Adiponectin receptor 1	AdipoR1	1.01 ± 0.17	1.29 ± 0.29	1.21 ± 0.25
Adiponectin receptor 2	AdipoR2	0.92 ± 0.16	1.20 ± 0.22	1.28 ± 0.26
Lipogenesis				
Fatty acid synthase	FAS	1.36 ± 0.21	0.79 ± 0.12	1.69 ± 0.46[*†]
Acetyl-CoA carboxylase	ACC	1.27 ± 0.10	0.80 ± 0.05	1.49 ± 0.37[*†]
Sterol regulatory element-binding protein-1	SREBP-1	3.76 ± 0.51[*]	1.93 ± 0.23[‡]	4.69 ± 0.17[*‡†]
Peroxisome proliferator activated receptor γ1	PPARγ1	1.45 ± 0.42	1.58 ± 0.48	1.29 ± 0.26
Peroxisome proliferator activated receptor γ2	PPARγ2	1.88 ± 0.33	12.92 ± 6.04[‡]	50.18 ± 3.61[*†]
PPARγ coactivator-1β	PGC-1β	0.81 ± 0.10	0.61 ± 0.15[‡]	0.59 ± 0.12[‡†]
Fatty acid oxidation				
Peroxisome proliferator activated receptor α	PPARα	0.51 ± 0.23	1.25 ± 0.48	1.28 ± 0.41
Carnitine palmitoyl transferase-1	CPT-1	0.63 ± 0.11	1.03 ± 0.34	1.08 ± 0.57
Triglyceride excretion				
Microsomal triglyceride transfer protein	MTP	1.04 ± 0.16	0.92 ± 0.11	0.91 ± 0.11
Apolipoprotein B	ApoB	1.11 ± 0.15	1.20 ± 0.13	1.15 ± 0.09
Cholesterol metabolism				
Sterol regulatory element-binding protein-2	SREBP-2	0.87 ± 0.15	1.66 ± 0.25	1.69 ± 0.34
Hydroxymethylglutaryl-CoA synthase-1	HMGCs1	1.03 ± 0.11	1.60 ± 0.26	4.56 ± 0.73[*‡†]
Hydroxymethylglutaryl-CoA reductase	HMGCr	0.99 ± 0.13	1.22 ± 0.32	2.93 ± 0.44[*‡†]
Apolipoprotein A-1	ApoA1	0.93 ± 0.09	1.26 ± 0.21	0.61 ± 0.11[*‡†]

All results are expressed as the relative fold change compared to the low-fat control diet group ± 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; $p < 0.05$.

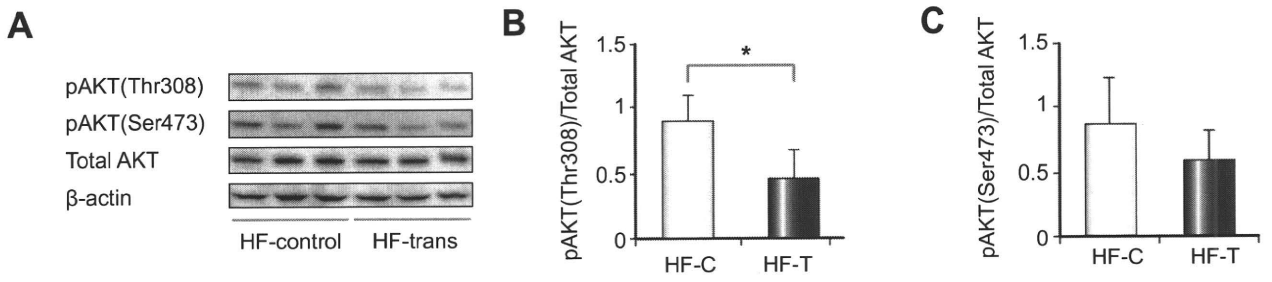

Fig. 3. Effect of excessive TFA consumption of AKT in the high-fat diet-fed mice liver. (A) Representative pictures of phospho-AKT (Thr308 and Ser473), total AKT and β-actin Western blots as well as densitometric analysis of the (B) pAKT(Thr308) or (C) pAKT(Thr473)/total AKT ratio. *p <0.05.

though a few studies have reported that hepatic steatosis [30] and ALT elevation [17] were induced by a TFA-rich diet in mice, the mechanisms remain to be clarified. In agreement with this, the HF-T group showed severe steatosis with a significant transaminase elevation, while HF-C-fed mice only showed moderate steatosis without liver injury, in addition we showed that relatively small amounts of TFA-rich oil intake do not induce severe steatosis and liver injury in the current study. Interestingly, the plasma cholesterol fraction was significantly altered even in the LF-T group in association with the elevation of the total: HDL-cholesterol ratio, a risk factor index of coronary artery disease [22]. The alteration of the plasma cholesterol fraction might be partially explained by changes in the cholesterol metabolism-related gene expressions in the HF-T group, but not in the LF-T group. We could not determine why the cholesterol fraction was altered in the LF-T group, but it might be partially due to the modified membrane fluidity induced by TFA intake [14]. That a relatively small TFA intake could affect the cholesterol fraction but not the liver might be the reason why less attention has been paid to the liver until recently.

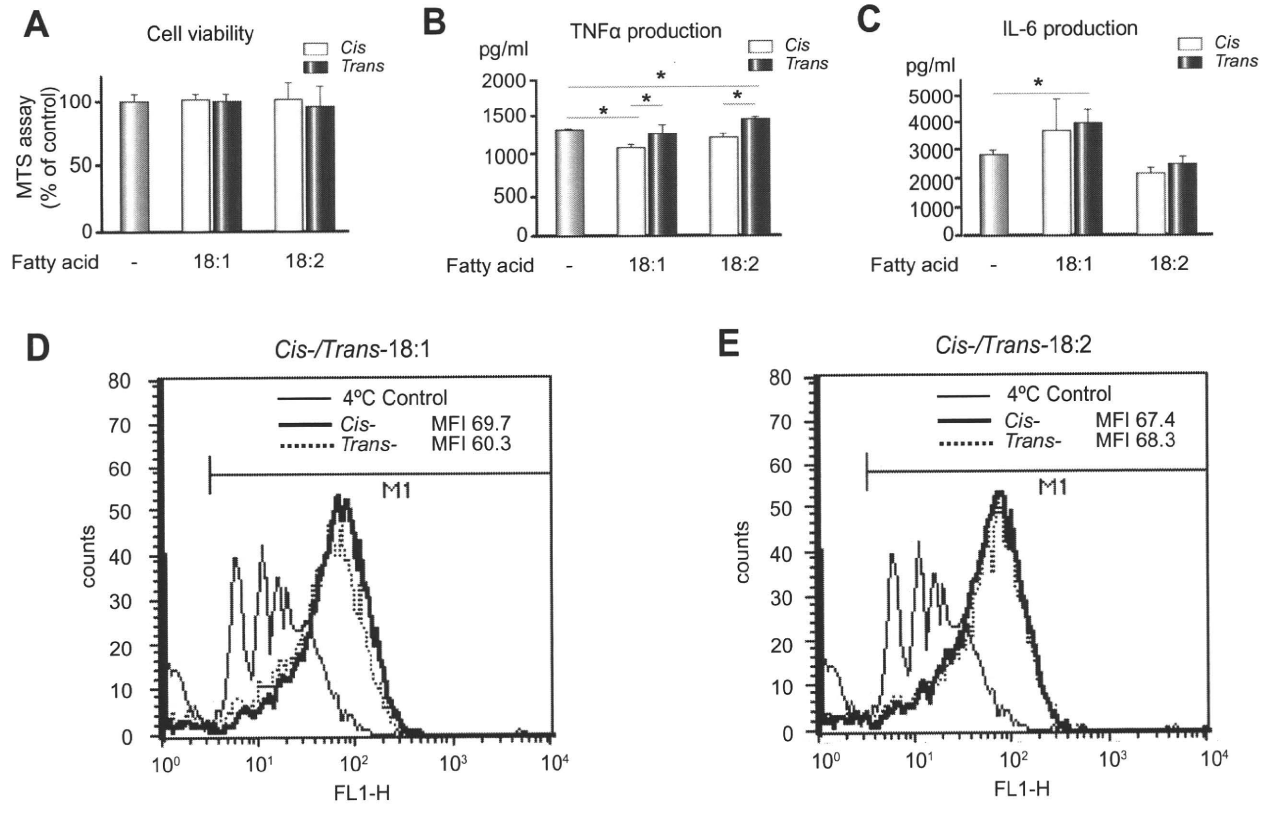

Fig. 4. The impact of *cis*-/*trans*-fatty acid on the cytokine production and phagocytotic ability of KCs. (A) No fatty acids (200 μM) shows cytotoxicity for primary KCs after 24 h incubation, but (B) increases TNFα production in both C18:1 and C18:2-TFA-containing medium compared to its *cis*-structural isomer. (D) IL-6 production increases in C18:1-containing medium and remains unchanged in C18:2-containing medium, whereas the *cis*- or *trans*-structural difference does affect the results. The influence of the C18:1 (D) and C18:2 (E) *cis*-/*trans*-fatty acid on the phagocytotic ability of KCs is measured by flow cytometry analysis. MFI: mean fluorescence intensity (n = 8 for each group, A–C). *p <0.05.

One of the noted biochemical changes seen in the HF-T group was the elevation of plasma FFA, almost all of which is derived from adipose tissue and accumulates in the liver as TG in a dose-dependent fashion [7]. The increase of plasma FFA might be due to the TFA incorporation in the adipocyte plasma membrane resulting in decreased membrane fluidity, accompanied by increased adipose tissue insulin resistance as evidenced by increased lipolysis, decreased antilipolysis and decreased glucose uptake in rat adipocytes [14]. In addition, in contrast to TG, the circulating FFAs [21] and accumulation of FFAs in liver [32] are known to exert lipotoxicity to hepatocytes, so the significant increase of the plasma FFA in the HF-T group might affect the pathophysiology. On the other hand, hepatic FFA was higher in HF than LF irrespective of the dietary lipid sources, and it was reported that forced high-fat feeding induced NASH in mice, while usual high-fat intake does not [6]. Hepatic FFA accumulation remained unaltered among healthy, NAFLD and NASH subjects liver [24], so it might not be necessary for the progression to NASH but could be a exacerbating factor in the case of excess fat consumption. Therefore, elevated plasma FFA and accumulated hepatic lipid peroxide [23] would contribute synergistically to the liver injury in this model.

A previous study reported that TFA intake decreased the arachidonic acid level and induced insulin resistance in adipose tissue, probably due to the decreased membrane fluidity [14], and the present study also revealed a decrease of the arachidonic acid level in the liver and lower phosphorylation status of AKT which could reflect the hepatic insulin resistant status in the HF-T group. In addition, a human lipidomic analysis of NAFLD/NASH liver described a decrease of arachidonic acid and unaltered levels of precursor linoleic acid, but the study did not address TFA consumption [24]. Although, the mechanisms of the arachidonic acid decrease and involvement in the insulin resistance and progression to NASH remain to be clarified, these common findings might suggest that TFA intake influences both the liver and adipose tissue in a somewhat similar fashion in NASH patients and people who consume excess TFAs.

Typical pathological findings in human NAFLD/NASH patients such as macrovesicular lipid deposit and inflammation are usually identified around zone 3, though the mice liver showed lipid deposits around zone 1 even in the control lipid-fed group. Microvesicular lipid droplets were remarkable in the HF-T group, so it might be difficult to assess the pathological changes using a human scoring system such as NAS. The pathological differences might be due to a specific problem, since the pediatric NAFLD is known to show histological findings around zone 1 [26], which might be related to the dietary habit of consuming excess TFAs from snacks and first food.

Although the expression of proinflammatory cytokines such as TNFα [30] and IL-1β [17] have been shown to be induced in the mice liver by TFA-rich diets, this was not the case in the current study. However, a previous study reported that, among hypercholesterolemic subjects, the production of TNFα by cultured mononuclear cells was increased by a TFA-rich soy bean margarine diet compared with a natural soybean oil diet [12], and we showed KCs increased TNFα production in TFA-containing medium compared to that of *cis*-structural isomer-containing medium. Accordingly, pathophysiological conditions induced by TFA consumption could be partially due to alterations in the monocyte/macrophage ability in proinflammatory cytokine production and phagocytosis, and KCs in particular may play important roles in the local circumstances.

With regard to the adipokines, the adiponectin levels were not changed between the HF-C and HF-T group, but plasma leptin was significantly increased in the HF-T group. Leptin is an appetite-suppressing and body weight-regulating adipokine, and is even related to liver regeneration and fibrosis [13]; hyperleptinemia might be related directly to the elevation of type1 collagen α1 mRNA expression in the liver.

In terms of the severe steatosis of the model, the lipogenetic genes such as FAS, ACC, SREBP-1 and PPARγ2 were coordinately induced, but PGC-1β was not. PGC-1β is known to be induced in the liver by short term high-fat feeding, to coactivate SREBP-1, but to reduce hepatic fat accumulation. However, since it was not investigated in this study, the decrease of PGC-1β gene expression might be due to long term feeding or any other factors, such as the relatively low carbohydrate diet. It was reported that a TFA-rich diet suppressed the PPARγ gene expression in rat adipose tissue [8], and that lipogenic gene expressions in the liver and the adipose tissue are reciprocal modulations [4], which might reflect the core mechanisms of the heterotopic fat accumulation *in vivo*.

In summary, excess TFA consumption induces significant hepatic steatosis accompanied by augmentation of the hepatic lipogenic gene expressions, FFA influx into the liver, and the hepatic accumulation of lipid peroxide. The hepatic accumulation of TFA and the reduction of the arachidonic acid content were the lipidomic properties in this model. Together with their potential induction of local cytokines by KCs, lipid species including TFA may play a pivotal role in the development of non-alcoholic fatty liver diseases.

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Conflicts of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2010.02.029.

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MAJOR ARTICLE

Hepatitis B Virus Replication Could Enhance Regulatory T Cell Activity by Producing Soluble Heat Shock Protein 60 From Hepatocytes

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Background. HBcAg-specific regulatory T (T_{reg}) cells play an important role in the pathogenesis of chronic hepatitis B. Soluble heat shock proteins, especially soluble heat shock protein 60 (sHSP60), could affect the function of T_{reg} cells via Toll-like receptor.

Methods. We analyzed the relationship between soluble heat shock protein production and hepatitis B virus (HBV) replication with both clinical samples from HBeAg-positive patients with chronic hepatitis B ($n = 24$) and HBeAb-positive patients with chronic hepatitis B ($n = 24$) and in vitro HBV-replicating hepatocytes. Thereafter, we examined the biological effects of sHSP60 with isolated T_{reg} cells.

Results. The serum levels of sHSP60 in patients with chronic hepatitis B were statistically significantly higher than those in patients with chronic hepatitis C ($P < .01$), and the levels of sHSP60 were correlated with the HBV DNA levels ($R = 0.532$; $P < .001$) but not with the alanine aminotransferase levels. Moreover, the levels of sHSP60 in HBV-replicating HepG2 cells were statistically significantly higher than those in control HepG2 cells. Preincubation of $CD4^+$ $CD25^+$ cells with recombinant HSP60 (1 ng/mL) statistically significantly increased the frequency of HBcAg-specific interleukin 10–secreting T_{reg} cells. The frequency of $IL7R^-CD4^+CD25^+$ cells, the expression of Toll-like receptor 2, and the suppressive function of T_{reg} cells had declined during entecavir treatment.

Conclusion. The function of HBcAg-specific T_{reg} cells was enhanced by sHSP60 produced from HBV-infected hepatocytes. Entecavir treatment suppressed the frequency and function of T_{reg} cells; this might contribute to the persistence of HBV infection.

Hepatitis B virus (HBV) is a noncytopathic DNA virus that causes chronic hepatitis and hepatocellular carcinoma as well as acute hepatitis and fulminant hepatitis [1]. HBV now affects more than 400 million people worldwide [2], and persistent infection develops in ~5% of adults and 95% of neonates who become infected with HBV.

It has been shown that the cellular immune system, including cytotoxic T lymphocytes, $CD4^+$ T helper 1 cells, and $CD4^+C25^+FoxP3^+$ regulatory T (T_{reg}) cells, plays a central role in the control of viral infection [3–6]. The hyporesponsiveness of HBV-specific T helper 1 cells and the excessive regulatory function of T_{reg} cells in peripheral blood in patients with chronic hepatitis B has been shown elsewhere [7–10]. Lamivudine treatment of chronic hepatitis B has been reported to restore both $CD4^+$ T cells and cytotoxic T lymphocyte hyporesponsiveness following the decrease of serum levels of HBV DNA and HBV-derived Ag [8, 11–13]. In our previous study, we observed that HBcAg-specific interleukin 10 (IL-10)–secreting T_{reg} cells could play an important role in the immunopathogenesis of chronic hepatitis B [9].

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Table 1. Clinical Characteristics of Patients with Chronic Hepatitis B or Chronic Hepatitis C Included in This Study

Characteristic	Patients with chronic hepatitis B		Patients with chronic hepatitis C
	HBeAg-positive, HBeAb-negative patients	HBeAg-negative, HBeAb-positive patients	
Age, years	45.16 (12.46)	48.21 (10.23)	48.63 (7.96)
Sex, no. of patients			
Male	12	12	12
Female	12	12	12
ALT level, IU/L	76.91 (39.82)	75.96 (45.90)	76.21 (33.77)
HBV DNA level, log copies/mL	7.83 (0.86)	6.00 (0.81)	NA
Genotype, % of patients			
A	0	4.17	NA
B	12.5	8.33	NA
C	87.5	87.5	NA

NOTE. Data are mean values (standard deviations), unless otherwise indicated. ALT, alanine aminotransferase; HBV, hepatitis B virus; NA, not applicable.

Many research groups have reported the possible induction of anergy by T_{reg} cells, which constitutively express CD25 (the interleukin 2 receptor α chain) in the physiological state [14–16]. In humans, this population of T_{reg} cells, as defined by $CD4^+CD25^+CTLA4^+$ cells, $CD4^+CD25^+FoxP3^+$ cells, or $CD4^+CD25^+IL7R^-$ cells, constitutes 5%–10% of peripheral $CD4^+$ T cells and has a broad repertoire that recognizes various self and nonself antigens. It has been reported that T_{reg} cells have several different mechanisms in suppressing various kinds of immune cells [17, 18]. The important mechanisms are cell to cell contact and secretion of cytokines including IL-10 and transforming growth factor β (TGF-β) [19, 20]. HBcAg derived from HBV might induce T_{reg} cells to escape from immunological pressure, as reported in persistent infection with Epstein-Barr virus, hepatitis C virus (HCV), and human immunodeficiency virus type 1 [21–23]. Some results have indicated that reduction of HBV replication could reduce the frequency and/or function of T_{reg} cells in patients with chronic hepatitis B [4, 5, 8]. However, the key factors that affect HBcAg-specific T_{reg} cells in the replication of HBV remain unclear.

The mammalian 60-kDa heat shock protein is a many-faceted molecule. In addition to serving as a chaperone, heat shock protein 60 (HSP60) is expressed by different types of cells following their exposure to stress or immune responses and is present in the blood during inflammation [24–27]. Recently, HSP60 was reported to enhance the function of $CD4^+CD25^+$ regulatory T cell function via Toll-like receptor 2 (TLR2) signaling [28].

In this study, we investigated the serum level of HSP60 in patients with chronic hepatitis B and the relevance of HBcAg-specific IL-10-secreting T_{reg} cells and HSP60. We report evidence of the production of soluble HSP60 (sHSP60) from HBV-replicating hepatocytes, by use of clinical samples from patients with chronic hepatitis B and an in vitro HBV replication system. In addition, reductions of $CD4^+CD25^+IL7R^-$ T_{reg} cells and TLR2 expression on T_{reg} cells were observed during entecavir therapy. This study could contribute to better understanding of the immunopathogenesis of chronic hepatitis B and the development of immune-based treatment.

MATERIALS AND METHODS

Patients. Forty-eight patients with chronic hepatitis B were enrolled in this study (Table 1). The patients had serum levels of HBV DNA of >5.0 log copies/mL and had elevated alanine aminotransferase (ALT) levels (reference range, <40 IU/L) for >6 months prior to the study. To focus the analysis on the active phase of chronic hepatitis B, we excluded asymptomatic carriers and patients with immune tolerance by age (<30 years old), ALT values (<40 IU/L), and HBV DNA levels (<5.0 log copies/mL). Twenty-four patients were seropositive for HBeAg, and 24 patients were seropositive for anti-HBeAb. None of the patients tested positive for antibodies to hepatitis C virus or had liver disease due to other causes, such as alcohol, drugs, congestive heart failure, and autoimmune disease. Twenty-four patients with chronic hepatitis C and 10 healthy subjects were included as control subjects. Permission for the study was obtained from the Ethics Committee at Tohoku University Graduate School of Medicine (permission no. 2006-194]). Written informed consent was obtained from all the participants enrolled in this study. Participants were monitored for 6 months, and peripheral blood samples were obtained and assessed at 1, 2, 3, and 6 months. At each assessment, patients were evaluated for serum levels of HBV DNA, HBeAg, and anti-HBe, blood chemistry, and hematology. Levels of HBsAg, anti-HBs, total and immunoglobulin anti-HBc, HBeAg, anti-HBe, and anti–

hepatitis C virus were determined by means of commercial enzyme immunoassay kits (Abbott Laboratories). Serum levels of HBV DNA were measured by means of an Amplicor polymerase chain reaction (PCR) assay (lower limit of detection, 2.6 log copies/mL; Roche). High titers of HBV DNA were measured by means of a transcription-mediated amplification-hybridization protection assay (TMA; lower limit of detection, 3.7 log genome equivalents per milliliter). Data were adjusted by means of the following formula: Amplicor value = 0.83 × (TMA value) + 0.67.

Reagents. The following antibodies were used: CD3–allophycocyanin (APC), CD4–peridinin chlorophyll protein complex (PerCP), CD25–fluorescein isothiocyanate (FITC), CD25-phycoerythrin (PE), CD127-PE, Alexa Fluor 488 mouse anti–human CD282 (TLR2), CD284 (Toll-like receptor 4 [TLR4]), and isotype-matched control antibodies purchased from BD Bioscience. Recombinant HBcAg was obtained from Biodesign International. Recombinant HSP60 (rHSP60) was purchased from Stressgen.

Quantification of sHSP60 and soluble heat shock protein 70 (sHSP70) levels. Levels of HSP60 and heat shock protein 70 (HSP70) were quantified by use of HSP60 and HSP70 enzyme-linked immunosorbent assay (ELISA) kits (Stressgen). The serum samples from patients and supernatants from cell cultures were collected at sampling points and stocked at −20°C. The ELISA procedure was performed according to the manufacturer's protocol. First, 100-μL prepared samples were added to wells of anti-HSP60-coated plates. Then the reaction of the anti-HSP60 and horseradish peroxidase conjugate was performed after incubation and washing. Absorbance was measured at 450 nm. The HSP60 sample concentration was calculated by use of a standard curve.

Isolation of peripheral blood mononuclear cells (PBMCs) and T_{reg} cells. PBMCs were isolated from fresh heparinized blood by means of Ficoll-Hypaque density gradient centrifugation. T_{reg} cells were isolated by use of a Dynabeads regulatory $CD4^+CD25^+$ T cell kit (Invitrogen). T_{reg} cells were isolated according to the manufacturer's protocol. In brief, $CD4^+$ cells were isolated from PBMCs by means of negative selection. The remaining cells included the PBMCs depleted of $CD4^+$ cells. Then the $CD4^+CD25^+$ cells were selected positively by use of $CD25^+$ antibody combined with beads. Finally, the beads were detached by means of Detachabead (Invitrogen), because the function of T_{reg} cells might be modified by anti-CD25 antibody.

Coculture of γ-irradiated HBcAg-presenting antigen-presenting cells (APCs) and T_{reg} cells. During the isolation of T_{reg} cells, PBMCs depleted of $CD4^+$ cells could be obtained for use as APCs. PBMCs depleted of $CD4^+$ cells were stimulated at 1×10^6 cells/mL in Roswell Park Memorial Institute 1640 medium containing 10% fetal bovine serum with HBcAg (10 μg/mL) for 12 h at 37°C. Then these γ-irradiated cells were coincubated with 1×10^5 isolated T_{reg} cells that were untreated pretreated with TLR2 and TLR4 neutralizing antibody and rHSP60 (1 ng/mL) (Figures 1*A* and 2).

IL-10 secretion assay. Isolated T_{reg} cells were stimulated with HBcAg-presenting autologous γ-irradiated APCs for 12 h at 37°C. IL-10-secreting cells were stained by adding 10 μL of IL-10-detection antibody (PE-conjugated) together with anti-CD4-PerCP, anti-CD25-FITC, and anti-CD3-APC.

Flow cytometry. PBMCs were stained with CD3-APC, CD4-PerCP, CD25-FITC, and CD127-PE antibodies for 15 min on ice to analyze the frequency of $CD4^+CD25^+IL7R^-$ cells. CD4-PerCP, CD25-PE, and Alexa Fluor 488 mouse anti–human CD282 (TLR2) or CD284 (TLR4) were used for the analysis of TLR2 and TLR4 expression on $CD4^+CD25^+$ cells. Isotype-matched control antibodies were used for adjustment of the fluorescence intensity.

Construction of plasmids. The HBV plasmids was constructed as described elsewhere, with minor modifications [29]. In brief, a serum sample from one of the consecutive patients with fulminant hepatitis B (fulminant hepatitis clone 2), whose serum level of HBV DNA was the highest of the 5 patients, was used to extract total DNA (QIAamp DNA blood mini kit; Qiagen), which was subjected to nested PCR for 2 overlapping fragments; the amplified fragments were nucleotides 1051–3215/1–327 (2492 nucleotides; fragment A) and nucleotides 180–1953 (1774 nucleotides; fragment B). Then the vectors were digested with XbaI, and the XbaI-XbaI site of fragment A-pUC118 was ligated to the XbaI-XbaI site of fragment B-pUC118. Finally, a plasmid containing a 1.3-fold HBV genome (nucleotides 1051–3215/1–1953) was constructed and named pBFH2.

Cell culture and transfection. Human hepatoma HepG2 cells were incubated in Dulbecco modified Eagle medium supplemented with 10% bovine serum at 37°C and 5% carbon dioxide. For the assay of HBV replication, 6-well plates were seeded with 5×10^5 HepG2 or Huh7 cells each. On the next day, 1.5 μg of plasmid DNA were transfected to these cells by use of TransIT LT-1 transfection reagent (Mirus), and the culture supernatant and cells were collected 3 d later. The transfection efficiency was evaluated with a Great EscAPe secreted alkaline phosphatase reporter system 3 (Clontech), in which 10 ng/mL of a reporter plasmid expressing secreted alkaline phosphatase was cotransfected. Experiments were performed at least in triplicate.

Quantification of extracellular HBV DNA, HBsAg, and HBeAg levels. To digest the input plasmid DNA in the culture supernatant, 5 μL of the supernatant was treated with 5 U of DNase I (TaKaRa Bio) at 37°C for 1 h, and the reaction was stopped with edetic acid. Then total DNA was extracted with a QIAamp DNA blood mini kit, and 10 μL of 200-μL DNA solution was subjected to real-time PCR by use of a LightCycler