Research Article

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Characteristics of splenic CD8$^+$ T cell exhaustion in patients with hepatitis C

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Summary

There is increasing interest in the role of T cell exhaustion and it is well known that the natural history of chronic hepatitis C virus infection (HCV) is modulated by CD8$^+$ T cell immunobiology. There are many pathways that alter the presence of exhaustive T cells and, in particular, they are functionally impaired by inhibitory receptors, such as programmed death-1 (PD-1) and T cell immunoglobulin and mucin domain-containing protein 3 (Tim-3). We obtained spleen, liver and peripheral blood (before and after splenectomy) lymphoid cells from 25 patients with HCV-related cirrhosis undergoing liver transplantation for end-stage disease or splenectomy for portal hypertension. In all samples we performed an extensive phenotypic study of exhaustion markers [PD-1, Tim-3, interferon (IFN)-γ) and their ligands (PD-L1, PD-L2, galectin-9] in CD8$^+$ T cell subpopulations (both total and HCV-specific) and in antigen-presenting cells (APC; monocytes and dendritic cells). In the spleen, total and HCV-specific CD8$^+$ T cells demonstrated enhanced markers of exhaustion, predominantly in the effector memory subpopulation. Similarly, splenic APC over-expressed inhibitory receptor ligands when compared to peripheral blood. Finally, when peripheral blood CD8$^+$ T cells were compared before and after splenectomy, markers of exhaustion were reduced in splenic CD8$^+$ T cells and APC. Our data in HCV-related cirrhosis suggest that CD8$^+$ T cells in the spleen manifest a significantly higher exhaustion compared to peripheral blood and may thus contribute to the failure to control HCV. Counteracting this process may contribute to inducing an effective immune response to HCV.

Keywords: hepatitis C, liver cirrhosis, PD-1, portal hypertension, splenectomy, Tim-3

Introduction

During the course of chronic viral infection, T cells may undergo both memory and exhaustion as part of the natural history in the modulation of infection, as well exemplified by hepatitis C virus (HCV) in both liver and peripheral blood [1,2]. In this respect, the spleen is often ignored despite its key role in the regulation of the immune response to infectious agents [3,4]. Importantly, a significant proportion of HCV-infected patients develop hypersplenism, which is a contributing factor to their co-morbidities of liver cirrhosis and portal hypertension [5–7]. We note an increasing role for CD8$^+$ T cells in clearing infection, as a mediator in autoimmunity and, in particular, the observation that 70% of infected individuals are unable to clear HCV [8,9]. These observations suggest that T cell exhaustion is part of the natural history of HCV. CD8$^+$ T cell exhaustion is co-regulated by multiple inhibitory receptors, and the interaction between cellular receptors and inhibitory receptor ligands on antigen-presenting cells (APC) regulate virus persistence [2,10–13]. In fact, as part of the hypersplenic syndrome, the spleen is often removed in patients with HCV-mediated liver cirrhosis and portal hypertension with thrombocytopenia because of their association with thrombocytopenia and the subsequent risk of further reductions in platelet counts following anti-viral therapies; the risk associated with spleen removal includes an increased risk of infection [14–16]. Our labora-

tory has demonstrated recently that splenectomy in HCV patients is followed by an increase of interferon (IFN)-γ production and a reduction of programmed death 1 (PD-1) expression by $CD4^+$ T cells in peripheral blood of patients with HCV-related cirrhosis [17]. To extend these observations, we report herein the phenotype of exhausted $CD8^+$ T cells in the spleen of patients with HCV-related cirrhosis undergoing splenectomy. Our data have significant implications for understanding the cellular alterations that occur in HCV-related cirrhosis and the subsequent loss of spleen.

Materials and methods

Subjects

Sixteen patients with HCV-related liver cirrhosis undergoing splenectomy for severe thrombocytopenia were studied. In all patients, the spleen was removed because of severe thrombocytopenia that was a contraindication for interferon (IFN)-α therapy. In addition, there were nine patients with HCV-related liver cirrhosis who underwent liver transplantation. Liver, spleen and peripheral blood mononuclear cells were isolated from patients. All subjects gave their written informed consent and experimental protocols were conducted under the Guidelines of the Research Ethics Committee of Kyushu University.

Isolation of mononuclear cells and $CD8^+$ T cells

Peripheral blood mononuclear cells (PBMC) were separated from heparinized fresh blood by gradient centrifugation on Ficoll-Isopaque, while spleen mononuclear cells (SMC) and liver mononuclear cells (LMC) were isolated from fresh explanted tissues using established protocols [18]. Briefly, spleen tissues were digested mechanically and dissociated cells filtered through 100-μm nylon mesh, while liver specimens were first digested with 1 mg/ml of collagenase type IV (Sigma-Aldrich, Tokyo, Japan) and filtered through 100-μm nylon mesh. Digested spleen and liver cells were separated by Ficoll-Isopaque gradient centrifugation to obtain SMC and LMC. $CD8^+$ T cells were negatively isolated using magnetic beads (CD8 isolation kit II; Miltenyi Biotec, Auburn, CA, USA) from PBMC, SMC and LMC; >95% viability by trypan blue dye exclusion and >90% purity by flow cytometry were considered as acceptable. All cells were washed and cryopreserved in fetal cow serum containing 10% dimethylsulphoxide (DMSO) and stored in liquid nitrogen until used.

Mononuclear cell immunophenotyping

PBMC, LMC and SMC (1×10^6) were stained for cell surface antigen expression at 4°C in the dark for 30 min, washed twice in 2 ml phosphate-buffered saline containing 1% bovine serum albumin and 0·01% sodium azide, and fixed in 500 μl of 1% paraformaldehyde. Cells were stained for CD8, CD14, PD-1, PD-L1, PD-L2 (BD Biosciences, San Diego, CA, USA), CD45RA, CCR7, CD11c (e-Biosciences, San Diego, CA, USA), T cell immunoglobulin and mucin domain-containing protein-3 (Tim-3) (R&D Systems, Minneapolis, MN, USA) and galectin-9 (Biolegend, San Diego, CA, USA). Of note, we stained peripheral blood, liver and spleen antigen-presenting cells (APC) identified as $CD14^+$ (i.e. monocytes) or $CD11c^+$ (i.e. myeloid dendritic cells) for the PD-1 ligands L1 and L2 and the Tim-3 ligand galectin-9 [19,20]. $CD8^+$ T cells in PBMC, LMC and SMC were arrayed as naive T cells ($CCR7^+CD45RA^+$), central memory T cells ($CCR7^+CD45RA^-$), effector memory T cells ($CCR7^-CD45RA^-$) and terminal differentiated effector memory T cells that re-expressed CD45RA–EMRA ($CCR7^-CD45RA^+$) [21].

$CD8^+$ T cell cytokine staining and tetramers

For intracellular cytokine staining of $CD8^+$ T cells, fresh PBMC, LMC and SMC were cultured *in vitro* for 6 h in plates precoated with anti-CD3 (10 μg/ml; R&D Systems) and anti-CD28 (5 μg/ml; R&D systems) monoclonal antibodies. Cells were washed once with fluorescence activated cell sorter (FACS) buffer and stained with T cell markers at 4°C in the dark for 30 min and then fixed and permeabilized with the Cytofix/Cytoperm Kit (BD Biosciences), washed twice with permeabilization buffer, and stained using anti-IFN-γ (BD Biosciences). Multiparameter flow cytometry was performed using a FACSCaliber Flow Cytometer (BD Biosciences) equipped with FlowJo software (Tree Star, Ashland, OR, USA).

Fluorochrome-labelled HLA-A0201 tetramers for $CD8^+$ T cell staining [Medical and Biological Laboratories (MBL), Nagoya, Japan] included HCV NS3 1073 (CINGVCWTV), NS3 1406 (KLVALGINAV) and NS5B 2594 (ALYDVVSKL), while HLA-A2402 tetramers included HCV E2 717 (EYVLLLFLL), NS3 1292 (TYSTYGKFL) and NS5B 2870 (CYSIEPLDL). After incubation with human Fc receptor blocking reagent (MBL) at room temperature for 5 min, cryopreserved mononuclear cells (1×10^6) were stained for tetramers at 4°C in the dark for 30 min, and stained for CD8, PD-1 and Tim-3.

Statistical analysis

All continuous variables were expressed as mean ± standard deviation (s.d.) and compared between groups by Student's *t*-test. All analyses were two-tailed and *P*-values < 0·05 were considered statistically significant.

Results

Subjects

The characteristics of patients undergoing splenectomy for portal hypertension or during liver transplantation are

Table 1. Clinical characteristics of patients with hepatitis C virus (HCV)-related liver cirrhosis undergoing splenectomy for portal hypertension and thrombocytopenia or during liver transplantation.

	Portal hypertension ($n = 16$)	Liver transplantation ($n = 9$)
Age (years)	61 ± 8·3	60 ± 6·3
Male sex (%)	8 (50%)	4 (44%)
Platelet count (/mm^3)	5000 ± 1900	7300 ± 1900*
ALT (IU/l)	53 ± 30	53 ± 54
Total bilirubin (mg/dl)	1·3 ± 0·4	3·1 ± 2·3*
Albumin (g/dl)	3·5 ± 0·3	2·8 ± 0·7*
Prothrombin activity (%)	74 ± 10	58 ± 16*
Child–Pugh class A–B	16 (100%)	3 (33%)*

Variables are expressed as mean ± standard deviation. *$P < 0·05$. ALT: alanine aminotransferase.

summarized in Table 1. As expected, patients undergoing splenectomy during the course of liver transplantation had signs of more advanced disease represented by higher bilirubin levels, lower prothrombin activity and Child Pugh class C. Conversely, patients undergoing splenectomy for portal hypertension had significantly lower platelet counts.

Exhaustion markers in CD8$^+$ T cells and APC in different organs

CD8$^+$ T cells were positive for exhaustion markers (i.e. expressing both PD-1 and Tim-3) more frequently in the spleen (8·8 ± 5·8%) and the liver (17·2 ± 15·3%) compared to the peripheral blood (3·9 ± 5·0%, $P < 0·01$ *versus* the liver and the spleen) of patients with HCV-related liver cirrhosis (Fig. 1a). Upon stimulation with anti-CD3/CD28, IFN-γ expression was lower in spleen- and liver-derived cells (12·2 ± 6·3 and 10·1 ± 5·8%, respectively) compared to peripheral blood-derived cells (19·6 ± 9·2%; $P < 0·05$, for both comparisons) (Fig. 1a).

The frequency of CD14$^+$ monocytes expressing PD-1 ligands (PD-L1, PD-L2) and Tim-3 ligand (galectin-9) was higher in the spleen (PD-L1; 69·9 ± 14·8%, PD-L2; 71·5 ± 15·1%, galectin-9; 83·4 ± 13·9%) and liver (PD-L1; 87·7 ± 12·3%, PD-L2; 85·8 ± 13·3%, galectin-9; 93·4 ± 5·3%) compared to peripheral blood (PD-L1; 24·7 ± 6·3%, PD-L2; 8·9 ± 7·1%, galectin-9; 54·0 ± 22·1%, $P < 0·01$ for all comparisons) (Fig. 1b). Similar differences were observed in ligand expression on CD11c$^+$ dendritic cells from the spleen (PD-L1; 37·0 ± 15·1%, PD-L2; 43·5 ± 14·8%, galectin-9; 65·4 ± 15·1%), liver (PD-L1; 77·7 ± 14·6%, PD-L2; 74·6 ± 14·8%, galectin-9; 82·7 ± 8·4%) and peripheral blood (PD-L1; 13·5 ± 15·6%, PD-L2; 5·4 ± 4·1%, galectin-9; 34·2 ± 12·6%; $P < 0·01$ for all comparisons) (Fig. 1c).

CD8$^+$ T cell differentiation markers

The frequency of naive T cells in peripheral blood (22·8 ± 15·7%) and spleen (16·9 ± 16·1%) was significantly

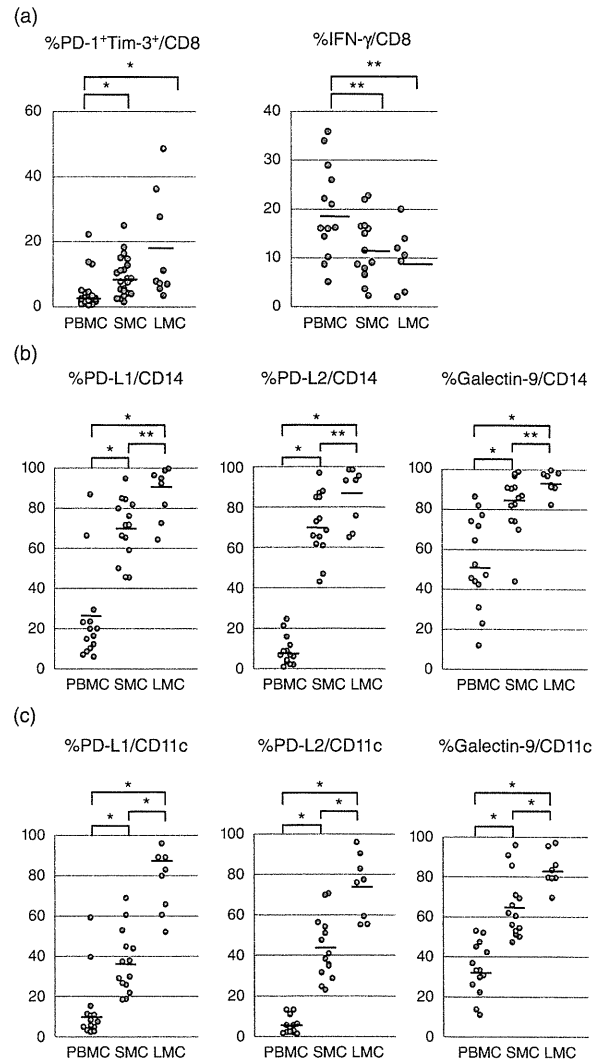

Fig. 1. Phenotype of CD8$^+$ T cells and antigen-presenting cells (APC) in different tissues from patients with hepatitis C virus (HCV)-related cirrhosis. (a) Expression of exhaustion markers programmed death 1 (PD-1) and T cell immunoglobulin and mucin domain-containing protein-3 (Tim-3) on CD8$^+$ T cells from spleen, liver and peripheral blood, and interferon (IFN)-γ production from CD8$^+$ T cells upon CD3 and CD28 stimulation. The frequency of dual PD-1$^+$ and Tim-3$^+$ (i.e. exhausted) T cells in the spleen and the liver are significantly higher compared to the peripheral blood, while the IFN-γ production from CD8$^+$ T cells in the spleen and the liver are decreased. *$P < 0·01$ and **$P < 0·05$. (b) Expression of the PD-L1, PD-L2 and galectin-9 ligands on CD14$^+$ monocytes from different organs. The frequency of PD-L1$^+$, PD-L2$^+$ and galectin-9$^+$ cells in the spleen and the liver is higher compared to the peripheral blood. (c) Expression of the PD-L1, PD-L2 and galectin-9 ligands on CD11c$^+$ dendritic cells from different organs. The frequency of PD-L1$^+$, PD-L2$^+$ and galectin-9$^+$ cells in the spleen and the liver is higher compared to the peripheral blood. *$P < 0·01$ and **$P < 0·05$.

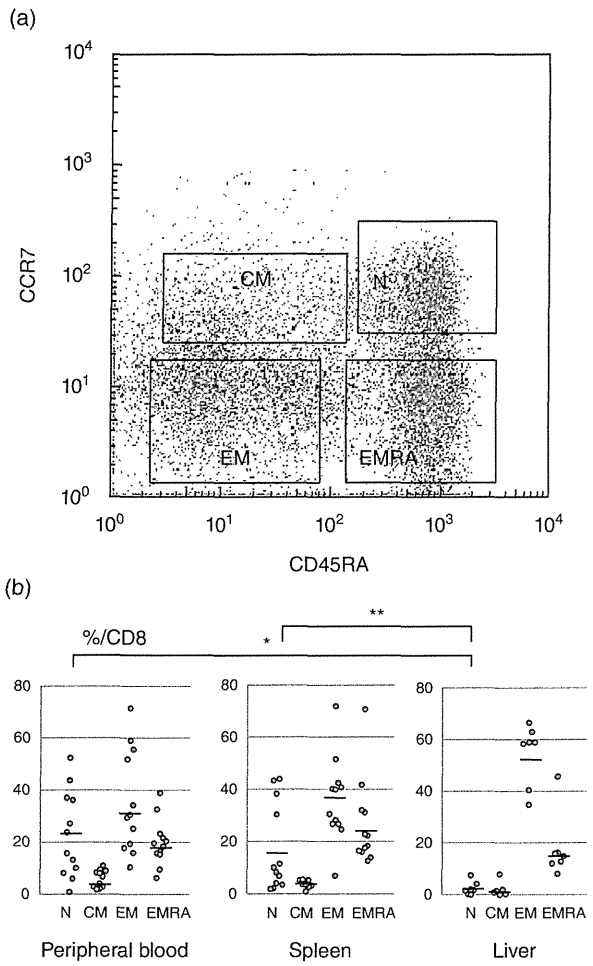

Fig. 2. Differentiation of CD8+ T cells in different organs. (a) CD8+ T cells are classified as naive (N: CCR7+CD45RA+), central memory (CM: CCR7+CD45RA−), effector memory (EM: CCR7− CD45RA−) and terminal effectors with CD45 RA-positive (EMRA: CCR7− CD45RA+). (b) The frequency of naive T cells in the peripheral blood and the spleen are higher in comparison with the liver. $*P < 0.01$ and $**P < 0.05$.

higher compared to the liver (2·4 ± 2·4%; $P < 0.05$ for LMC *versus* SMC, $P < 0.01$ for LMC *versus* PBMC) (Fig. 2b). Exhausted effector memory CD8+ T cells identified by PD-1+Tim-3+ co-expression were represented significantly more in spleen (7·5 ± 7·3%) and liver (10·8 ± 7·9%) compared to peripheral blood (2·7 ± 2·9%; $P < 0.05$ for both comparisons), and the same tendency was observed for central memory cells (liver; 5·8 ± 5·5%, spleen; 2·5 ± 2·5%, peripheral blood; 0·6 ± 0·8%). For both EMRA and naive T cells, the frequency of exhausted cells was similar in the three tissues (Fig. 3).

HCV-specific spleen CD8+ T cells

HCV-specific tetramer positive T cells were represented more significantly in the spleen (0·60 ± 0·15%) compared to the peripheral blood (0·20 ± 0·11%, $P < 0.05$) and the latter tissue also had lower expression of exhaustion markers (82·5 ± 9·5 *versus* 58·3 ± 21·6% in peripheral blood; $P < 0.05$) (Fig. 4).

Effect of splenectomy on CD8+ T cells and APC

Following splenectomy, the frequency of exhausted peripheral blood CD8+ T cells was reduced significantly (2·6 ± 1·5 *versus* 1·7 ± 1·2%; $P < 0.05$), while the IFN-γ production was increased (15·7 ± 7·8 *versus* 20·9 ± 9·3%; $P < 0.05$) (Fig. 5a). Similarly, splenectomy was associated with a reduced expression of PD-L2 (2·5 ± 2·2 *versus* 7·4 ± 5·4% before; $P < 0.05$) and galectin-9 (29·7 ± 21·2 *versus* 60·5 ± 20·1% before; $P < 0.01$) on CD14+ monocytes

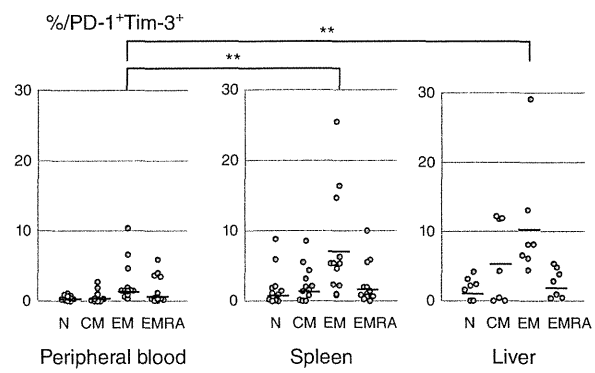

Fig. 3. Exhaustion and differentiation markers in CD8+ T cells from different organs. The frequency of dual programmed death 1 (PD-1)+ and T cell immunoglobulin and mucin domain-containing protein-3 (Tim-3)+ effector memory T cells in the spleen and liver are higher in comparison with the peripheral blood. $**P < 0.05$.

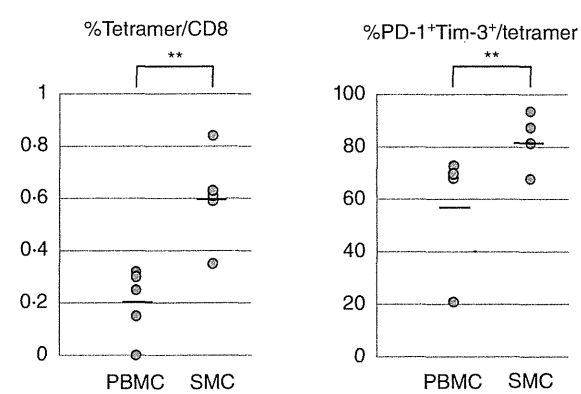

Fig. 4. Hepatitis C virus (HCV)-specific T cells from different organs are studied with human leucocyte antigen (HLA) class I tetramers. (a) HCV-specific CD8+ T cells are enriched in the spleen compared to peripheral blood. (b) Dual programmed death 1 (PD-1)+ and T cell immunoglobulin and mucin domain-containing protein-3 (Tim-3) expression was increased in the spleen compared to peripheral blood. $**P < 0.05$.

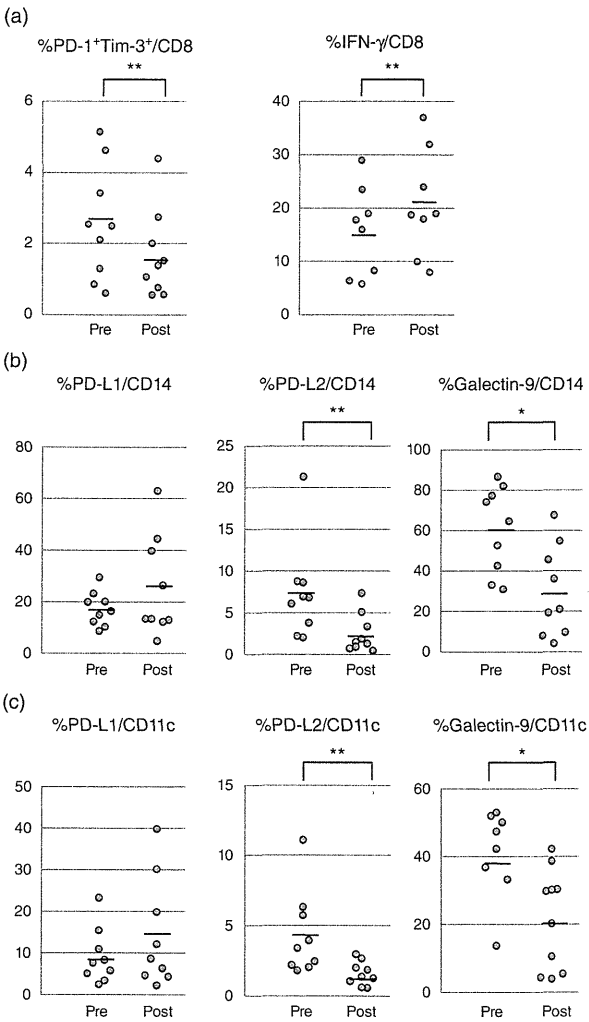

Fig. 5. $CD8^+$ T cell exhaustion and function markers are studied before and after splenectomy. (a) Dual programmed death 1 $(PD\text{-}1)^+$ and T cell immunoglobulin and mucin domain-containing protein-3 (Tim-3) expression is decreased following splenectomy, while interferon (IFN)-γ production is increased compared to pre-splenectomy. (b) The frequency of the ligands PD-L2 and galectin-9-expressing monocytes $(CD14^+)$ is decreased following splenectomy compared to pre-splenectomy. (c) The frequency of PD-L2 and galectin-9 on dendritic cells $(CD11c^+)$ is decreased following splenectomy. $^*P < 0{\cdot}01$ and $^{**}P < 0{\cdot}05$.

(Fig. 5b) and $CD11c^+$ dendritic cells (PD-L2: $1{\cdot}6 \pm 0{\cdot}8$ *versus* $4{\cdot}3 \pm 2{\cdot}8\%$ before; $P < 0{\cdot}05$; galectin-9: $20{\cdot}7 \pm 14{\cdot}4$ *versus* $39{\cdot}9 \pm 12{\cdot}2\%$ before; $P < 0{\cdot}01$) (Fig. 5c).

Discussion

The factors impairing HCV clearance and allowing chronic infection remain largely enigmatic, despite enormous research efforts to dissect potential therapeutic targets. Indeed, virus-mediated T cell exhaustion limits T cell function, thus promoting chronic disease, and we report for the first time that spleen effector memory T cells manifest significant exhaustion while spleen APC over-express inhibitory receptor ligands when compared to peripheral blood in patients with HCV-related cirrhosis. Further, splenectomy leads to a reduction of exhaustion markers and an increase of IFN-γ production along with a reduced APC expression of inhibitory receptor ligands.

There is a relative paucity of data on the issue of T cell exhaustion, and the majority of studies focus on patients with viral infections. There are significant data on the clinical significance of $CD8^+$ cells and their subsets, including the related issue of epitope spreading [22–30]. T cell exhaustion has been characterized as over-expression of several inhibitory receptors, including PD-1 [2,31] and Tim-3 [11,12]. The expression of both Tim-3 and PD-1 on $CD8^+$ T cells is thus the established marker for exhaustion and may contribute to the perpetuation of HCV infection [13]. During HCV chronic hepatitis, splenomegaly occurs following portal hypertension [7], and splenectomy may reduce portal hypertension and increase the number of white blood cells and platelets [14,32], along with an established risk of overwhelming post-splenectomy infections (OPSI) by encapsulated bacteria such as *Streptococcus pneumonia* [15]. Most recently, new therapeutic approaches limited the need for surgery in these cases, as represented by the use of eltrombopag before anti-viral induction [33], and make the present study design unlikely to be recapitulated in the future. We performed a detailed investigation of T cell phenotypes in the spleen, liver and peripheral blood of patients with HCV-related cirrhosis and portal hypertension, and clarified that spleen T cell phenotypes are not so different despite the observation that peripheral naive T cells are decreased and peripheral effector memory T cells are increased when compared to healthy subjects [34].

The surgical removal of lymphoid compartments, as in the case of tonsillectomy, improves autoimmune disease based on T cell changes [35], but it remains to be determined whether lymphoid compartments regulate T cell exhaustion, and only a few studies have investigated spleen $CD8^+$ T cells in HCV-related liver cirrhosis. In HCV chronic infection, exhaustion markers are expressed highly in liver HCV-specific $CD8^+$ T cells [2] and we have reported previously that spleen $CD4^+$ T cells become exhausted and functionally impaired [17]. HCV-specific T cells within the liver over-express PD-1 [2] and we report herein a similar observation in the spleen. Further, effector and memory T cells heterogeneity includes separate models of precursors, decreasing potential, signal strength and asymmetric cell fate [36], but we could not identify differences in exhaustion markers. Indeed, we report that PD-1 and Tim-3 double-positive naive T cells are found in the spleen and liver but not in peripheral blood, thus suggesting that this specific homing may contribute to chronic infection establishment. Indeed, HCV antigens are over-expressed in liver,

and thus our data that exhausted T cells are expressed more frequently in liver and spleen, compared to peripheral blood, is consistent with a local immune response. Future studies should focus on whether such CD8+ T cells are viral specific.

We should also note that we studied APC ligands interacting with exhaustion markers on T cells to regulate the T cell response [19,20], as represented by the effects of both PD-1 and Tim-3 ligands. Myeloid dendritic (CD11c+) cells in the peripheral blood from patients with chronic HCV infection over-express the PD-1 ligands (PD-L1, PD-L2) and induce the proliferation of regulatory T cells [37,38], while the Tim-3 ligand galectin-9 is well represented in the serum and liver (particularly Kupffer cells) during chronic HCV infection [39]. We report that both myeloid dendritic cells and monocytes in the spleen express all three ligands significantly more compared to peripheral blood and hypothesize that this may contribute to CD8+ T cell dysfunction in the spleen. We are particularly intrigued by the possibility that antibodies against PD-1 and Tim-3 ligands may restore the *in-vitro* cytotoxicity of virus antigen-specific T cells [2,13], thus counteracting exhaustion, but the anti-viral efficacy of this approach remains inconclusive [40]. In a similar fashion, transcription factors such as T-bet or eomesdermin (Eomes) [41] may control T cell exhaustion, and the resulting poor effector function and gene therapy may target this pathway [42,43] or the cytokine signalling 3 suppressor (SOCS3) through interleukin (IL)-7 [44].

Lastly, we investigated whether or not T cell exhaustion could be modified by splenectomy performed to allow antiviral treatment by increasing the platelet count [14]. Of note, T cell function improves following splenectomy, as represented by the decrease in CD8+ T cell exhaustion markers and APC PD-1 and Tim-3 ligands in peripheral blood and we speculate that splenectomy may reduce the efflux of exhaustion ligands to T cells. In conclusion, this is the first study aimed at identifying markers of T cell exhaustion in the spleen of patients with HCV-related cirrhosis and portal hypertension and our data suggest cumulatively that the spleen may act as a rheostat for modulating this phenomenon impairing T cell functions. Based on the consistent lines of evidence reported, we suggest that this pathway constitutes an optimal therapeutic target in chronic HCV infection, particularly at advanced stages.

Disclosure

None.

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Anticholestatic Effects of Bezafibrate in Patients with Primary Biliary Cirrhosis Treated with Ursodeoxycholic Acid

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Bezafibrate is a widely used hypolipidemic agent and is known as a ligand of the peroxisome proliferator-activated receptors (PPARs). Recently this agent has come to be recognized as a potential anticholestatic medicine for the treatment of primary biliary cirrhosis (PBC) that does not respond sufficiently to ursodeoxycholic acid (UDCA) monotherapy. The aim of this study was to explore the anticholestatic mechanisms of bezafibrate by analyzing serum lipid biomarkers in PBC patients and by cell-based enzymatic and gene expression assays. Nineteen patients with early-stage PBC and an incomplete biochemical response to UDCA (600 mg/day) monotherapy were treated with the same dose of UDCA plus bezafibrate (400 mg/day) for 3 months. In addition to the significant improvement of serum biliary enzymes, immunoglobulin M (IgM), cholesterol, and triglyceride concentrations in patients treated with bezafibrate, reduction of 7α-hydroxy-4-cholesten-3-one (C4), a marker of bile acid synthesis, and increase of 4β-hydroxycholesterol, a marker of CYP3A4/5 activity, were observed. *In vitro* experiments using human hepatoma cell lines demonstrated that bezafibrate controlled the target genes of PPARα, as well as those of the pregnane X receptor (PXR); down-regulating CYP7A1, CYP27A1, and sinusoidal Na^+/taurocholate cotransporting polypeptide (NTCP), and up-regulating CYP3A4, canalicular multidrug resistance protein 3 (MDR3), MDR1, and multidrug resistance-associated protein 2 (MRP2). *Conclusion*: Bezafibrate is a dual PPARs/PXR agonist with potent anticholestatic efficacy in early-stage PBC patients with an incomplete biochemical response to UDCA monotherapy. (HEPATOLOGY 2013;57:1931-1941)

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Primary biliary cirrhosis (PBC) is a chronic liver disease that is presumably caused by autoimmunity. The detection of serum antimitochondrial antibodies (AMA) and increased levels of immunoglobulin M (IgM) are biochemical features of this disease. Histopathologically, it is characterized by portal inflammation and the slow progressive destruction of the portal interlobular bile ducts due to chronic nonsuppurative cholangitis. The loss of bile ducts leads to cholestasis, which leads to further hepatic damage, fibrosis, cirrhosis, and ultimately, liver failure.[1]

Ursodeoxycholic acid (UDCA) is the only Food and Drug Administration (FDA)-approved drug and the first-line medicine for the treatment of PBC.[2] UDCA has been shown to improve serum levels of biliary enzymes and IgM, and may slow the histologic progression to liver cirrhosis.[3-6] The mechanisms of the

Abbreviations: ABC, ATP-binding cassette transporter; BSEP, bile salt export pump; C4, 7α-hydroxy-4-cholesten-3-one; CA, cholic acid; CAR, constitutive androstane receptor; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; FGF, fibroblast growth factor; FXR, farnesoid X receptor; 4β-HC, 4β-hydroxycholesterol; 24S-HC, 24S-hydroxycholesterol; 27-HC, 27-hydroxycholesterol; HMGCR, HMG-CoA reductase; HNF4α, hepatocyte nuclear factor 4α; LCA, lithocholic acid; LXRα, liver X receptor α; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; NF-κB, nuclear factor-κB; NTCP, Na^+/taurocholate cotransporting polypeptide; PBC, primary biliary cirrhosis; PPAR, peroxisome proliferator-activated receptor; PXR, pregnane X receptor; PGC1α, peroxisome proliferator-activated receptor-γ coactivator-1α; UDCA, ursodeoxycholic acid.

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anticholestatic and antiinflammatory effects of UDCA have been reported to be due to the activation of the canalicular bile salt export pump (BSEP), canalicular multidrug resistance protein 3 (MDR3; ATP-binding cassette transporter B4 [ABCB4]) and basolateral multidrug resistance-associated protein 4 (MRP4 [ABCC4]).[7] In addition, the replacement of hydrophobic bile acids with hydrophilic UDCA appears to attenuate the damage to hepatocytes and biliary cells.[2] It has been reported that about two-thirds of patients treated with UDCA in the early stage of the disease could have a normal life expectancy without additional therapies.[8] However, the remaining patients are not sufficiently controlled with UDCA monotherapy and additional therapeutic approaches have been necessary.

Immunosuppressive medication is not recommended as the first-line, alternative drug for PBC, but budesonide, a nonhalogenated glucocorticoid with a high first-pass metabolism, and/or mycophenolate mofetil, an inhibitor of the purine biosynthetic pathway which is critical to lymphocytic proliferation and activation, are sometimes used in patients who fail to respond to UDCA.[9,10] However, the effects of these immunosuppressive agents remain controversial.[11,12] The farnesoid X receptor (FXR; NR1H4) agonist, 6-ethyl-chenodeoxycholic acid, has been administered to PBC patients that exhibit incomplete responses to UDCA in a phase II clinical trial. This trial exhibited anticholestatic effects and serum alkaline phosphatase (ALP) levels were reduced, but pruritus occurs at the higher doses.[13]

In 1999, Iwasaki et al.[14] introduced the effectiveness of a hypolipidemic agent, bezafibrate, on the reduction of serum ALP and IgM levels in precirrhosis PBC patients, and recently, combination therapy with UDCA and bezafibrate is being recognized as a beneficial treatment for PBC that is refractory to UDCA monotherapy.[15,16] Although the mechanisms of anticholestatic action by bezafibrate have not been elucidated completely, it is believed that the induction of MDR3 through activation of the peroxisome proliferator-activated receptor α (PPARα; NR1C1)[17] is the main mechanism, because fibrate class agents are ligands of the PPARs.[18] However, because MDR3 is activated by both the addition of bezafibrate as well as by UDCA monotherapy,[7] the roles of bezafibrate in the combination therapy remain unknown.

The current study was undertaken to explore the mechanisms of the remission of cholestasis by bezafibrate in PBC patients who failed to respond to UDCA monotherapy. Our *in vivo* and *in vitro* studies demonstrated that bezafibrate was a dual PPARs/pregnane X receptor (PXR; NR1I2) agonist with potent anticholestatic efficacy.

Patients and Methods

Patients. Thirty-one Japanese patients with asymptomatic and untreated PBC (4 males and 27 females; ages 37-81 years) were enrolled in the study. The diagnosis of PBC was established by laboratory and histological findings, and all patients were classified as early-stage PBC (Scheuer's classification I or II). Informed consent was obtained from all subjects and the study protocol was approved by the Ethics Committee of Tokyo Medical University Ibaraki Medical Center.

Study Design. All patients (n = 31) were treated with UDCA (600 mg/day; 10-13 mg/kg/day) alone for at least 3 months (maximum 6 months) until serum ALP and gamma glutamyl transpeptidase (GGT) became stable (Supporting Figure). Then bezafibrate (400 mg/day) was administered with UDCA (600 mg/day) to patients (n = 19; 1 male and 18 females) who exhibited an incomplete biochemical response to UDCA monotherapy (defined as ALP or GGT level of above the upper limit of normal) and treated for 3 months. Before and after UDCA monotherapy and after the addition of bezafibrate, blood samples were collected in the morning before breakfast after an overnight fasting, and serum was stored at −20°C until analyzed. Control sera from 49 healthy Japanese volunteers (11 males and 38 females; ages 22-79 years) were obtained from another study group (courtesy of Prof. T. Teramoto, Teikyo University School of Medicine, Tokyo, Japan) and were stored as mentioned above.

Determination of Serum Markers for Cholesterol and Bile Acid Metabolism. Serum sterol concentrations were determined by liquid chromatography, tandem mass spectrometry (LC-MS/MS) as described.[19] Serum fibroblast growth factor 19 (FGF19) levels were measured using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quantikine Human

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Additional Supporting Information may be found in the online version of this article.

FGF-19 Immunoassay, R&D Systems, Minneapolis, MN). Serum bile acid profiles were determined by LC-MS/MS according to the method of Ando et al.[20]

Cell Culture. The human hepatoma cell line, HepaRG, was obtained from Biopredic International (Rennes, France). On day 0 a 24-well plate was seeded with 4.8×10^5 differentiated HepaRG cells/well using HepaRG Thawing and Seeding Medium 670. On day 3 the medium was replaced with 500 μL/well of HepaRG Induction Medium 640 containing bezafibrate, rifampicin, carbamazepine, or GW4064 dissolved in 1% acetonitrile. Cells were incubated for 48 hours at 37°C in a humidified incubator containing 5% CO_2 and 95% air.

Assays of CYP3A4 Activity and PXR Activation. - CYP3A4 activities were measured by cell-based P450-Glo CYP3A4 Assay Kit (Luciferin-IPA) purchased from Promega (Madison, WI). The activation of PXR was determined by a Human PXR Activation Assay System (Puracyp, Carlsbad, CA) utilizing DPX2 hepatoma cells harboring the human PXR and luciferase-linked CYP3A4 promoters.

RNA Measurements. Total RNA was extracted from the HepaRG cells using an RNeasy Plus Mini Kit (Qiagen, Tokyo, Japan). Reverse transcription and real-time quantitative polymerase chain reaction (PCR) were performed as described.[21] The sequences of some primer pairs have been described in the same report.[21] The other primer sequences used in this study are listed in the Supporting Table.

Statistics. Data are reported as the mean ± SEM for human data and as the mean ± SD for cell data. The statistical significance of differences between the results in the different groups was evaluated by non-parametric Mann-Whitney test for human data (Tables 1, 2) and Student's two-tailed t test for cell data (Figs. 4, 5). On the other hand, the data obtained before and after treatment were compared by Wilcoxon signed-ranks test (Figs. 1-3). In all statistical tests significance was accepted at the level of $P < 0.05$.

Results

The characteristics of the PBC patients enrolled in the present study are shown in Table 1. In patients before UDCA treatment (n = 31) and those who responded to UDCA insufficiently and before additional bezafibrate treatment (n = 19), serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), GGT, ALP, and IgM levels were significantly elevated compared with healthy controls. Serum low-density lipoprotein (LDL) cholesterol and triglyceride concentrations were increased and HDL cholesterol concentration was decreased significantly in the patients before UDCA treatment compared with controls. In the patients before additional bezafibrate treatment a similar tendency was observed, but the differences were not statistically significant.

Baseline biomarker levels for lipid metabolism in the three groups are compared in Table 2. In this study cholesterol metabolism in PBC patients was assayed by measuring serum sterol biomarkers. Because most non-cholesterol sterols are transported in serum with cholesterol, the expression of each sterol level relative to the total cholesterol concentration tends to be more reliable compared with the absolute concentration, especially when dyslipidemia is present.[22] Serum concentrations

Table 1. Characteristics of Patients with PBC Enrolled in the Present Study

Laboratory Data	Control (n=49)	Before UDCA Treatment (n=31)	Before BF Treatment (n=19)
Age (yrs)	57.8±1.6 [22-79]	60.3±1.8 [37-81]	58.8±1.6 [45-73]
Gender (Male/Female)	11/38	4/27	1/18
AST (IU/L)	21±1 [11-34]	64±18‡ [19-120]	45±5‡ [20-101]
ALT (IU/L)	17±1 [7-30]	82±34‡ [12-138]	51±9‡ [18-152]
GGT (IU/L)	25±2 [7-58]	196±27‡ [30-757]	178±59‡ [47-445]
ALP (IU/L)	230±9 [126-336]	517±43‡ [229-1163]	597±51‡ [266-952]
Total bilirubin (mg/dL)	0.7±0.1 [0.3-1.2]	0.7±0.2 [0.3-1.3]	0.6±0.1 [0.3-1.1]
IgM (mg/dL)	97±12 [56-161]	288±27‡ [90-637]	306±60† [130-466]
Total cholesterol (mg/dL)	199±4 [130-257]	213±9 [120-356]	228±18 [118-343]
LDL cholesterol (mg/dL)	115±4 [46-194]	138±7* [91-254]	149±18 [54-228]
HDL cholesterol (mg/dL)	65±2 [33-111]	53±4* [13-95]	55±5 [13-89]
Triglycerides (mg/dL)	91±6 [33-214]	107±7* [47-199]	113±11 [40-243]

Data are expressed as mean ± SEM [range].

Before UDCA treatment, all PBC patients before treatment with UDCA; Before BF treatment, PBC patients who exhibited an incomplete biochemical response to the UDCA monotherapy (600 mg/day) and before additional treatment with bezafibrate.

*$P < 0.05$, significantly different from control.

†$P < 0.005$, significantly different from control.

‡$P < 0.0001$, significantly different from control.

Table 2. Baseline Biomarker Levels for Cholesterol Metabolism in Enrolled Patients with PBC

Serum Biomarkers	Control (n=49)	Before UDCA Treatment (n=31)	Before BF Treatment (n=19)
Bile acid metabolism			
C4 (ng/mg CHOL)	15.7±2.9 [2.3-118]	12.1±1.8 [0.8-49]	11.8±2.1 [1.5-38]
FGF19 (pg/ml)	336±51 [50-1662]	309±49 [74-1543]	353±57 [114-930]
Cholesterol metabolism			
Lathosterol (μg/mg CHOL)	2.8±0.3 [0.9-11.7]	2.2±0.2 [0.7-5.8]	2.2±0.3 [0.8-6.1]
Sitosterol (μg/mg CHOL)	1.6±0.1 [0.4-3.8]	2.0±0.2* [0.8-3.9]	2.4±0.2† [1.1-4.3]
Campesterol (μg/mg CHOL)	1.8±0.1 [0.4-5.1]	2.0±0.1 [0.7-3.7]	1.9±0.2 [0.7-3.3]
Oxysterol metabolism			
4β-HC (ng/mg CHOL)	29±3 [11-135]	44±4‡ [24-140]	51±5‡ [20-92]
24S-HC (ng/mg CHOL)	31±2 [17-74]	34±2 [22-69]	41±2†[20-64]
27-HC (ng/mg CHOL)	77±3 [35-140]	75±4 [48-124]	75±4 [39-102]

Data are expressed as mean ± SEM [range].

Before UDCA treatment, all PBC patients before treatment with UDCA; Before BF treatment, PBC patients who exhibited an incomplete biochemical response to the UDCA monotherapy (600 mg/day) and before additional treatment with bezafibrate; C4, 7α-hydroxy-4-cholesten-3-one; CHOL, cholesterol; FGF19, fibroblast growth factor 19; 4β-HC, 4β-hydroxycholesterol; 24S-HC, 24S-hydroxycholesterol; 27-HC, 27-hydroxycholesterol.

*$P < 0.05$, significantly different from control.

†$P < 0.005$, significantly different from control.

‡$P < 0.0001$, significantly different from control.

of sitosterol, 4β-hydroxycholesterol (4β-HC), and 24S-hydroxycholesterol (24S-HC) expressed relative to total cholesterol were significantly elevated in both patient groups compared with controls. However, other sterols, 7α-hydroxy-4-cholesten-3-one (C4), lathosterol, campesterol, and 27-hydroxycholesterol (27-HC), and FGF19 concentrations did not differ significantly among the three groups.

Effects of UDCA and Bezafibrate on Serum Liver Enzymes and Lipids. As shown in Fig. 1A, serum AST, ALT, GGT, ALP, and IgM levels were all reduced significantly by treatment with UDCA. In patients

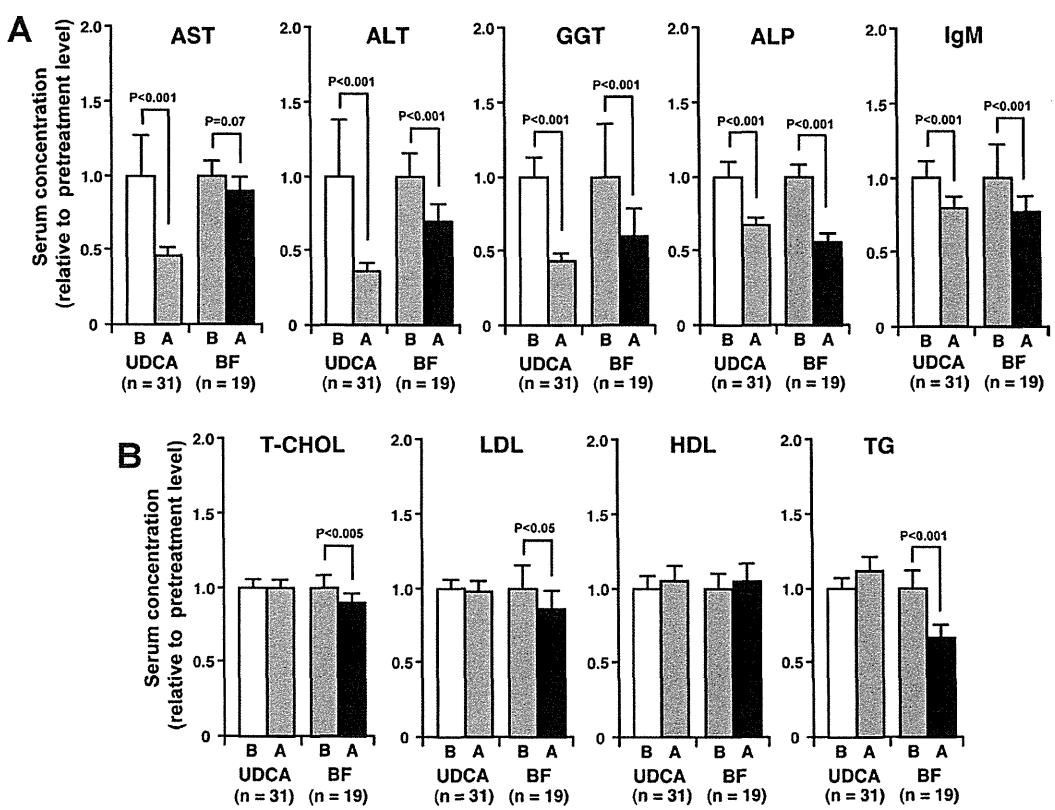

Fig. 1. Effects of UDCA and additional bezafibrate treatment on serum liver enzymes (A) and lipids (B). B, before treatment; A, after treatment; BF, bezafibrate; T-CHOL, total cholesterol; LDL, LDL cholesterol; HDL, HDL cholesterol; TG, triglyceride. The mean concentrations before treatment were set to 1.0, and the absolute concentrations before treatment are shown in Table 1. Data are expressed as the mean ± SEM.

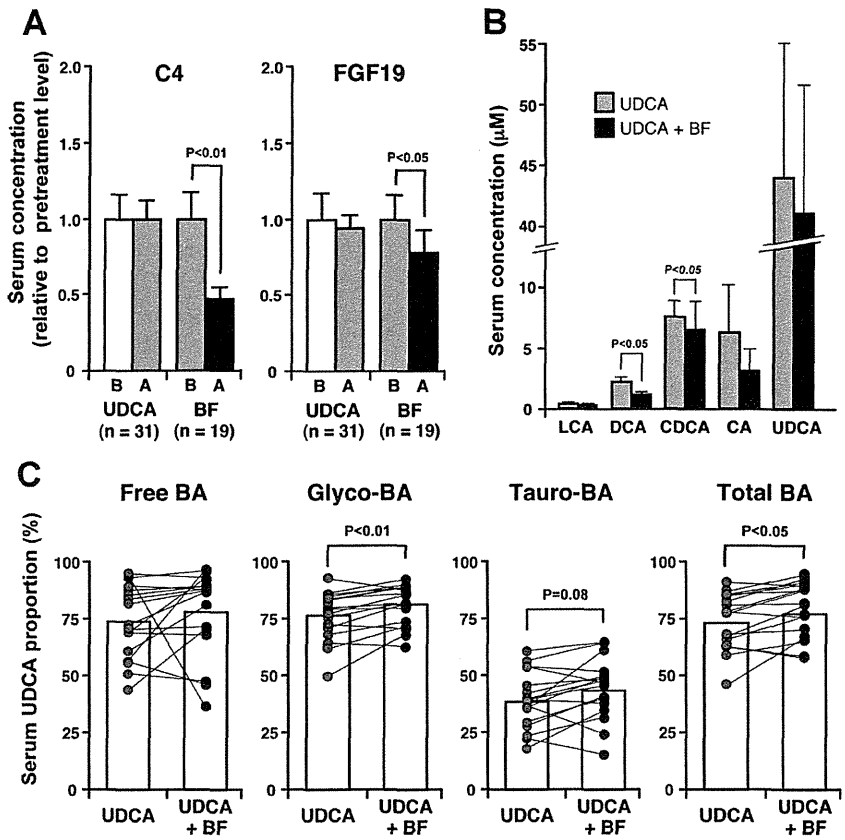

Fig. 2. Effects of UDCA and additional bezafibrate treatment on bile acid metabolism. (A) C4, 7α-hydroxy-4-cholesten-3-one; FGF19, fibroblast growth factor 19; B, before treatment; A, after treatment; BF, bezafibrate. Mean concentrations before treatment (ng/mg cholesterol for C4 and pg/ml for FGF19) were set to 1.0, and the absolute concentrations before treatment are shown in Table 2. Data are expressed as the mean ± SEM. (B) Serum concentrations of bile acids in UDCA-treated patients before and after addition of bezafibrate (n = 17). (C) Serum proportions of UDCA in UDCA-treated patients before and after addition of bezafibrate (n = 17). The mean value for each group is indicated by the columns. Free BA, unconjugated bile acids; Glyco-BA, glycine-conjugated bile acids; Tauro-BA, taurine-conjugated bile acids.

who responded incompletely to UDCA monotherapy, the combination of bezafibrate and UDCA further reduced serum levels of ALT, GGT, ALP, and IgM. The changes in serum lipid concentrations by UDCA and bezafibrate treatment are presented in Fig. 1B. UDCA monotherapy did not change the serum lipid levels significantly. However, the addition of bezafibrate significantly decreased serum concentrations of total cholesterol, LDL cholesterol, and triglyceride in those patients whose cholestasis was not sufficiently improved by UDCA alone.

Effects of UDCA and Bezafibrate on Bile Acid Metabolism. C4 and FGF19 are markers of bile acid production[23] and transintestinal flux,[24] respectively. As shown in Fig. 2A, UDCA did not change C4 or FGF19 concentrations, but bezafibrate significantly reduced both C4 and FGF19 levels. In Fig. 2B,C, serum bile acid concentrations and UDCA proportion in UDCA-treated patients before and after addition of bezafibrate are shown. The addition of bezafibrate significantly reduced the serum chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) concentrations. The serum cholic acid (CA) and lithocholic acid (LCA) concentrations also tended to be reduced by bezafibrate, but the differences were not statistically significant. The serum proportion of UDCA was significantly increased by the addition of bezafibrate compared with UDCA monotherapy, presumably due to its inhibitory effect on *de novo* bile acid biosynthesis. The proportion of UDCA in serum is usually higher than that in bile in patients treated with UDCA, but it appears to reflect the biliary proportion of UDCA to some extent.[25]

Effects of UDCA and Bezafibrate on Sterol Metabolism. Cholesterol biosynthesis and intestinal absorption were studied by measuring serum concentrations of lathosterol and plant sterols (sitosterol and campesterol), respectively. As shown in Fig. 3A, UDCA treatment did not affect cholesterol biosynthesis but significantly increased cholesterol absorption. In contrast, bezafibrate significantly inhibited cholesterol biosynthesis but did not change cholesterol absorption.

Serum concentrations of major oxysterols that are potential ligands of liver X receptor α (LXRα, NR1H3) were compared between UDCA and bezafibrate treatments (Fig. 3B). UDCA treatment did not affect serum 4β-HC or 24S-HC concentrations but increased the 27-HC concentration significantly. Treatment with bezafibrate clearly increased serum 4β-HC

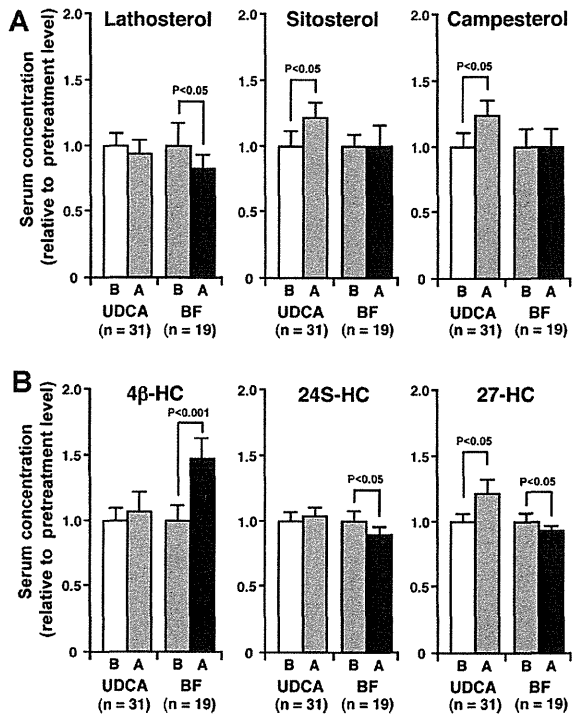

Fig. 3. Effect of UDCA and additional bezafibrate treatment on cholesterol (A) and oxysterol (B) metabolism. B, before treatment; A, after treatment; BF, bezafibrate; 4β-HC, 4β-hydroxycholesterol; 24S-HC, 24S-hydroxycholesterol; 27-HC, 27-hydroxycholesterol. Mean concentrations before treatment (μg/mg cholesterol or ng/mg cholesterol) were set to 1.0, and the absolute concentrations before treatment are shown in Table 2. Data are expressed as the mean ± SEM.

levels, whereas it significantly reduced the 24S-HC and 27-HC levels.

Effects of Bezafibrate on CYP3A4. Differentiated HepaRG cells exhibit a gene expression pattern similar to primary human hepatocytes and human liver tissues and maintain significant levels of hepatic cell functions, including CYP and transporter activities.[26] Rifampicin and carbamazepine are classical inducers of CYP3A4 by way of the activation of PXR,[27] whereas GW4064 is one of the most potent agonists of FXR.[28] As shown in Fig. 4A, bezafibrate, as well as rifampicin and carbamazepine, induced both CYP3A4 mRNA expression and activity in a dose-dependent manner.

Effects of Bezafibrate on PXR Activation. The DPX2 cell-based luciferase reporter gene assay demonstrated that in comparison with rifampicin, bezafibrate was a weak but significant activator of human PXR as well as carbamazepine (Fig. 4B). It is noteworthy that GW4064 activated human PXR at concentrations higher than 3 μM.

Effects of Bezafibrate on Gene Expression of Nuclear Receptors, Transporters, and Enzymes. Among the nuclear receptors and related coactivators (Fig. 5A), PXR expression was induced by bezafibrate to a greater degree than that by rifampicin, which suggests that PXR is a target gene of PPARs, as reported.[29] In contrast, the small heterodimer partner (SHP; NR0B2), a

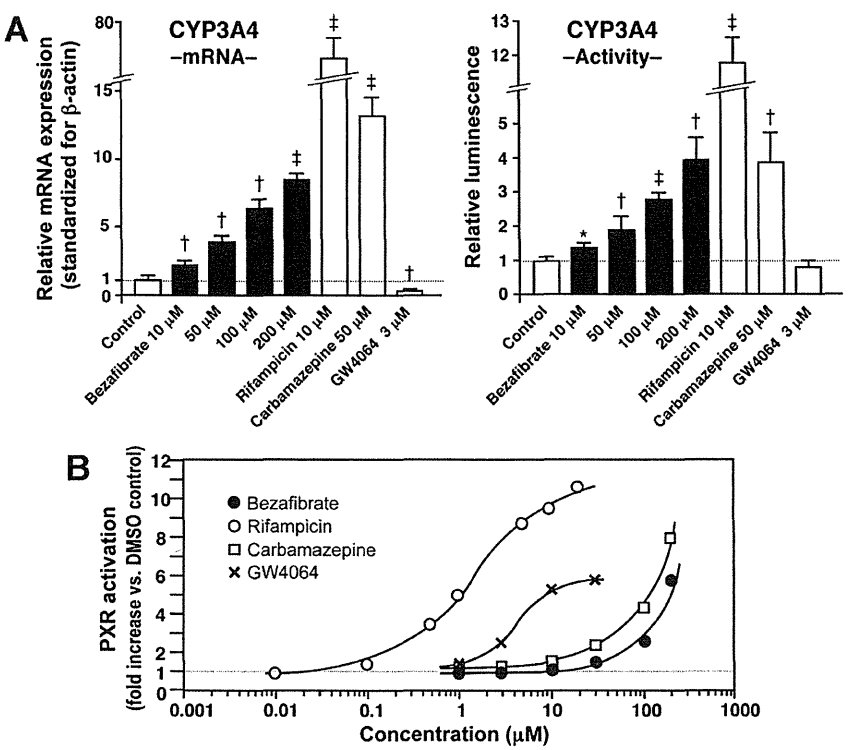

Fig. 4. Effects of bezafibrate, rifampicin, carbamazepine, and GW4064 on the activation of CYP3A4 and human PXR. (A) HepaRG cells were treated with each compound for 48 hours in triplicate. mRNA expression levels were standardized to those of β-actin. The mean expression and activity levels in control cells were set to 1.0. Data are expressed as the mean ± SD. Effects of bezafibrate are shown as solid bars. $*P < 0.05$, $\dagger P < 0.005$, $\ddagger P < 0.001$, significant difference from controls. (B) DPX2 cells were treated with each compound for 24 hours in triplicate. Activation of human PXR was determined by a cell-based luciferase reporter gene assay. The average relative luminescent units (RLU) obtained with the dimethyl sulfoxide (DMSO) solvent control was set to 1.0.

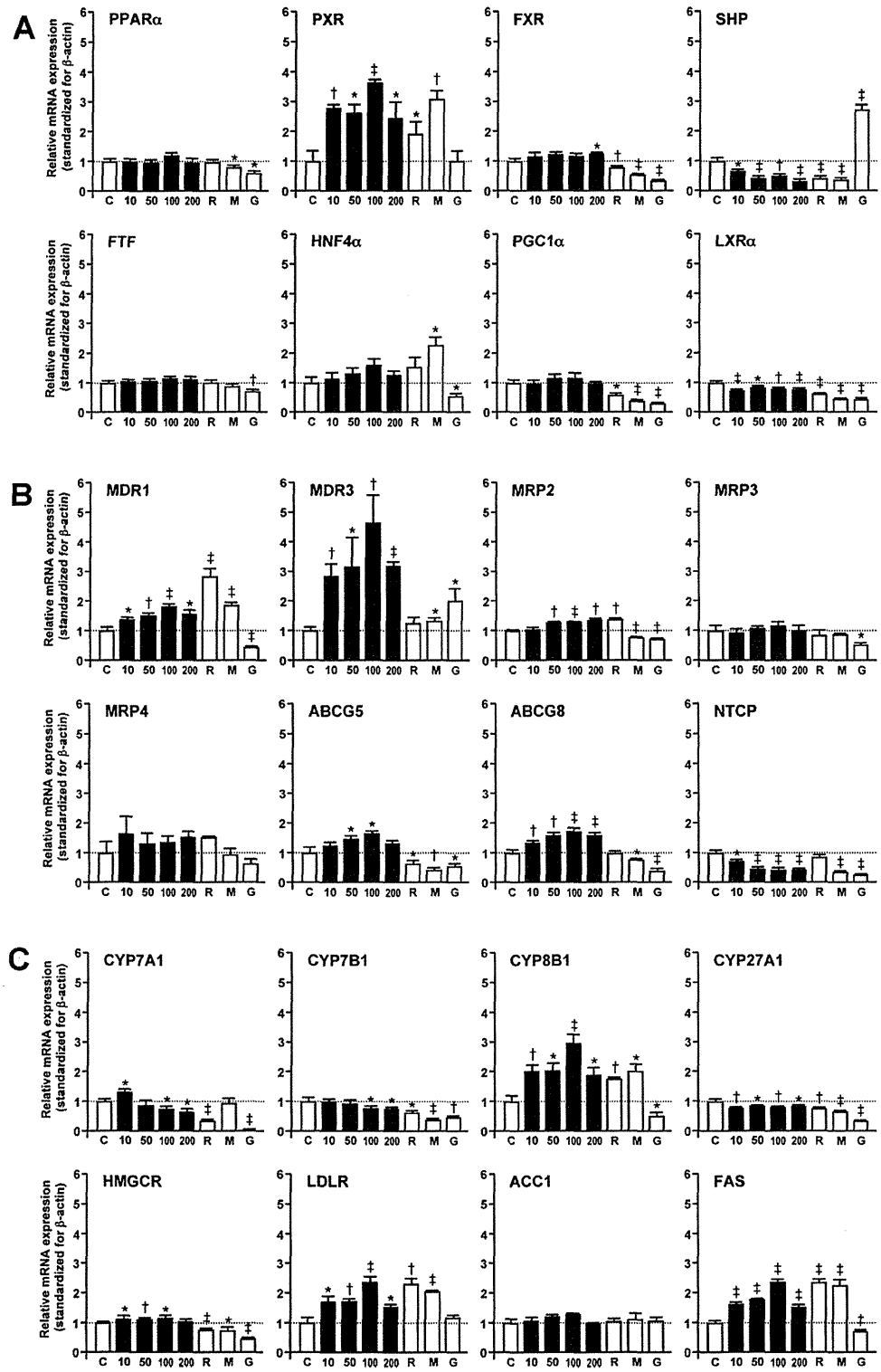

Fig. 5. Effects of bezafibrate, rifampicin, carbamazepine, and GW4064 on mRNA expression levels of nuclear receptors and a related coactivator (A), transporters (B), and enzymes and LDL receptor (C) in HepaRG cells. The cells were treated with each compound for 48 hours, in triplicate. mRNA expression levels were standardized to those of β-actin. The mean expression and activity levels in control cells were set to 1.0. Data are expressed as the mean $\pm$ SD. The effects of bezafibrate are shown as the solid bars. C, control; 10, bezafibrate 10 μM; 50, bezafibrate 50 μM; 100, bezafibrate 100 μM; 200, bezafibrate 200 μM; R, rifampicin 10 μM; M, carbamazepine 50 μM; G, GW4064 3 μM. PPARα, peroxisome proliferator-activated receptor α; PXR, pregnane X receptor; FXR, farnesoid X receptor; SHP, small heterodimer partner; FTF, α-fetoprotein transcription factor; HNF4α, hepatocyte nuclear factor 4α; PGC1α, peroxisome proliferator-activated receptor γ coactivator 1α; LXRα, liver X receptor α; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; ABC, ATP-binding cassette transporter; NTCP, Na^+/taurocholate-cotransporting polypeptide; CYP7A1, cholesterol 7α-hydroxylase; CYP7B1, oxysterol 7α-hydroxylase; CYP8B1, 7α-hydroxy-4-cholesten-3-one 12α-hydroxylase; CYP27A1, sterol 27-hydroxylase; HMGCR, HMG-CoA reductase; LDLR, LDL receptor; ACC1, acetyl-CoA carboxylase 1; FAS, fatty acid synthase. *$P < 0.05$, †$P < 0.005$, ‡$P < 0.001$, significant difference from control.

target of FXR, and LXRα were down-regulated by bezafibrate, as well as rifampicin and carbamazepine. FXR and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α) expressions were significantly down-regulated by rifampicin and carbamazepine but not by bezafibrate.

The MDR1 (ABCB1) and MRP2 (ABCC2) transporters (Fig. 5B) were up-regulated by bezafibrate, similar to rifampicin, whereas MDR3, ABCG5, and ABCG8 were up-regulated by bezafibrate but not by rifampicin. In addition, Na^+/taurocholate cotransporting polypeptide (NTCP) was down-regulated by bezafibrate but did not change significantly by rifampicin. It is notable that significant messenger RNA (mRNA) expression of BSEP was observed in HepaRG cells treated with GW4064, whereas only a trace amount of BSEP expression was detected in control cells and those treated with other compounds.

Enzymes involved in cholesterol, bile acid, and fatty acid syntheses and LDL receptor expression are summarized in Fig. 5C. CYP7A1, CYP7B1, and CYP27A1 were down-regulated and CYP8B1, fatty acid synthase (FAS), and LDL receptor (LDLR) were up-regulated by bezafibrate, which was the same as the effects of rifampicin. HMG-CoA reductase (HMGCR), the rate-limiting enzyme in the cholesterol biosynthetic pathway, was down-regulated by rifampicin but was slightly up-regulated by bezafibrate.

Discussion

Our results clearly showed that the combination therapy of bezafibrate and UDCA significantly improved cholestasis in early-stage PBC patients who were refractory to UDCA monotherapy. The mean levels of ALP and GGT during UDCA monotherapy were further reduced from 597 ± 51 to 324 ± 27 IU/L and 178 ± 59 to 99 ± 41 IU/L, respectively, by the additional administration of bezafibrate (Fig. 1). It is known that UDCA not only improves cholestasis but also serum IgM concentrations.[4,6] The combination therapy of bezafibrate and UDCA further reduced the IgM concentration from 306 ± 60 (UDCA alone) to 232 ± 41 mg/dL (UDCA + bezafibrate), consistent with the findings reported by Iwasaki et al.[16] Furthermore, our results showed that the combination therapy significantly reduced serum total cholesterol, LDL cholesterol, and triglyceride concentrations compared with UDCA alone.

The mechanisms of the anticholestatic effect of bezafibrate remain unclear. Because MDR3 is a target gene of PPARα[17] and bezafibrate is a ligand of PPARα, β/δ, and γ,[18] stimulation of biliary phospholipid secretion due to the up-regulation of MDR3 has generally been believed to be the main mechanism of the action. In fact, our experiment using HepaRG cells showed significantly elevated expression of MDR3 mRNA following the addition of bezafibrate (Fig. 5B). However, MDR3 is activated by both bezafibrate as well as UDCA.[7] Furthermore, recent reports have demonstrated that the expression of MDR3 was already markedly up-regulated in PBC patients[30] and it was not significantly affected by bezafibrate treatment.[31] Therefore, the anticholestatic effect of bezafibrate may be caused by mechanisms independent of phospholipid secretion.

Other possible anticholestatic mechanisms of bezafibrate by way of PPARα activation include down-regulation of NTCP,[17] CYP7A1,[32,33] and CYP27A1.[33] NTCP transports basolateral (sinusoidal) bile acids into hepatocytes, whereas CYP7A1 and CYP27A1 are key enzymes in the classic and alternative bile acid biosynthetic pathways, respectively. Coordinate down-regulation of these three proteins leads to a decrease in hepatic bile acid concentration and may protect hepatocytes against cytotoxic bile acids. In addition, the reduction of hepatic bile acid levels attenuates the activity of FXR. It is known that deactivation of FXR up-regulates MRP4,[34] one of the important basolateral transporters for the efflux of bile acids from hepatocytes to the sinusoid in cholestasis. The transcription of MRP4 is positively controlled by the constitutive androstane receptor (CAR; NR1I3)[35] and a CAR responsive element is embedded within an FXR responsive element in the human MRP4 promoter. Therefore, activated FXR competes with CAR for binding to this overlapping binding site, which down-regulates MRP4.[36]

The most striking results among our serum biomarker analyses are the elevation of 4β-HC, as well as the reduction of C4 during treatment with bezafibrate. Serum 4β-HC concentration is considered a biomarker of CYP3A4/5 activity,[37] whereas C4 is a marker of CYP7A1 activity or *de novo* bile acid synthesis.[23] Therefore, the changes in 4β-HC and C4 concentrations during bezafibrate treatment suggest that bezafibrate up-regulates CYP3A4/5 and down-regulates CYP7A1. In fact, our experiments using HepaRG cells clearly demonstrated that bezafibrate induced CYP3A4 mRNA expression and activity (Fig. 4A) and inhibited the expression of CYP7A1 mRNA (Fig. 5C) in a dose-dependent manner. Significant up-regulation of CYP3A4 was caused by at least 10 μM of bezafibrate, whereas the serum peak concentration (C_{max}) values after oral administration of 400 mg bezafibrate were 9.1-22.7 μM.[38]

Because the expression of CYP3A4 is mainly controlled by PXR,[39] it was strongly suggested that bezafibrate was a ligand of this nuclear receptor, and this hypothesis was proved by the reporter gene assay (Fig. 4B). In addition to PPARα, PXR also regulates hepatic enzyme and transporter activities to exert protective effects against cholestasis. First, the induced CYP3A4 detoxifies xenobiotics and endogenous substances, including the toxic bile acid LCA.[40,41] The C-6α or C-6β position of LCA is hydroxylated by CYP3A4 and nontoxic hyodeoxycholic acid (6α-OH) or murideoxycholic acid (6β-OH) is formed. Second, the activation of PXR up-regulates MDR1[42] and MRP2,[43] which was also observed in our HepaRG cells treated with rifampicin and bezafibrate (Fig. 5B). MDR1 transports various toxic metabolites and xenobiotics, whereas MRP2 transports organic anions from hepatocytes to bile canaliculi.

These results further suggest that the down-regulation of CYP7A1 by bezafibrate is caused not only by the activation of PPARα but also by the activation of PXR. Li and Chiang[44] demonstrated that hepatocyte nuclear factor 4α (HNF4α; NR2A1) interacts with several coactivators including PGC1α, and that the complex activates the transcription of *CYP7A1* in the absence of ligands.[45] Ligands for PXR activate PXR to promote its interaction with HNF4α, which disrupts the interaction between HNF4α and PGC1α and results in suppression of CYP7A1 expression.

Rifampicin is a more potent ligand of human PXR than bezafibrate (Fig. 4), and has also been shown to have anticholestatic effects in PBC patients.[46] However, continuous administration of rifampicin can sometimes result in severe hepatitis.[47] In addition to rifampicin and bezafibrate, budesonide, but not prednisolone, is also an agonist of the human PXR.[48] Therefore, the therapeutic effects of budesonide on PBC patients may be caused at least in part by the anticholestatic effects by way of the activation of PXR.

Hypercholesterolemia and hypertriglyceridemia are often observed in PBC patients. Although it remains controversial whether or not the lipid abnormalities in this disease increase atherosclerotic risk,[49] the administration of bezafibrate significantly reduced the serum concentrations of LDL cholesterol and triglycerides. The mechanism of the cholesterol-lowering effect of bezafibrate has not yet been completely elucidated, and at the very least, it is not likely due to a direct inhibition of HMGCR[50] (Fig. 5C). Because the concentration of serum lathosterol, a marker for *de novo* cholesterol biosynthesis, was decreased significantly during bezafibrate therapy, inhibition of other enzymes involved in the pathway is strongly suggested. Another mechanism of the cholesterol-lowering effect of bezafibrate may be due to the stimulation of cholesterol efflux from hepatocytes to the bile canaliculi by way of the activation of PPARs. Our experiment using HepaRG cells showed significantly up-regulated expression of ABCG5 and ABCG8 mRNA after bezafibrate but not rifampicin treatment (Fig. 5B). A similar effect of bezafibrate on ABCG5 in human liver has been reported previously.[51]

Because of the inhibition of bile acid synthesis and presumably the stimulation of cholesterol excretion into bile, bezafibrate significantly increases biliary cholesterol saturation.[52] Indeed, increased risk of gallstone formation has been reported in hyperlipidemic patients treated with another fibrate, fenofibrate.[53] However, combination therapy of UDCA and bezafibrate appears to attenuate the adverse effect of bezafibrate, because UDCA markedly lowers biliary cholesterol saturation and dissolves cholesterol gallstones.[2] On the other hand, bezafibrate may augment the anticholestatic and antilithogenic actions of UDCA by inhibiting bile acid synthesis and increasing the proportion of UDCA (Fig. 2C).

In addition to anticholestatic effects, activation of PXR[54] and the PPARs[55] has been reported to suppress inflammation through the inhibition of proinflammatory genes, including nuclear factor-κB (NF-κB), tumor necrosis factor-α, and interleukin-1α. In this study, although we did not evaluate the contribution of the anti-inflammatory effects of bezafibrate to the improvement of biochemical markers, bezafibrate is suggested to be an ideal drug with anticholestatic, hypolipidemic, and even antiinflammatory actions on PBC by way of the activation of both PXR and PPARs.

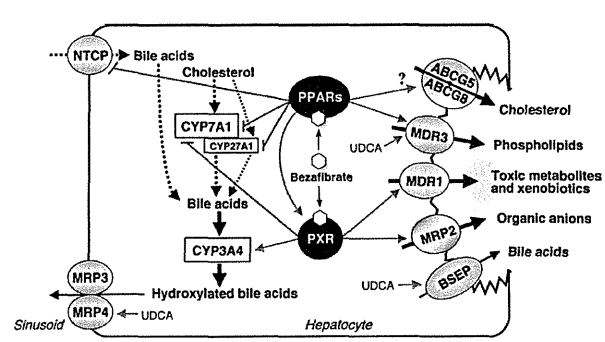

Fig. 6. Regulation of hepatic transporter activities and bile acid metabolism by PPARs, PXR, and UDCA. Bezafibrate is a dual agonist of both PPARs and PXR. The activation of PPARs inhibits CYP7A1, CYP27A1, and NTCP, and up-regulates MDR3, PXR and presumably ABCG5/G8. The activation of PXR inhibits CYP7A1 and stimulates CYP3A4, MDR1, and MRP2. Genes that are down-regulated by PPARs or PXR are indicated by the red lines, whereas those that are up-regulated by PPARs, PXR, or UDCA are indicated by the green arrows.

In summary, bezafibrate exhibited anticholestatic efficacy on PBC patients who showed an incomplete response to UDCA monotherapy. Although UDCA replaces hydrophobic bile acids and activates canalicular BSEP and MDR3 and basolateral MRP4,[7] bezafibrate inhibits hepatic synthesis and uptake of bile acids, enhances bile acid detoxification, and stimulates canalicular MDR3, MDR1 and MRP2 activities as a dual PPARs/PXR agonist (Fig. 6). These data lend support to the idea that combination therapy with UDCA and bezafibrate is an excellent method for the treatment of early-stage PBC patients who exhibit an incomplete biochemical response to UDCA monotherapy.

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Hepatology Research

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Original Article

Chronologic changes of explanted liver volume and the use of ursodeoxycholic acid in patients with end-stage primary biliary cirrhosis

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Aim: The clinical presentation of Primary biliary cirrhosis (PBC) at the time of liver transplantation (LT) may have changed, due to the long-term use of ursodeoxycholic acid (UDCA). The aim of this retrospective study was to investigate whether the clinical characteristics of LT recipients with PBC have changed over the years.

Methods: Of all 421 adults undergoing LT from 1997 to 2012 at our center, we included 85 recipients with PBC into the present study. The 85 recipients were divided into three groups according to the year LT was performed: group 1 (1997–2001, n = 29), group 2 (2002–2005, n = 29) and group 3 (2006–2012, n = 27).

Results: There were no significant differences in sex, recipient age, Model for End-Stage Liver Disease score, updated Mayo risk score for PBC, or liver-related complications except for esophageal varices among the three groups. Patients in group 1 were complicated with esophageal varices less frequently than those in the other two groups. In older cases, the ratio of explanted liver volume to standard liver volume (ELV/SLV) was significantly higher, and the duration of pre-LT UDCA treatment was significantly shorter. The duration of UDCA treatment was significantly correlated with ELV/SLV.

Conclusion: Recent LT patients were characterized by more frequent portal hypertension and more severe liver atrophy, with longer UDCA therapy prior to LT, which might have prevented the somewhat rapid progression of liver failure characterized by hepatomegaly with insignificant fibrosis or portal hypertension.

Key words: explanted liver, hepatomegaly, liver transplantation, living donor liver transplantation, primary biliary cirrhosis

INTRODUCTION

PRIMARY BILIARY CIRRHOSIS (PBC) is a chronic and cholestatic liver disease characterized by inflammatory destruction of the intrahepatic bile ducts that is thought to be autoimmune mediated.[1,2] The mechanism underlying the development of PBC remains controversial.[3] Cholestatic liver cirrhosis leading to hepatic failure is the most severe clinical manifestation of PBC, resulting in death or requiring liver transplantation (LT).[2,4] Potential subtypes of PBC disease progression, which are hepatic failure type and portal hypertension type, were recently proposed, and are represented by the presence or absence of autoantibodies, such as anti-gp210 and anticentromere.[5–7]

First introduced into clinical practice in the 1990s, the efficacy of ursodeoxycholic acid (UDCA) for suppressing the disease progression of PBC is well established. Although the mechanisms of action of UDCA are not clarified, the prognosis of PBC has improved since the introduction of UDCA, which decreases the need for LT.[8,9] Some patients with progressive PBC, however, still require LT despite adequate treatment with UDCA.[10] We hypothesized that there would be some different or

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