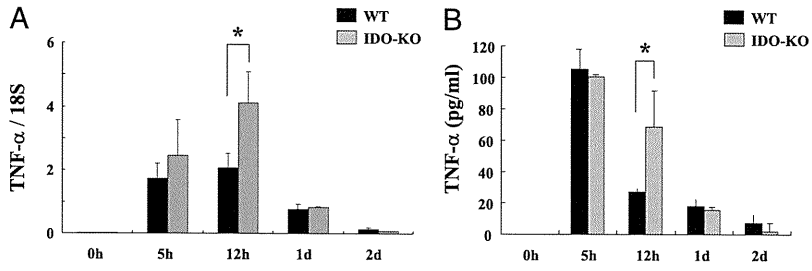

FIGURE 3. α-GalCer–induced TNF-α production in WT and IDO-KO mice. *A*, TNF-α mRNA expression in the livers of WT and IDO-KO mice that were administered α-GalCer. The mRNA level of TNF-α was normalized to that of 18S mRNA. Representative charts were derived from the analyses of three mice per group. *B*, Serum TNF-α concentration was determined by ELISA in WT and IDO-KO mice after α-GalCer injection. Each value is represented by the mean (SEM) of three mice. $*p < 0.05$.

ever, the concentration of TNF-α in IDO-KO mice was significantly increased compared with that in WT mice at 12 h after α-GalCer injection.

α-GalCer–induced cytokine and chemokine expression in WT mice and IDO-KO mice

As reported previously, NKT cells can produce a broad range of immunostimulatory or immunoregulatory cytokines and chemokines on activation, such as IL-2, IL-4, IL-10, IFN-γ, TNF-α, MIP-2, and KC (16–18). Therefore, we conducted a detailed time course analysis of the mRNA expression of intrahepatic cytokines (IFN-γ, IL-2, IL-4, IL-6, IL-10, and TGF-β) and chemokines (MIP-2, MCP-1, and KC) in WT and IDO-KO mice treated with α-GalCer (Fig. 4). Intrahepatic IL-6, MIP-2, and KC mRNA expression in IDO-KO mice was significantly increased compared with that in WT mice at 12 h after α-GalCer treatment. The marked differences between WT and IDO-KO mice were not observed with the mRNA expression of other cytokines and chemokines. Next, we measured the serum IL-6, MIP-2, and KC concentrations in WT and IDO-KO mice treated with α-GalCer. Although intrahepatic mRNA expressions of these

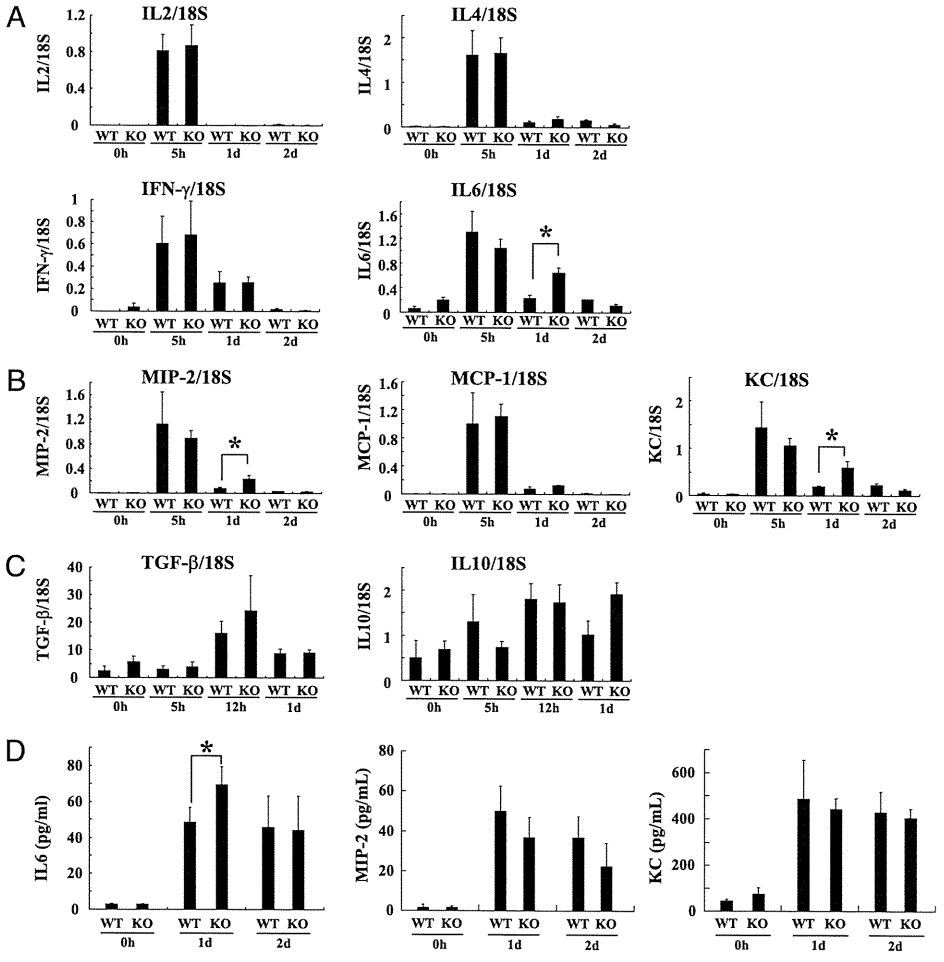

FIGURE 4. α-GalCer–induced cytokine and chemokine expression in WT mice and IDO-KO mice. *A*, IL-2, IL-4, IL-6, and IFN-γ mRNA expression in the livers of WT and IDO-KO mice that were administered α-GalCer. *B*, mRNA expression of chemokines (MIP-2, MCP-1, and KC) in the livers of WT and IDO-KO mice that were administered α-GalCer. *C*, TGF-β and IL-10 mRNA expression in the livers of WT and IDO-KO mice that were administered α-GalCer. The mRNA levels of cytokines and chemokines were normalized to those of 18S mRNA. *D*, Serum IL-6, MIP-2, and KC concentrations in WT and IDO-KO mice after α-GalCer injection were determined by ELISA. Representative charts derived from the analyses of three mice per group. $*p <$ 0.05.

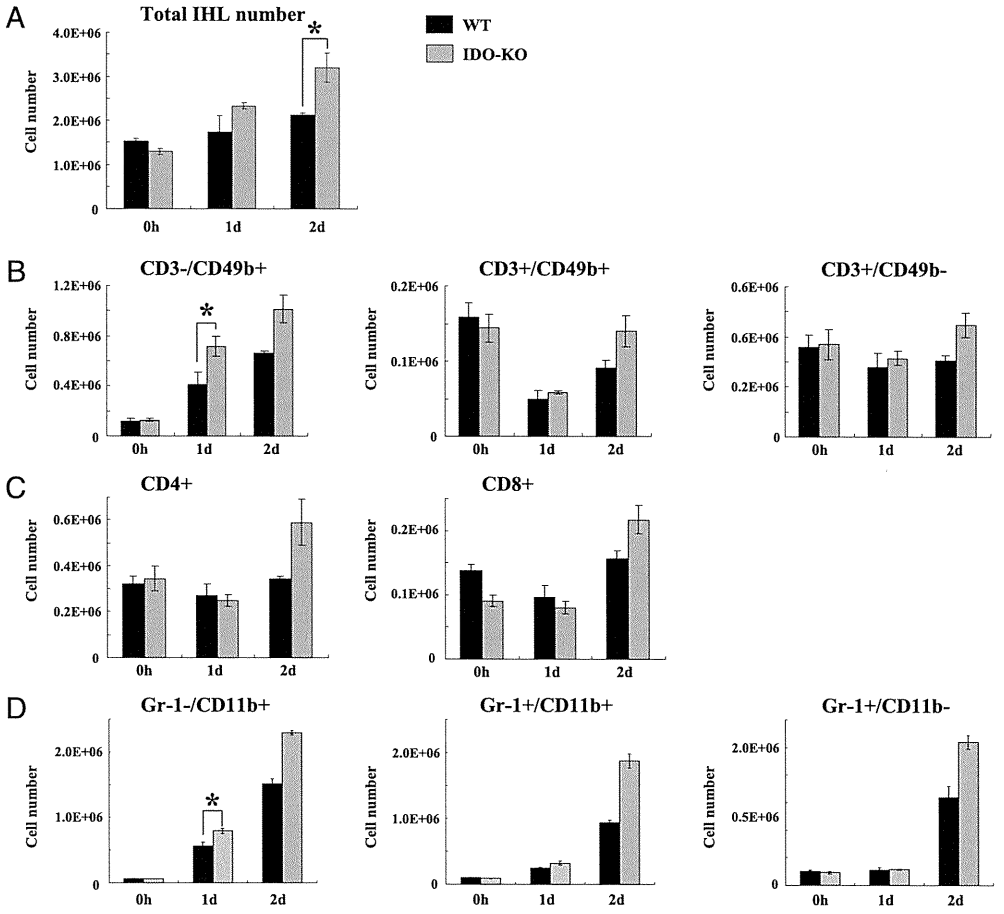

FIGURE 5. Kinetics and lymphocyte phenotypes of hepatic MNCs after α-GalCer injection into WT and IDO-KO mice. Hepatic MNCs from WT (black bars) and IDO-KO (gray bars) mice were obtained at 0, 1, and 2 d after α-GalCer injection. Cell numbers were quantified using FACScan analysis. *A*, Total number of hepatic MNCs after α-GalCer injection. *B*, Number of $CD3^-CD49b^+$ cells, $CD3^+CD49b^+$ cells, and $CD3^+CD49b^-$ cells after α-GalCer injection. *C*, Number of $CD4^+$ and $CD8^+$ cells after α-GalCer injection. *D*, Number of $CD11b^+Gr\text{-}1^-$ cells, $CD11b^+Gr\text{-}1^+$ cells, and $CD11b^-Gr\text{-}1^+$ cells at 1 d after α-GalCer injection. Results are presented as the mean number of cells of each cell type of hepatic MNCs for at least three mice per group. Error bars indicate the SEM. $*p < 0.05$. IHL, intrahepatic lymphocyte.

chemokines and cytokine in IDO-KO mice were increased compared with those in WT mice, the concentration of IL-6 in IDO-KO mice only elevated in serum.

Lymphocyte phenotypes of hepatic MNCs after α-GalCer injection into WT and IDO-KO mice

Next, we examined the lymphocyte phenotypes of hepatic MNCs from WT and IDO-KO mice treated with α-GalCer. The total number of MNCs in the livers of WT mice and IDO-KO mice was increased after α-GalCer injection. The increase in cell number was more enhanced in IDO-KO mice compared with WT mice at 2 d after α-GalCer injection (Fig. 5*A*). In particular, the frequency of hepatic $CD49b^+$ cells in IDO-KO mice significantly increased at 1 d after α-GalCer injection (Fig. 5*B*), coinciding with the peak of serum (Fig. 2). Although the number of $CD4^+$ and $CD8^+$ cells in the liver of IDO-KO mice increased compared with that in WT mice, the peak time was delayed compared with the peak time of liver injury in this model (Fig. 5*C*). Moreover, we measured the number of monocytes/macrophages in the liver from WT and IDO-KO mice after α-GalCer injection (Fig. 5*D*). The number of $CD11b^+Gr\text{-}1^-$ cells from IDO-KO mice was significantly increased compared with that from WT mice.

α-GalCer–induced TNF-α production in $CD49b^+$ and $CD11b^+$ cells

As shown in Fig. 5, we have confirmed that the number of $CD49b^+$ and $CD11b^+$ cells in the liver from IDO-KO mice treated with α-GalCer increased. Therefore, we measured the production of TNF-α on $CD49b^+$ and $CD11b^+$ cells by using intracellular cytokine staining (Fig. 6). $CD49b^+$ and $CD11b^+$ cells in the livers of both WT and IDO-KO mice significantly produced TNF-α at 12 h after α-GalCer injection. In particular, the number of TNF-α–producing cells in IDO-KO mice significantly increased compared with that in WT mice.

The IDO inhibitor 1-methyl-D-tryptophan enhances α-GalCer–induced liver injury

1-Methyl-D-tryptophan (1-MT) is a potent inhibitor of IDO, and hence we used this agent to substantiate data obtained with IDO-KO mice. WT mice were administered 1-MT orally at 0 or 5 mg/ml in drinking water for 3 d before α-GalCer stimulation. This experiment was performed twice independently. In both experiments, the serum ALT level in WT mice treated with 1-MT was significantly elevated compared with that in WT mice not treated with 1-MT at 1 d after α-GalCer stimulation (Fig. 7). Therefore,

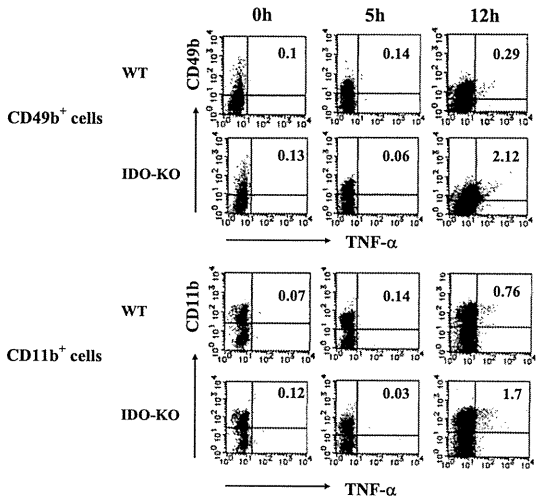

FIGURE 6. α-GalCer–induced TNF-α production in CD49b$^+$ and CD11b$^+$ cells. Flow cytometric analysis of intracellular TNF-α produced by hepatic CD49b$^+$ and CD11b$^+$ cells obtained from mice at 0, 5, and 12 h after α-GalCer injection and cultured for 4 h in brefeldin A. Data are representative of at least three independent experiments with similar results.

pharmacological inhibition of IDO with 1-MT is consistent with the exacerbated situation observed using IDO-KO mice.

Discussion

In this study, we report that α-GalCer–induced liver injury was exacerbated in IDO-KO mice, and that the exacerbation was accompanied by an increase in the number of intrahepatic TNF-α–producing MNCs. The serum ALT level was significantly augmented in IDO-KO mice compared with WT mice after α-GalCer injection. In parallel, TNF-α expression induced by α-GalCer injection was more enhanced in the livers of IDO-KO mice. Moreover, the number of intrahepatic CD49b$^+$ and CD11b$^+$ cells in IDO-KO mice treated with α-GalCer significantly increased compared with that in WT mice, and the cells from IDO-KO mice produced a large amount of TNF-α. These data indicated that deficiency of IDO increased the number of intrahepatic TNF-α–producing cells and exacerbated α-GalCer–induced liver injury. To the best of our knowledge, this is the first report describing the effects of IDO on acute hepatic injury.

IDO is an enzyme that is ubiquitously distributed in mammalian tissues and cells; that is, from L-Trp to *N*-formylkynurenine, which is further catabolized to L-Kyn. IDO production is induced by an IFN-γ–dependent and/or an IFN-γ–independent mechanism and other proinflammatory cytokines in the course of an inflammatory response in different cells, including macrophages, fibroblasts, and epithelial cells (3, 4). Previous studies on IDO have demonstrated that the role that IDO plays in regulating immune responses has been the subject of intense investigation. The bulk of the literature has focused on investigating the suppressive effects of IDO activity, predominantly on the activation of T cells (1). The prevailing theory is that IDO expressed by dendritic cells inhibits T cell activation, either directly or indirectly, by driving the development of regulatory T cells.

Inhibition of IDO activity during immune-mediated colitis has recently been reported to markedly worsen disease in the gut (19). In sharp contrast, IDO can act as a mediator of inflammatory disease, particularly in ischemia-reperfusion injury (20). Additionally, it was reported that administration of 1-MT to K/BxN mice reduced the level of inflammatory cytokines and autoantibodies, resulting in an attenuated course of arthritis (21). Thus, IDO has contrasting effects on several types of inflammation models. However, the effects of IDO function in the acute murine liver injury model remain unknown. In this study, we used the α-GalCer–induced liver injury model to examine the role of IDO an acute liver injury.

We first confirmed the expression of IDO in the liver after α-GalCer administration (Fig. 1). IDO is induced by IFN-γ and other proinflammatory cytokines, whereas such proinflammatory cytokines are secreted in mice treated with α-GalCer (15, 17). In particular, immunohistochemical examination revealed that IDO expression was enhanced in hepatocytes after α-GalCer treatment (Fig. 1*D*). These results coincide with our previous data in which murine recombinant IFN-γ induced IDO mRNA expression in primary hepatocytes in vitro (8).

In this study, using IDO-KO mice, we clearly demonstrated that the IDO deficiency caused the exacerbation of liver injury in this murine α-GalCer–induced hepatitis model (Fig. 2). As shown in Fig. 1*D*, the expression of IDO in the liver was enhanced 1 d after α-GalCer treatment, and ALT level also increased simultaneously. These results indicate that IDO expression may partially regulate the liver injury. Moreover, 1-MT, a competitive inhibitor of IDO, also exacerbated liver injury in this hepatitis model (Fig. 7). Induction of IDO by α-GalCer treatment may thus suppress the increased immune response observed in acute liver injury and attenuate the liver injury caused in this model.

Previous studies have reported that TNF-α is an important mediator in the α-GalCer–induced liver injury model (12, 22, 23). Intrahepatic NKT cells and NK cells mainly secrete TNF-α after α-GalCer injection. Although intrahepatic macrophages/monocytes are known as a pivotal source of TNF-α, they are not essential for α-GalCer–mediated hepatotoxicity (12). In this study, TNF-α production in IDO-KO mice treated with α-GalCer was increased compared with that in WT mice, and TNF-α also played a critical role in this acute liver injury model (Fig. 3). In particular, TNF-α production in CD49b$^+$ and CD11b$^+$ cells from IDO-KO mice was

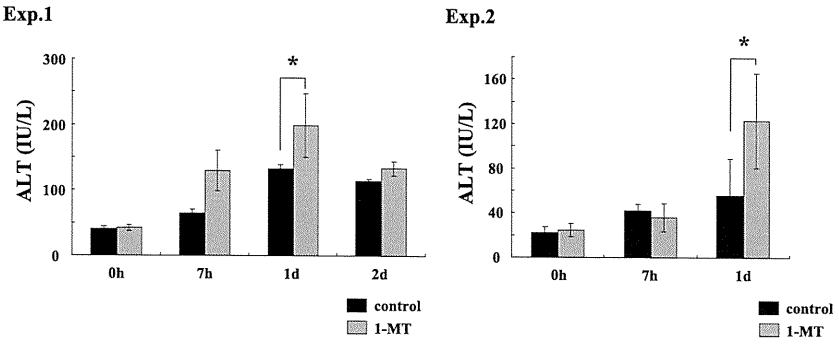

FIGURE 7. IDO inhibitor (1-MT) enhances α-GalCer–induced liver injury. IDO-WT mice were orally administered 1-MT at 0 or 5 mg/ml in drinking water for 3 d before α-GalCer stimulation. Serum ALT activity was analyzed at varying time points relative to the injection of α-GalCer into 1-MT–treated and nontreated mice. Representative charts were derived from the analyses of four or five mice per group. These experiments were independently performed twice (Exp. 1 and Exp. 2). Error bars indicate the SEM. $*p < 0.05$.

significantly increased compared with that from WT mice (Fig. 6). α-GalCer can enhance TNF-α production and cytotoxicity in NK cells and NKT cells (12, 24). The increase in the number of TNF-α–producing $CD49b^+$ cells (including NK cells and NKT cells) presumably contributes to the exacerbation of α-GalCer–induced liver injury. On the other hand, although the previous study demonstrated that macrophages/monocytes did not contribute to the progression of α-GalCer–mediated liver injury (12), TNF-α production in intrahepatic macrophages/monocytes from IDO-KO mice treated with α-GalCer was enhanced in our study. Therefore, we speculated that IDO may suppress TNF-α production in activated macrophages/monocytes.

It was previously reported that IDO induces inhibition of immune cell (e.g., NK cells and T cells) proliferation (25, 26). In our data, intrahepatic MNCs from IDO-KO mice treated with α-GalCer were apoptosis resistant with those from WT mice. Furthermore, the number of apoptotic splenocytes increased in the presence of WT hepatocytes on α-GalCer stimulation (Fig. 7). In contrast, apoptotic splenocytes did not increase in the presence of α-GalCer when splenocytes from IDO-KO mice were cultured with hepatocytes from IDO-KO mice. We suggested that activated intrahepatic MNCs may be resistant to apoptosis in the absence of IDO, and the number of intrahepatic MNCs in IDO-KO mice treated with α-GalCer increased compared with that in WT mice.

Real-time PCR analysis revealed that mRNA expression of IL-6, MIP-2, and KC in the liver of IDO-KO mice was upregulated at 24 h after α-GalCer injection (Fig. 4). There was no difference between WT and IDO-KO mice with regard to the α-GalCer reactivity, because the expression of cytokines and chemokines after α-GalCer injection was equally enhanced in the livers of WT and IDO-KO mice in the early phase (at 5 h) after α-GalCer injection. The lack of IDO promoted enhancement of IL-6, MIP-2, and KC expression in the late phase (at 24 h) after α-GalCer injection. However, there is no difference between WT and IDO-KO mice in serum MIP-2 and KC levels. These results indicated that expression of MIP-2 and KC may be affected by the expression of IDO only in the liver. Moreover, increased production of such cytokine and chemokines augments the number of intrahepatic NK cells and $CD11b^+$ cells, and they subsequently exacerbate liver injury in IDO-KO mice treated with α-GalCer. Taken together, we demonstrated that IDO inhibited not only the proliferation of NK cells and macrophages but also TNF-α production in these cells in the liver after administration of α-GalCer. IDO has the ability to suppress overactive immune response in the α-GalCer–induced hepatitis model.

In summary, this study demonstrated that IDO deficiency exacerbated liver injury in α-GalCer–induced hepatitis. IDO induced by proinflammatory cytokines may decrease the number of intrahepatic TNF-α–producing immune cells (NK cells and macrophages in particular) in acute hepatitis. Enhancement of IDO expression may suppress overactive immune response in the α-GalCer–induced hepatitis model. Accordingly, IDO regulation may be a therapeutic target in acute hepatitis.

Disclosures

The authors have no financial conflicts of interest.

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Role of Acid Sphingomyelinase of Kupffer Cells in Cholestatic Liver Injury in Mice

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Kupffer cells, resident tissue macrophages of the liver, play a key role in the regulation of hepatic inflammation, hepatocyte death, and fibrosis that characterize liver diseases. However, it is controversial whether Kupffer cells promote or protect from liver injury. To explore this issue we examined the role of Kupffer cells in liver injury, cell death, regeneration, and fibrosis on cholestatic liver injury in C57BL/6 mice using a model of partial bile duct ligation (BDL), in which animals do not die and the effects of BDL can be compared between injured ligated lobes and nonligated lobes. In cholestatic liver injury, the remaining viable cells represented tolerance for tumor necrosis factor alpha (TNF-α)-induced hepatocyte apoptosis and regenerative features along with AKT activation. Inhibition of AKT by adenovirus expressing dominant-negative AKT abolished the survival and regenerative properties in hepatocytes. Moreover, Kupffer cell depletion by alendronate liposomes increased hepatocyte damage and the sensitivity of TNF-α-induced hepatocyte apoptosis in ligated lobes. Kupffer cell depletion decreased hepatocyte regeneration and liver fibrosis with reduced AKT activation. To investigate the impact of acid sphingomyelinase (ASMase) in Kupffer cells, we generated chimeric mice that contained ASMase-deficient Kupffer cells and -sufficient hepatocytes using a combination of Kupffer cell depletion, irradiation, and the transplantation of ASMase-deficient bone marrow cells. In these mice, AKT activation, the tolerance for TNF-α-induced apoptosis, and the regenerative responses were attenuated in hepatocytes after BDL. *Conclusion:* Kupffer cells have a protective role for hepatocyte damage and promote cell survival, liver regeneration, and fibrosis in cholestatic liver disease. Kupffer cell-derived ASMase is crucial for AKT activation of hepatocytes that is required for the survival and regenerative responses. (HEPATOLOGY 2010;51:237-245.)

Chronic liver disease is associated with inflammatory cell infiltration, cytokine production, and liver cell death. Persistent hepatocyte death impairs hepatocyte regeneration accompanied with excessive production of extracellular matrix proteins causing liver fibrosis. Kupffer cells, resident tissue macrophages of the liver, function as both a promoter and a protector against liver injury. Lipopolysaccharide activates Kupffer cells and induces liver injury and inhibition of Kupffer cells prevents liver injury.[1] In addition, inhibition of Kupffer cell activation prevents liver injury induced by melphalan[2] and fumonisin B1.[3] In contrast, reduced Kupffer cell activity augments some kinds of liver injuries, such as hepatectomy- or acetaminophen-induced liver injury.[4,5] Activated Kupffer cells release various types of inflammatory cytokines and growth factors,[6] and these mediators are thought to regulate liver injury and regeneration. Especially, tumor necrosis factor alpha (TNF-α) from activated Kupffer cells plays a major role in the pathogenesis of various liver injuries.[7,8] Cholestasis is associated with many liver diseases. Bile duct ligation (BDL) causes hepatocyte damage, hepatic stellate cell (HSC) activation, and liver fibrosis accompanied by Kupffer cell activation leading to the production of a variety of cytokines and chemokines that are involved in liver damage and fibrosis.[9-11] Because these features are similar to human cholestatic diseases,

Abbreviations: α-SMA, alpha smooth muscle actin; Ale-lip, liposome-encapsulated alendronate; ASMase, acid sphingomyelinase; BDL, bile duct ligation; DN, dominant negative; GalN, D-galactosamine; GSK, glycogen synthase kinase; HSC, hepatic stellate cell; PBDL, partial BDL; TNF-α, tumor necrosis factor alpha; TUNEL, terminal deoxynucleotidyl transferase nick end-labeling.

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common BDL has been used as an animal model of chronic liver disease. However, in this model, common bile duct ligation causes total bile acid reflux to damage whole liver, and the animals show high mortality due to liver failure. We have previously established a partial BDL (PBDL) model, in which animals showed a typical liver injury only in the BDL lobes but no damage in the nonligated lobes with viable liver functions. In this study we examined the role of Kupffer cells in chronic liver injury using the PBDL model.

Acid sphingomyelinase (ASMase) hydrolyses sphingomyelin into ceramide and phosphorylcholine and is involved in various cell functions. Ceramide has been identified as a bioactive mediator of various cellular functions.[12] In addition, roles for sphingomyelin and ceramide in membrane lipid rafts have been reported,[13] which is related with transmitting signals across the plasma membrane. In macrophages, ASMase contributes to cytokine and chemokine release. Its inhibitor, sphingomyeline difluoromethylene analogue-7 (SMA-7), suppressed lipopolysaccharide-induced releases of TNF-α, interleukin (IL)-1β, and IL-6 from macrophages, and it reduces the severity of inflammatory bowel disease induced by dextran sodium sulfate.[14] In contrast, production of macrophage inflammatory protein-1α and -2 is increased in ASMase-deficient macrophages.[15] In addition, ASMase-deficient macrophage is impaired in killing bacteria.[16] Thus, ASMase contributes to various immunoresponses. In liver damage, although deficiency of ASMase leads to resistance to hepatocyte cell death induced by TNF-α,[17,18] the role of ASMase in Kupffer cells remains unclear.

In this study we assessed the roles of Kupffer cells and ASMase during chronic liver injury using PBDL mice. We found that Kupffer cells reduce liver damage, and induce hepatocyte survival and regeneration, and fibrosis. The protective and regenerative effects require AKT in hepatocytes by way of ASMase in Kupffer cells.

Materials and Methods

Animals and PBDL. ASMase knockout mice (ASMase$^{-/-}$) (C57Bl/6 background)[18] were bred for studies. Eight-week-old male wildtype C57Bl/6J mice were obtained from Japan SLC (Japan). The left hepatic duct was ligated for PBDL as reported.[19] The animals were fasted for 12 hours before sacrifice at 10 days after the surgery. As necessary, hepatocyte apoptosis was induced by mouse TNF-α (R&D Systems, Minneapolis, MN) (0.5 μg/mouse intravenously) with D-galactosamine (GalN) (Nacalai Tesque, Japan) (20 mg/mouse intraperitoneally) 10 days after the PBDL[20] and the animals were killed 6 hours after TNF-α administration. All procedures were approved by the Institutional Animal Care Committee of Gifu University.

Depletion of Kupffer Cells. Alendronate was reported to deplete Kupffer cells.[1] A single injection of liposome-encapsulated alendronate (Ale-lip) depleted F4/80-positive cells in the liver at 2-3 days after injection and the cells started to restore at 6 days (Supporting Fig. 1A). Ale-lip had no effect on hepatocytes with hematoxylin and eosin (H&E) (Supporting Fig. 1B) and alanine transaminase (ALT) (data not shown). The vitamin A autofluorescence and desmin-positive cells, characteristic features of HSCs, were not decreased by Ale-lip (Supporting Fig. 1CD). Ale-lip was injected to the operated mice 3 times at 1 day before surgery and 3 and 6 days after the surgery. Phosphate-buffered saline (PBS) encapsulated liposomes (PBS-lip) were used for control.

Bone Marrow Transplantation. Bone marrow transplantation was performed as reported.[11] The wildtype mice received Ale-lip injection twice at 1 and 4 days prior to lethal irradiation (11 Gy). Total bone marrow cells were collected from wildtype or ASMase$^{-/-}$ mice and injected to the irradiated recipient mice (10^7 cells). PBDL was performed 10 weeks after the transplantation.

Other Experimental Procedures. Other experimental procedures are described in the Supporting experimental procedures. These include preparation of liposome-encapsulated alendronate, adenovirus infection, histological analysis, western blot, quantitative real-time reverse-transcription polymerase chain reaction (RT-PCR), hydroxyproline measurement, and statistical analysis.

Results

Kupffer Cell Depletion Increases Hepatocellular Damage Induced by BDL. To examine the effect of Kupffer cell depletion on chronic liver damage induced by BDL, we initially injected Ale-lip three times to mice operated on with common BDL. Although the treatment with Ale-lip alone did not induce liver injury, the mortality of mice treated with common BDL and Ale-lip was extremely high; 40% 10 days after the surgery. In our established model, PBDL showed liver injury and fibrosis only in BDL lobes, which improved the survival rate up to 100% in Ale-lip-treated mice. Therefore, we decided to use PBDL for this study. In BDL lobes, F4/80-positive cells were increased. The Ale-lip treatment succeeded in deleting F4/80-positive cells (Fig. 1A). Thus, Ale-lip injection can be utilized as a new tool for Kupffer cell depletion. Inflammatory cytokines mainly produced from Kupffer cells were up-regulated in BDL lobes, whereas the

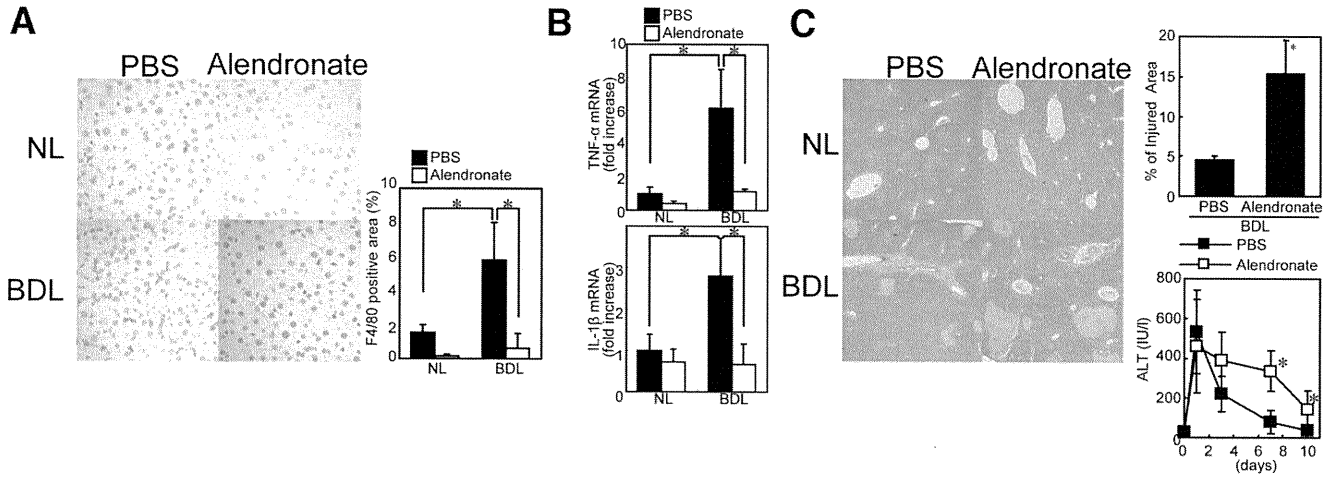

Fig. 1. Depletion of Kupffer cells increased liver injury after BDL. Wildtype mice were subjected to PBDL and treated with Ale-lip or PBS-lip. The animals were killed 10 days after the surgery. (A) Expression of F4/80 in the nonligated (NL) right (upper panel) and BDL left (lower panel) lobes was examined by immunohistochemistry. (Original magnification ×400; graph, right panel.) (B) Hepatic mRNA levels of TNF-α and IL-1β were determined by quantitative real-time RT-PCR. (C) The injured lesion in the ligated left lobes was assessed by H&E staining. (Original magnification ×40; graph, right upper panel.) Serum ALT levels were compared on the indicated time periods. Data are means ± SD from at least four independent experiments. *$P < 0.05$ using Student's t test.

Ale-lip treatment markedly inhibited the production of TNF-α and IL-1β (Fig. 1B). Kupffer cell-depleted mice showed an increase of injured lesion in BDL lobes and serum ALT level after the surgery (Fig. 1C). Interestingly, 24 hours after common BDL (Supporting Fig. 2) as well as PBDL (Fig. 1C), there were no significant differences in histological liver injury and elevated ALT activities between control and Kupffer cell-depleted mice. These findings indicate that Kupffer cells were not involved in the early stage of liver damage that occurs by BDL, but in the late stage.

Kupffer Cells Mediate Survival and Regeneration of Hepatocyte by BDL. As previously reported,[20] treatment with TNF-α plus GalN strongly induced hepatocyte destruction and massive hemorrhage with apoptotic cells in nonligated lobes of PBDL animals, whereas hemorrhagic damage and hepatocyte apoptosis were blunted in BDL lobes (Supporting Fig. 3A-C). Kupffer cell depletion itself did not induce hepatocyte apoptosis (Supporting Fig. 3D). In Kupffer cell-depleted livers, GalN plus TNF-α treatment induced hemorrhagic liver damage and hepatocyte apoptosis with the cleavage of poly (ADP-ribose) polymerase (PARP), which is the downstream target of caspase-3, both in nonligated and BDL lobes (Fig. 2A-C).

In the BDL lobes, proliferation cell nuclear antigen (PCNA) or Ki67-positive hepatocytes were increased with up-regulation of cyclin E expression (Fig. 2D-F), indicating that BDL induces hepatocyte regeneration. In Kupffer cell-depleted livers the expressions of PCNA, Ki67, and cyclin E were decreased (Fig. 2D-F). Thus, Kupffer cells are important for survival and regeneration of hepatocytes after BDL.

Kupffer Cells Are Required for Liver Fibrosis. Fibrosis was induced in BDL lobes as demonstrated by Sirius red staining, hydroxyproline content, expression of α-smooth muscle actin (α-SMA) and desmin, and messenger RNA (mRNA) expression of collagen-α1(I) and transforming growth factor (TGF)-β1 (Fig. 3). Kupffer cell-depleted mice showed reduced fibrosis in BDL lobes (Fig. 3). The number and the activation of HSCs were decreased by Kupffer cell depletion as assessed by desmin and α-SMA expression, respectively. These results suggest that the decrease in the fibrogenic response by Kupffer cell depletion is due to a lack of signal from Kupffer cells to activate and proliferate HSCs.

ASMase Deficiency in Kupffer Cells Diminishes the BDL-Induced Survival and Proliferative Effect. To further elucidate the mechanisms by which Kupffer cells contribute to BDL-mediated functional changes in liver injury, survival of hepatocyte, regeneration, and fibrosis, we focused on ASMase. The protein level of ASMase (Supporting Fig. 4) and ceramides (Supporting Table 1), the metabolite of ASMase, were increased in BDL lobes compared with those in nonligated lobes, suggesting the contribution of ASMase in the liver. To explore the involvement of ASMase in bone marrow-derived cells we generated ASMase-chimeric mice using a combination of alendronate-induced Kupffer cell depletion, irradiation, and bone marrow transplantation. To confirm the substitution of Kupffer cells in chimeric mice we initially generated the mice transplanted with bone marrow isolated

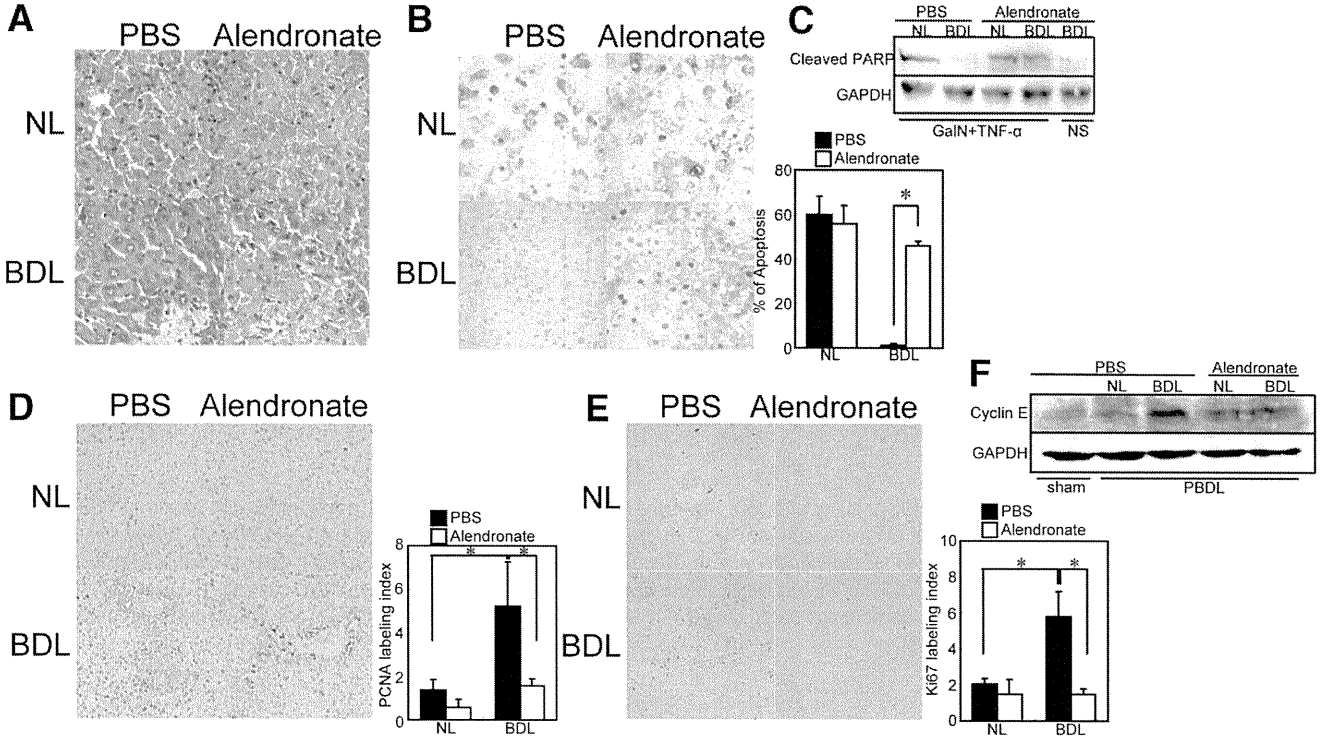

Fig. 2. Depletion of Kupffer cells abolished the survival effect of BDL and decreased hepatocyte regeneration after BDL. Wildtype mice were subjected to PBDL and treated with Ale-lip or PBS-lip. The animals were administered with (A-C) or without (D-F) GalN plus TNF-α (6 hours) on 10 days after the surgery. (A) Liver sections from the nonligated (NL) right (upper panel) and BDL left (lower panel) lobes were stained with H&E. (B) Apoptotic nuclei were identified using TUNEL staining. (Original magnification $\times$400; graph, right panel.) Expression of PCNA (D) and Ki67 (E) in the NL right (upper panel) and the BDL left (lower panel) lobes were examined by immunohistochemistry. (Original magnification $\times$200.) PCNA and Ki67 indexes were compared (right panel). Data are means $\pm$ SD from at least four independent experiments. *$P < 0.05$ using Student's t test. (C,F) The protein extracts from the NL and BDL lobes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting was performed with anti-PARP, cyclin E, and GAPDH antibodies. The results shown are representative of at least three independent experiments. NS, normal saline.

from β-actin promoter-driven green fluorescent protein (GFP)-transgenic mice. In the GFP-chimeric mouse liver all F4/80-positive cells were GFP-positive (Supporting Fig. 5A), suggesting that this protocol achieved full reconstitution of Kupffer cells to bone marrow-derived cells. The chimeric mice containing ASMase$^{-/-}$ bone marrow cells showed an increase of F4/80-positive cells, and TNF-α and IL-1β production after BDL, which were comparable to ASMase$^{+/+}$ bone marrow-transplanted mice (Supporting Fig. 5BC).

ASMase$^{-/-}$ bone marrow-transplanted mice showed an increase of liver injury in BDL lobes at the same degree as ASMase$^{+/+}$ bone marrow-transplanted mice (Fig. 4), suggesting that ASMase of Kupffer cell is not implicated in the liver injury. Hemorrhagic liver damage and apoptosis with PARP cleavage by GalN plus TNF-α were observed in BDL lobes of ASMase$^{-/-}$ bone marrow-transplanted mice but not in that of ASMase$^{+/+}$ bone marrow-transplanted mice (Fig. 5A-C). An increase of PCNA or Ki67-positive cells with cyclin E expression were blunted in BDL lobes of ASMase$^{-/-}$ bone marrow-transplanted mice (Fig. 5D-F). These results suggest that ASMase in Kupffer cells contribute to the protection against apoptosis and regeneration in BDL lobes. However, there was no difference between ASMase$^{-/-}$ bone marrow and ASMase$^{+/+}$ bone marrow-transplanted mice in mRNA expression of fibrogenic markers, Sirius red staining, and hydroxyproline content (Fig. 6). Thus, ASMase of Kupffer cell was not associated with liver fibrosis.

AKT Activation of Hepatocytes Is Required for Survival and Regeneration in BDL Lobes. Our previous study demonstrated that AKT was up-regulated in BDL lobes and was involved in hepatocyte survival from TNF-α-induced cell death.[20] In BDL lobes, phosphorylated-AKT and its downstream target, phosphorylated-glycogen synthase kinase (GSK)3β were increased (Supporting Fig. 6A). Immunohistochemical analysis identified that AKT in hepatocytes was phosphorylated (Supporting Fig. 6B). The AKT activation in BDL lobes was abrogated by the infection of Ad5 dominant negative (DN)-AKT (Supporting Fig. 6C). The inhibition of AKT abolished the survival effect (Supporting Fig. 6D) as re-

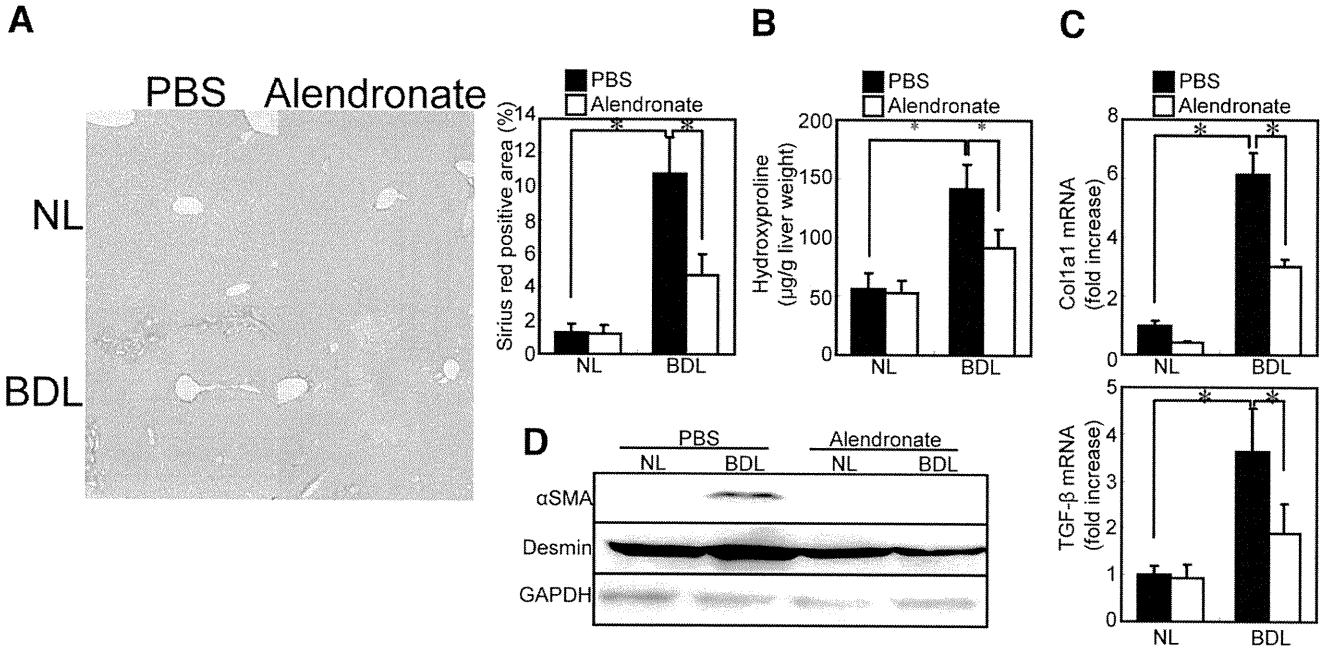

Fig. 3. Depletion of Kupffer cells reduced liver fibrosis after BDL. Wildtype mice were operated on with PBDL and treated with Ale-lip or PBS-lip. The animals were killed 10 days after the surgery. (A,B) Collagen deposition was assessed by Sirius red staining (original magnification ×100; graph, right panel) and measurement of hydroxyproline content. (C) mRNA levels of collagen α1(I) (col1α1) and TGF-β1 (TGF-β) in the livers were determined by quantitative real-time RT-PCR. Data are means ± SD from at least four independent experiments. *$P < 0.05$ using Student's t test. (D) The protein extracts from the livers were subjected to SDS-PAGE and immunoblotting was performed with anti-α-SMA, desmin, and GAPDH antibodies. The results shown are representative of at least three independent experiments.

ported,[20] and eliminated the induction of Ki67-positive cells and cyclin E (Fig. 7A,B) induced by BDL. These findings suggest that AKT activation in hepatocytes is essential for hepatocyte survival and regeneration observed in BDL lobes. In Kupffer cell-depleted mice (Fig. 7C) or ASMase$^{-/-}$ bone marrow-transplanted mice (Fig. 7D), the phosphorylation of AKT and GSK3β by BDL was inhibited. Thus, Kupffer cells and their ASMase are required for AKT-mediated survival and regeneration induced by BDL. Mcl-1 induction, which is a Bcl-2 family member and was regulated by AKT in hepatocytes (Supporting Fig. 6C,E),[21] was diminished in Kupffer cell-depleted mice or ASMase$^{-/-}$ bone marrow-transplanted mice, whereas Bcl-XL or Bfl-1 were not affected. These results suggest that survival may be mediated by Mcl-1 at the downstream of AKT.

Discussion

The present study specifically addressed the role of Kupffer cells and of ASMase in the cholestatic liver injury. Our results demonstrate that depletion of Kupffer cells

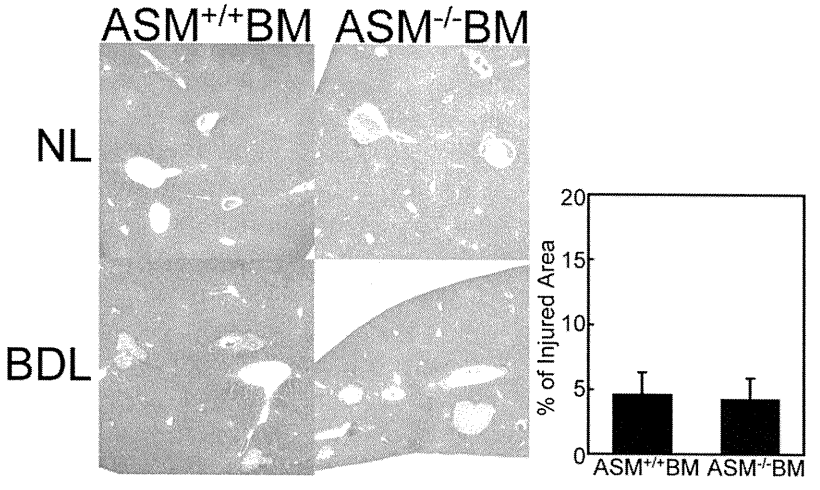

Fig. 4. Reconstitution of Kupffer cells with ASMase knockout cells did not affect on the liver injury induced by BDL. The ASMase$^{+/+}$ bone marrow or ASMase$^{-/-}$ bone marrow-transplanted mice were subjected to PBDL and killed 10 days after the surgery. Measurement of injured lesion in the BDL lobes was assessed with H&E. (Original magnification ×40; graph, right panel.) Data are means ± SD from at least four independent experiments.

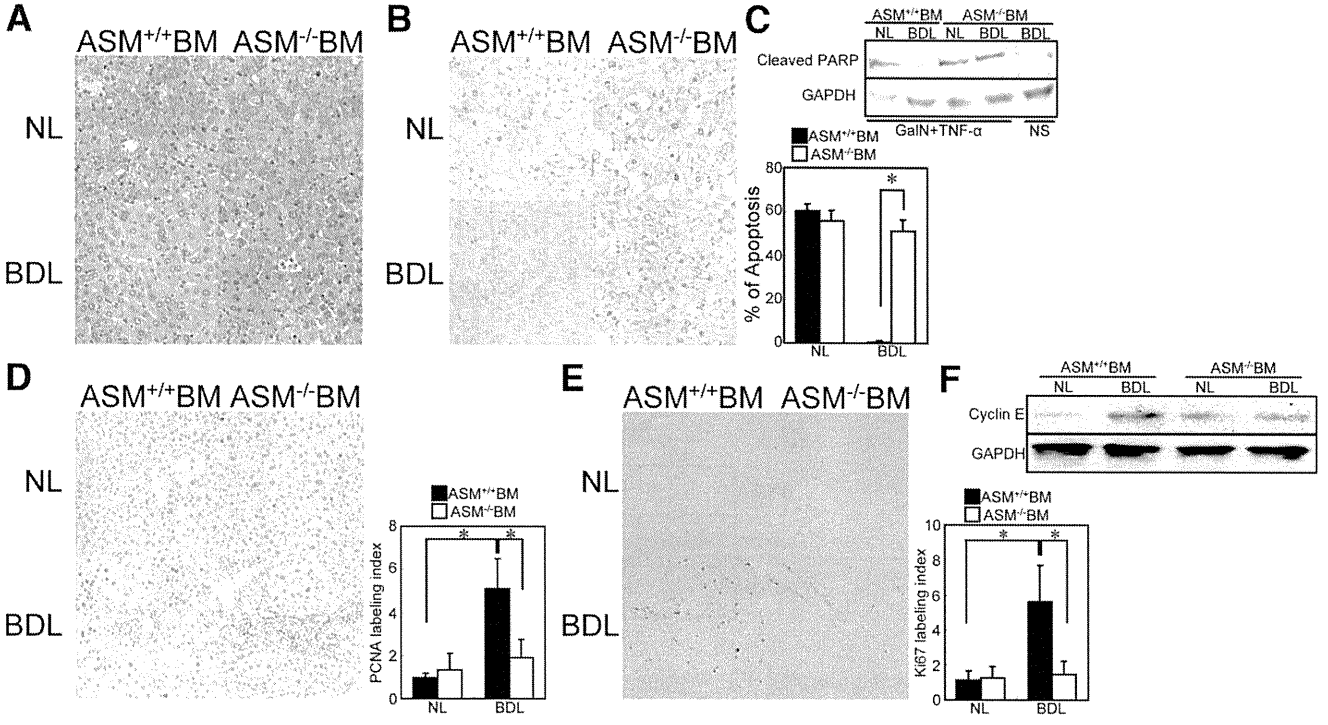

Fig. 5. Reconstitution of Kupffer cells to ASMase knockout cells abrogated the survival effect of BDL and decreased hepatocyte regeneration after BDL. The ASMase$^{+/+}$ bone marrow or ASMase$^{-/-}$ bone marrow-transplanted mice were operated on with PBDL and treated with (A-C) or without (D-F) GalN plus TNF-α treatment (6 hours) 10 days after the surgery. (A) Liver sections were stained with H&E. (B) Apoptotic nuclei were identified using TUNEL staining. (Original magnification $\times$400; graph, right panel.) Expression of PCNA (D) and Ki67 (E) in the nonligated (NL) right (upper panel) and the BDL left (lower panel) lobes was examined by immunohistochemistry. (Original magnification $\times$200.) PCNA and Ki67 indexes were compared (right panel). Data are means $\pm$ SD from at least four independent experiments. *$P < 0.05$ using Student's t test. (C,F) The protein extracts from the NL and BDL lobes were subjected to SDS-PAGE and immunoblotting was performed with anti-PARP, anti-cyclin E, and GAPDH antibodies. The results shown are representative of at least four independent experiments. NS, normal saline.

increased liver injury and susceptibility to TNF-α-induced hepatocyte apoptosis, and decreased hepatocyte regeneration and liver fibrosis with reduced AKT activation. Kupffer cell-derived ASMase was crucial for the AKT activation. The results raise novel therapeutic possibilities for treating liver injury.

After BDL, hepatocytes are exposed to elevated concentrations of bile acid, and hydrophobic bile acids lead to hepatocyte cell death[22] through various factors such as reactive oxygen species (ROS) generation from mitochondria[23] and activation of Fas signaling in a ligand-independent manner by altering cellular trafficking of Fas.[24] Indeed, expression of 4-hydroxy-2-nonenal (HNE), which is produced by lipid peroxidation, was increased on 1 day after the surgery of BDL (data not shown). Because Kupffer cell depletion did not increase the initial liver damage by BDL (1 day after the surgery), it is likely that this damage is induced by a direct toxic effect of bile acid rather than subsequent immune responses because Kupffer cells are not activated in this early stage. The initial hepatocyte cell death stimulates subsequent inflammatory responses leading to further liver injury and fibrosis.[25,26] In BDL liver, the engulfment of apoptotic or necrotic body in Kupffer cells is observed,[27] which leads to production of cytokines including TNF-α and TGF-β.[9] Either a promotive[9] or protective[10] effect of Kupffer cells on BDL-induced liver injury have been reported. In the present study, alendronate treatment, which depleted Kupffer cells in the livers, increased liver injury and reduced fibrosis 10 days after BDL, suggesting that Kupffer cells have a protective effect on the subsequent damage of hepatocytes and a promotive effect on fibrosis in the late stage. The increase of liver injury is probably explained by the diminished Kupffer cell functions, including the phagocytosis of injured tissue and the production of protective factors for hepatocytes. The reduced fibrosis is most likely due to decreased fibrogenic cytokines from Kupffer cells. Cytokines including TGF-β and TGF-α are released from Kupffer cells,[28] and HSCs are stimulated to induce collagen I α1 transcription by TGF-β.[29]

In the liver chronically injured by BDL, hepatocytes represented the survival and regenerative properties, and AKT was a critical factor for the survival and regeneration of the remaining viable hepatocytes. Indeed, overexpression of constitutive active-AKT led hepatocytes to be re-

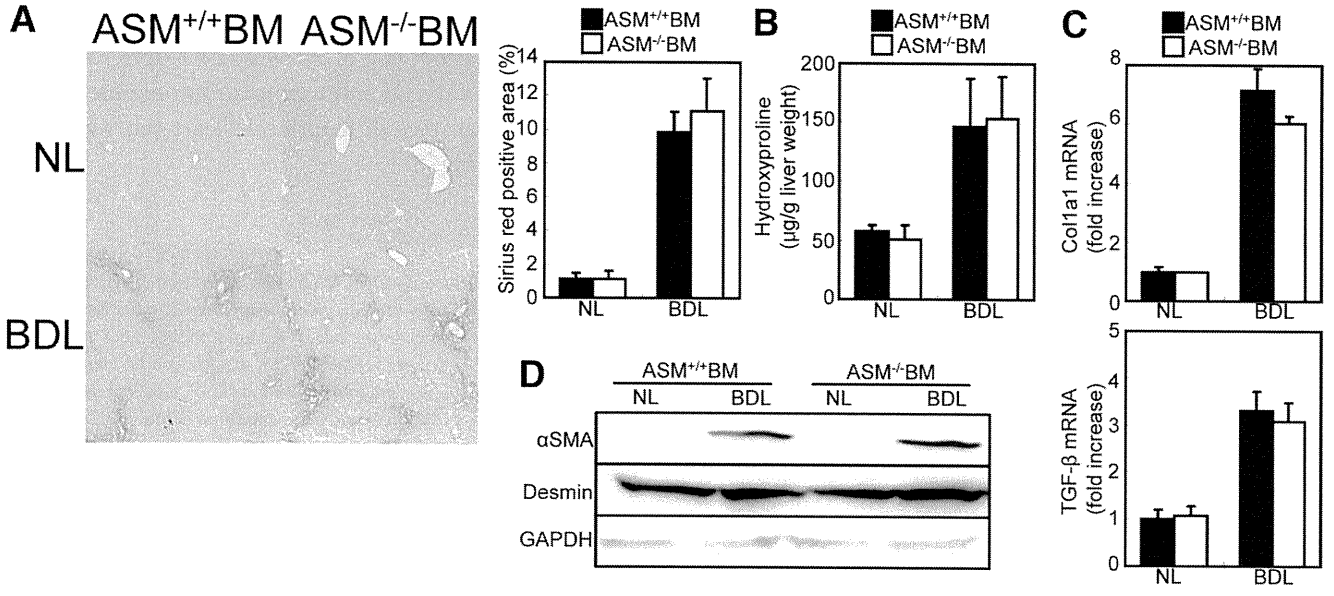

Fig. 6. Reconstitution of Kupffer cells to ASMase knockout cells did not affect the liver fibrosis induced by BDL. The ASMase$^{+/+}$ bone marrow or ASMase$^{-/-}$ bone marrow-transplanted mice were operated on with PBDL and killed 10 days after the surgery. (A,B) Collagen deposition was assessed by Sirius red staining (A) (original magnification ×100; graph, right panel) and measurement of hydroxyproline content (B). (C) mRNA levels of collagen α1(I) (col1a1) and TGF-β1 (TGF-β) in the livers were determined by quantitative real-time RT-PCR. Data are means ± SD from at least five independent experiments. (D) The protein extracts from the livers were subjected to SDS-PAGE and immunoblotting was performed with anti-α-SMA, desmin, and GAPDH antibodies. The results shown are representative of at least three independent experiments.

sistant against TNF-α-induced apoptosis in primary cultured hepatocytes (data not shown) and promoted hepatocyte proliferation by cyclin E.[30] Because depletion of Kupffer cells diminished the survival and regeneration of hepatocytes with reduced AKT activation, Kupffer cells could produce factors that activate AKT in hepatocytes. In our study, the survival and regenerative effects of AKT activation were abrogated in ASMase$^{-/-}$ bone marrow-transplanted mice, suggesting that ASMase in Kupffer cells requires the production of unknown factors that lead to the activation of AKT in hepatocytes. mRNA expression of TNF-α, IL-1β, and IL-6 in ASMase$^{-/-}$ bone marrow-transplanted mice were similar to those in ASMase$^{+/+}$ bone marrow-transplanted mice (Supporting Fig. 5 and data not shown) after BDL. mRNA levels of hepatocyte growth factor (HGF) and heparin-binding ep-

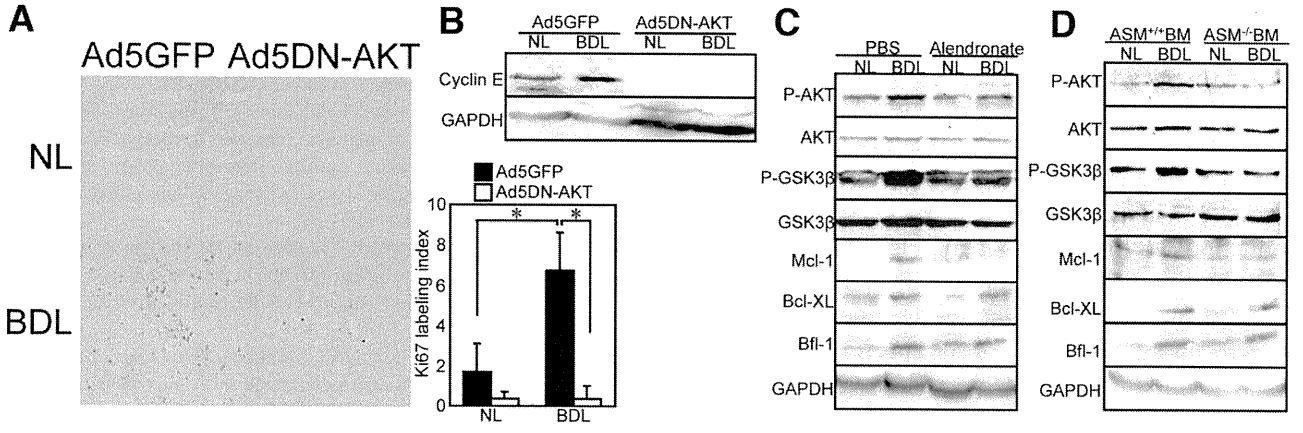

Fig. 7. Kupffer cells and its ASMase are required for AKT activation by BDL. Wildtype mice underwent PBDL and were infected with Ad5GFP or Ad5DN-AKT (A,B) or were treated with Ale-lip or PBS-lip (C). The ASMase$^{+/+}$ bone marrow or ASMase$^{-/-}$ bone marrow-transplanted mice were operated on with PBDL (D). The animals were killed 10 days after the surgery. Expression of Ki67 in the NL right (upper panel) and BDL left (lower panel) lobes were examined by immunohistochemistry (A). (Original magnification ×200.) Ki67 indexes were compared (right panel). Data are means ± SD from at least four independent experiments. *$P < 0.05$ using Student's t test. (B-D) The protein extracts from the livers were subjected to SDS-PAGE and immunoblotting was performed with anti-cyclin E, GAPDH, phosphorylated-AKT, AKT, phosphorylated-GSK3β, GSK3β, Mcl-1, Bcl-XL, and Bfl-1 antibodies. The results shown are representative of at least three independent experiments.

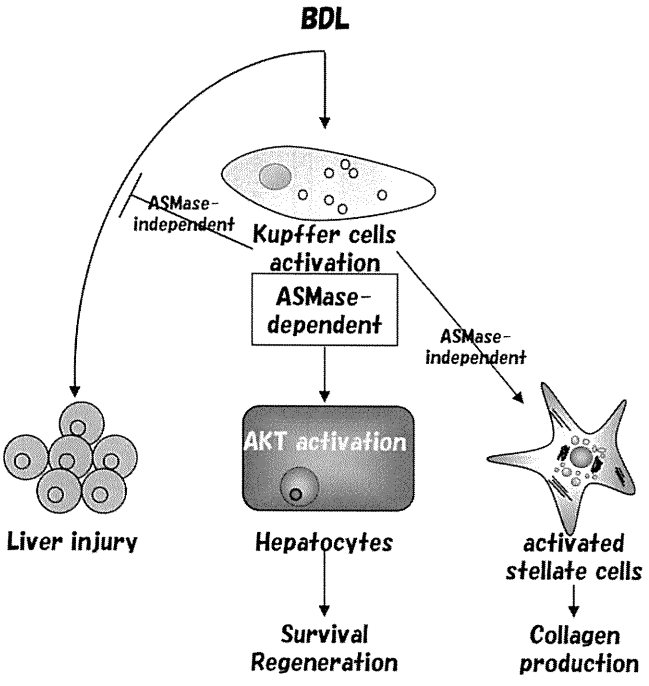

Fig. 8. Hypothetical relations between Kupffer cells and other cells.

ithelial growth factor (HB-EGF), which induce hepatocyte proliferation,[31,32] were not changed in ASMase$^{-/-}$ bone marrow-transplanted mice (Supporting Fig. 7). Accumulation of CD3-positive T cells in BDL lobes in ASMase$^{-/-}$ bone marrow-transplanted mice was also similar to those in ASMase$^{+/+}$ bone marrow-transplanted mice (data not shown). The factors that lead to AKT-dependent hepatocyte protection and regeneration are currently unknown. Further studies are needed to determine these factors.

ASMase has various roles in both parenchymal and nonparenchymal cells. ASMase in hepatocytes modulates hepatocyte apoptosis.[18] Although ASMase in Kupffer cells did not contribute to liver fibrosis, ASMase in HSCs promotes collagen production. Administration of ASMase to human HSCs increased collagen expression. ASMase plus TGF-β treatment further increased collagen production in HSCs (Supporting Fig. 8A). The collagen expression by ASMase is, at least in part, stimulated by way of the modulation of intracellular signals, Smad2/3, downstream targets of TGF-β receptor, and p38, which increases collagen α1(I) mRNA stability in HSCs.[33] The administration of ASMase also phosphorylated p38 (Supporting Fig. 8B). Moreover, exogenous membrane permeable ceramide exerts a stimulatory effect of basal and TGF-β-induced collagen promoter activity in foreskin fibroblast.[34]

In conclusion, Kupffer cells regulate liver injury, hepatocyte survival, regeneration, and fibrosis after chronic liver damage by BDL. AKT activation in hepatocytes, which is induced by way of ASMase of Kupffer cells, is required for the survival and regeneration of hepatocytes. The hypothetical roles of Kupffer cells are schematically summarized in Fig. 8.

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Applied nutritional investigation

Elevated serum tumor necrosis factor-α and soluble tumor necrosis factor receptors correlate with aberrant energy metabolism in liver cirrhosis

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Abstract

Objective: Protein–energy malnutrition is frequently observed in patients with liver cirrhosis and is associated with their poor prognosis. Tumor necrosis factor-α (TNF-α) is elevated in those patients and may contribute to the alterations of energy metabolism. Our aim was to characterize the aberrant energy metabolism in cirrhotic patients with regard to TNF-α.

Methods: Twenty-four patients (mean age 65 ± 6 y) with viral liver cirrhosis who did not have hepatocellular carcinoma or acute infections were studied. Twelve healthy volunteers were recruited after matching for age, gender, and body mass index with the patients and served as controls (59 ± 8 y). Serum levels of TNF-α, soluble 55-kDa TNF receptor (sTNF-R55), soluble 75-kDa TNF receptor (sTNF-R75), and leptin were determined by immunoassay. Substrate oxidation rates of carbohydrate and fat were estimated by indirect calorimetry after overnight bedrest and fasting.

Results: In cirrhotic patients, serum levels of TNF-α, sTNF-R55, and sTNF-R75 were significantly higher than those in the controls and correlated with the increasing grade of disease severity as defined by Child-Pugh classification. Serum leptin concentration was not different between cirrhotics and controls but correlated with their body mass index. The decrease in substrate oxidation rate of carbohydrate and the increase in substrate oxidation rate of fat significantly correlated with serum TNF-α, sTNF-R55, and sTNF-R75 concentrations.

Conclusion: Tumor necrosis factor-α might be associated with the aberrant energy metabolism in patients with liver cirrhosis.

Keywords: Liver cirrhosis; Tumor necrosis factor-α; Soluble 55-kDa tumor necrosis factor receptor; soluble 75-kDa tumor necrosis factor receptor; Leptin; Indirect calorimetry; Protein–energy malnutrition

Introduction

A significant proportion of patients with liver cirrhosis shows protein–energy malnutrition (PEM) [1,2], and PEM leads to poor prognosis in these cases [3–5]. Energy metabolism and nutritional status can be estimated, for example, by indirect calorimetry [5,6] and by anthropometry, such as triceps skinfold thickness (TSF) and arm muscle circumference (AMC) [7]. Impaired energy metabolism significantly correlates with a worse event-free survival of cirrhotics [5,7,8]. However, the mechanisms underlying energy malnutrition in cirrhosis have not been elucidated enough [2]. Candidate causes for this state include enhanced secretion of cytokines such as tumor necrosis factor-α (TNF-α) and adipokines represented by leptin in liver cirrhosis, because both induce anorexia and increase energy expenditure, leading to physical wasting [9–11].

Tumor necrosis factor-α, or cachectin, is a proinflammatory cytokine and is released mainly from monocytes and lymphocytes in response to inflammatory stimuli [12]. TNF-α is also postulated to play a role in the development of anorexia or physical wasting and to regulate energy

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Table 1
Baseline demographic characteristics, body composition, blood biochemistry, and calorimetric data in cirrhotic patients and control subjects*

	Controls ($n = 12$)	Cirrhosis ($n = 24$)	$P^{\dagger}$	Child A ($n = 9$)	Child B ($n = 9$)	Child C ($n = 6$)	$P^{\ddagger}$
Age (y)	58.5 (48–68)	64 (64–75)	0.31	63 (58–69)	67 (63–75)	61 (54–76)	0.10
Male/female	8/4	16/8	0.58	6/3	5/4	5/1	0.90
Height (cm)	168 (153–175)	159 (143–173)	<0.05	159 (145–170)	156 (143–166)	161 (144–173)	0.67
Weight (kg)	62 (54–66)	59 (47–87)	0.36	64 (48–74)	56 (39–67)	59 (47–87)	0.51
Body mass index (kg/m^2)	22.0 (20.8–24.3)	21.3 (17.3–26.9)	0.42	22.9 (19.0–26.9)	22.1 (17.0–29.0)	18.5 (17.7–22.5)	0.11
Arm muscle circumference (%)	101 (85–107)	96 (78–107)	0.59	104 (87–112)	91 (72–108)	95 (78–105)	<0.01
Triceps skinfold thickness (%)	139 (82–200)	92 (46–180)	<0.01	104 (84–181)	92 (46–138)	80 (55–168)	0.05
Total bilirubin (mg/dL)	0.7 (0.4–0.9)	1.4 (0.7–6.6)	<0.01	1.2 (0.7–1.9)	1.4 (0.4–2.5)	3.5 (1.4–6.5)	<0.01
Albumin (g/dL)	4.6 (4.2–4.8)	3.1 (2.4–4.4)	<0.01	3.3 (2.4–4.4)	3.0 (2.5–3.3)	2.8 (2.0–3.4)	0.07
Alanine aminotransferase (IU/L)	18 (8–25)	50 (22–140)	<0.01	71 (22–140)	39 (26–106)	46 (38–69)	0.10
Prothrombin time (%)	98 (94–102)	69 (30–150)	<0.01	83 (55–150)	62 (47–87)	50 (30–65)	<0.01
TNF-α (ng/L)	3.0 (2.0–3.5)	8.3 (5.0–19.0)	<0.01	6.6 (5.0–11.0)	9.4 (7.4–15.1)	12.3 (7.0–19.0)	<0.01
sTNF-R55 (ng/L)	1025 (773–1450)	2505 (1390–5000)	<0.01	1575 (1350–5000)	2680 (2000–5000)	3240 (2840–5000)	<0.01
sTNF-R75 (ng/L)	1805 (1460–2360)	4255 (1840–5000)	<0.01	3210 (1840–5000)	4900 (3920–5000)	4800 (4000–5000)	<0.01
Leptin (μg/L)	4.3 (3.2–5.4)	4.7 (1.5–17.5)	0.95	6.3 (2.9–17.5)	3.8 (1.5–13.9)	2.8 (1.7–8.3)	0.09
REE (kcal/d)	1330 (1140–1450)	1188 (892–1830)	0.41	1507 (981–1830)	1188 (1011–1826)	1655 (1011–1342)	0.36
BMR (kcal/d)	1355 (1163–1428)	1170 (1077–1760)	0.09	1250 (1110–1530)	1160 (850–1380)	1170 (1077–1760)	0.47
npRQ	0.91 (0.87–0.94)	0.84 (0.70–0.97)	<0.01	0.88 (0.84–0.97)	0.83 (0.77–0.86)	0.75 (0.70–0.80)	<0.01
CHO (%)	50.4 (42.0–56.3)	39.9 (18.4–61.6)	<0.01	42.3 (25.7–61.6)	39.7 (18.4–48.7)	25.4 (19.9–30.6)	<0.01
FAT (%)	29.1 (22.6–37.2)	37.5 (23.0–61.6)	<0.01	31.0 (25.9–53.9)	38.0 (23.0–55.5)	61.0 (45.9–61.6)	<0.01
PRO (%)	20.7 (11.6–26.4)	22.7 (5.2–32.3)	0.43	23.8 (11.8–33.0)	25.2 (5.2–32.3)	14.5 (12.9–23.5)	0.08

BMR, basal metabolic rate predicted by the Harris-Benedict formula; Child, Child-Pugh grade; CHO, substrate oxidation rate of carbohydrate; FAT, substrate oxidation rate of fat; PRO, substrate oxidation rate of protein; REE, resting energy expenditure; TNF-α, tumor necrosis factor-α; npRQ, non-protein respiratory quotient; sTNF-R55, soluble 55-kDa tumor necrosis factor receptor; sTNF-R75, soluble 75-kDa tumor necrosis factor receptor.

* Values are expressed as median (range).

† Between controls and cirrhosis by Fisher's exact test or Mann-Whitney U test.

‡ For distribution among Child-Pugh grades by chi-square test or one-way analysis of variance.

metabolism [9,10]. The actions of TNF-α are mediated by two distinct cell-surface receptors, soluble 55-kDa tumor necrosis factor receptor (sTNF-R55) and soluble 55-kDa tumor necrosis factor receptor (sTNF-R75) [12–14]. These two receptors also exist in soluble form after their extracellular domains have been cleaved proteolytically from the cell surface. These soluble receptors can block TNF-α activity by competing with cell-surface TNFR or prolong the biological effect of TNF-α as a buffer system. Because circulating TNFR levels remain elevated for a longer period than TNF-α, it is proposed that sTNFR levels may serve as a more sensitive means of monitoring the activity of the TNF-α system. In previous reports, serum TNF-α, sTNF-R55, and sTNF-R75 concentrations were elevated in cirrhotic patients [15,16], correlating with severity of liver damage [16–18]. However, little has been studied regarding the relation between TNF-α and alternations of energy metabolism in cirrhosis [19].

Leptin, a 16-kDa protein product by adipocytes, is postulated to regulate energy balance by suppressing appetite and increasing energy expenditure [11]. In several studies, serum leptin concentrations were elevated in cirrhotic patients, which suggested that leptin might play a role in the development of anorexia or physical wasting associated with cirrhosis [20–22].

Thus, in this study we aimed to test whether elevated serum concentrations of TNF-α, sTNF-R55, sTNF-R75, and leptin are associated with the aberrant energy metabolism in patients with liver cirrhosis.

Materials and methods

Patients

Twenty-four patients with liver cirrhosis (16 men and 8 women, mean age 65 ± 6 y) participated in this study. Liver cirrhosis was diagnosed by clinical and laboratory profiles and by histologic examination of liver biopsy specimens. Child-Pugh grade of disease severity [23] was A in nine cases, B in nine cases, and C in six cases. Hepatitis C virus was the cause of cirrhosis in all cases. Patients with physically detectable ascites or peripheral edema of moderate to severe grade [8] were excluded. Patients with hepatocellular carcinoma, fever, human immunodeficiency virus infection, overt infectious disease (septicemia, pneumonia, urinary tract infection), renal insufficiency, or under immunomodulatory therapy were also excluded. Clinical profiles of the patients are presented in Table 1. Twelve healthy volunteers were recruited after matching for age, gender, and body mass index (BMI) with the patients and served as controls (eight men and four women, mean age 59 ± 8 y). The study protocol was approved by the institutional review board for human research, and informed consent was obtained from all participants. The study protocol was in agreement with the 1975 Helsinki Declaration as revised in 1983. We matched the patients and the controls not only by age and gender but also by BMI, because obesity is reported to affect significantly the histologic grade of hepatic fat deposition, inflammation, and fibrosis even in

chronic viral hepatitis [24,25], thus reducing the functional reserve of the liver.

Methods

Blood was drawn in the early morning for fasting serum concentrations of TNF-α, sTNF-R55, sTNF-R75, leptin, and routine laboratory examinations on the day of metabolic studies. Serum albumin, total bilirubin, alanine aminotransferase, prothrombin activity, and urinary nitrogen (UN) were measured with a standard clinical analyzer at the central laboratory in our hospital. Serum TNF-α, sTNF-R55, and sTNF-R75 were determined in duplicate with an enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, USA). Serum leptin was measured in duplicate by radioimmunoassay (Linco Research, Inc., St. Louis, MO, USA).

Metabolic studies were carried out using an indirect calorimeter (Deltatrac Metabolic Monitor, Datax Division Instrumentarium Corp., Helsinki, Finland) to estimate non-protein respiratory quotient (npRQ), resting energy expenditure (REE), and substrate oxidation rates of carbohydrate (%CHO), fat (%FAT), and protein (%PRO) from measured oxygen consumption per minute (Vo_2), carbon dioxide production per minute (Vco_2) and total UN using the following equations [26–28].

$$REE = 5.50\ Vo_2 + 1.76\ Vco_2 - 1.99\ UN$$

$$CHO(g/24\ h) = 5.926\ Vo_2 - 4.189\ Vco_2 - 2.539\ UN$$

$$FAT(g/24\ h) = 2.432\ Vo_2 - 2.432\ Vco_2 - 1.943\ UN$$

$$PRO(g/24\ h) = 6.250\ UN$$

$$npRQ = (1.44 \times Vco_2 - 4.890\ UN)/(1.44 \times Vo_2 - 6.04\ UN)$$

$$\%CHO = CHO \times 4.18/REE \times 100$$

$$\%FAT = FAT \times 9.46/REE \times 100$$

$$\%PRO = PRO \times 4.32/REE \times 100$$

Measurements were performed between 07:00 and 09:00 h while the patients were still lying in bed. The last meal was served 18:00 h on the previous day. The basal metabolic rate was predicted by the Harris-Benedict formula [29].

We measured height and body weight and calculated BMI. Arm circumference and TSF were measured with an Insertape and Adipometer (Abbott Japan Co., Ltd., Tokyo, Japan), and AMC was estimated. Anthropometric data were standardized according to age- and gender-stratified Japanese anthropometric reference data [30] and expressed as percentages of TSF and AMC.

Statistical analysis

Values were expressed as median and range. Comparisons between groups were analyzed using Mann-Whitney U or Kruskal-Wallis non-parametric test. Comparison between measured REE and corresponding Harris-Benedict prediction was performed by paired *t* test. The relation among blood test parameters and substrate oxidation rates or Child-Pugh grade was determined by Spearman's correlation.

All analyses were carried out using JMP 5.0 (SAS Institute, Cary, NC, USA) and statistical significance was accepted at $P < 0.05$.

Results

Serum concentrations of TNF-α, sTNF-R55, sTNF-R75, and leptin

Cirrhotic patients had significantly lower %TSF and %AMC in parallel with the increasing Child-Pugh score (Table 1), suggesting the presence of PEM in these subjects.

The median serum concentrations of TNF-α, sTNF-R55, and sTNF-R75 were significantly higher in cirrhotic patients than in controls (Table 1). Serum TNF-α, sTNF-R55, and sTNF-R75 levels correlated with the increasing grade of disease severity as defined by the Child-Pugh classification in patients with liver cirrhosis (Table 1, Fig. 1).

Serum leptin concentration did not differ between cirrhotic patients and controls or correlate with Child-Pugh score (Table 1), but correlated with their BMI (Fig. 2).

Energy metabolism

Measured REE (1188 kcal/d, 892–1830 kcal/d) was significantly higher than the basal metabolic rate (1170 kcal/d, 1077–1760 kcal/d) predicted by the Harris-Benedict formula in liver cirrhosis ($P < 0.01$), whereas both agreed well in control subjects.

The npRQ in patients with liver cirrhosis (0.84, 0.70–0.97) was significantly lower than that in control subjects (0.91, 0.87–0.94, $P < 0.01$). Decrease in npRQ was brought about by a significantly lower oxidation rate of carbohydrate and a higher oxidation rate of fat in patients with liver cirrhosis compared with control subjects (Fig. 3). A decrease in %CHO and an increase in %FAT significantly correlated with the progression of disease severity in patients with liver cirrhosis as defined by the Child-Pugh classification (Fig. 3).

Correlation between serum cytokine levels and oxidation rates of nutrients in patients with liver cirrhosis

Inverse correlations were found between %CHO and serum concentrations of TNF-α, sTNF-R55, and sTNF-R75 (Fig. 4, Table 2). Significant correlations were observed between %FAT and serum concentrations of TNF-α, sTNF-R55,

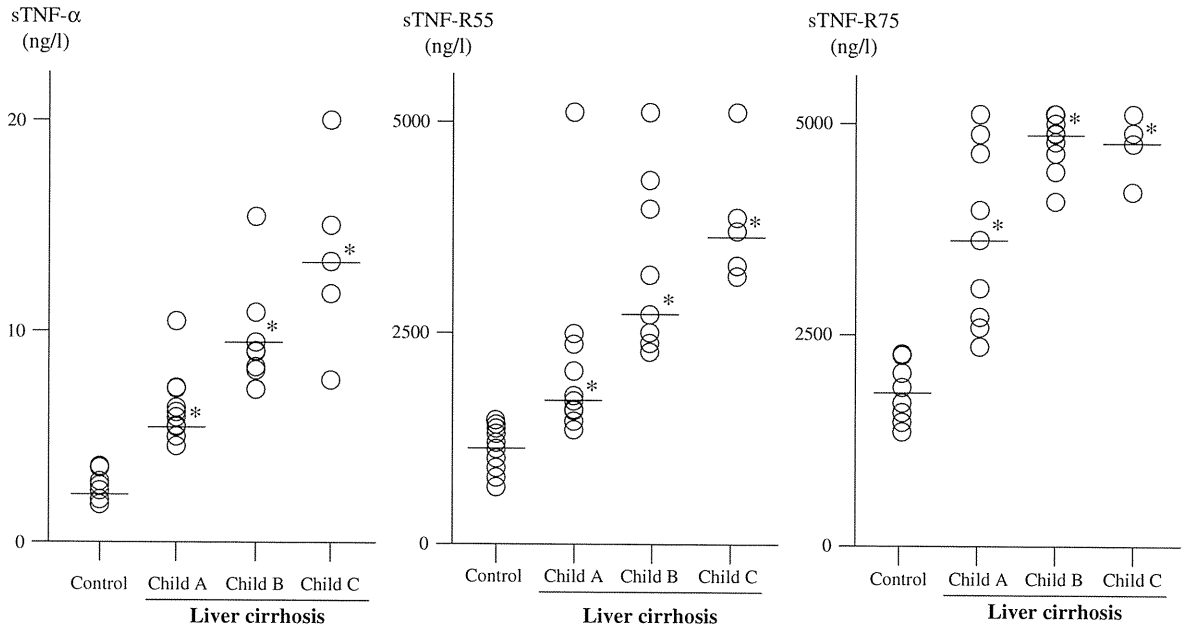

Fig. 1. Serum levels of TNF-α, sTNF-R55, and sTNF-R75 in control subjects ($n = 12$) and in patients with liver cirrhosis ($n = 24$) graded by Child-Pugh classification ($n = 9, 9$, and 6 for grades A, B, and C, respectively). Horizontal line indicates the median. * $P < 0.05$ compared with control. Child, Child-Pugh grade; sTNF-R55, soluble 55-kDa tumor necrosis factor receptor; sTNF-R75, soluble 75-kDa tumor necrosis factor receptor receptor; TNF-α, tumor necrosis factor-α.

and sTNF-R75 (Fig. 5, Table 2). However, %PRO did not correlate with serum concentrations of these cytokines.

Other intercorrelations among cytokines, leptin, substrate oxidation rates, and Child- Pugh grade in patients with liver cirrhosis are presented in Table 2. Serum leptin did not correlate with %CHO or %FAT. Serum leptin did not correlate with TNF-α, sTNF-R55, or sTNF-R75.

Correlations of serum cytokine and leptin levels with parameters of liver damage in cirrhotic patients

The serum concentrations of TNF-α, sTNF-R55, and sTNF-R75 correlated significantly with Child-Pugh score (Table 2), prothrombin time, total bilirubin, and albumin in patients with liver cirrhosis. However, serum leptin level showed no correlation with such parameters (data not shown).

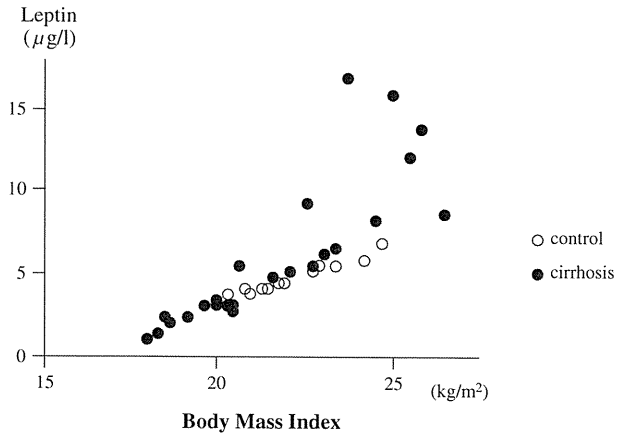

Fig. 2. Correlation between body mass index and serum leptin level in cirrhotic patients (closed circles) and controls (open circles; $n = 36$, $r = 0.735$, $P < 0.05$).

Discussion

Because the liver plays a central role in fuel and energy metabolism, PEM is common in patients with liver cirrhosis [1,2]. Recently, several studies have elucidated the relation between PEM and event-free survival in patients with liver cirrhosis [3–5,31,32]. Thus, PEM is an important outcome marker and a therapeutic target in cirrhotic patients.

It has been reported that the fasting oxidation rate of glucose is decreased and that of fat is increased in patients with liver cirrhosis [5,6,26]. Such aberrant energy metabolism consequently decreases npRQ in cirrhotics [5,6,26]. It also has been reported that this decrease of npRQ closely correlates with survival in patients with liver cirrhosis [5]. As confirmed in this study, npRQ in liver cirrhosis was significantly lower than that in control subjects. The decrease in npRQ was brought about by a significantly lower oxidation rate of carbohydrate and higher oxidation rate of fat in patients with liver cirrhosis compared with control subjects. A decrease in %CHO and an increase in %FAT significantly correlated with the progression of disease severity in patients with liver cirrhosis as defined by the Child-Pugh classification. These characteristics of energy metabolism in liver cirrhosis agree well with those in previous reports [5,6,26], suggesting that the patients recruited in the present study can be regarded as representative of a general cirrhotic cohort. Regarding

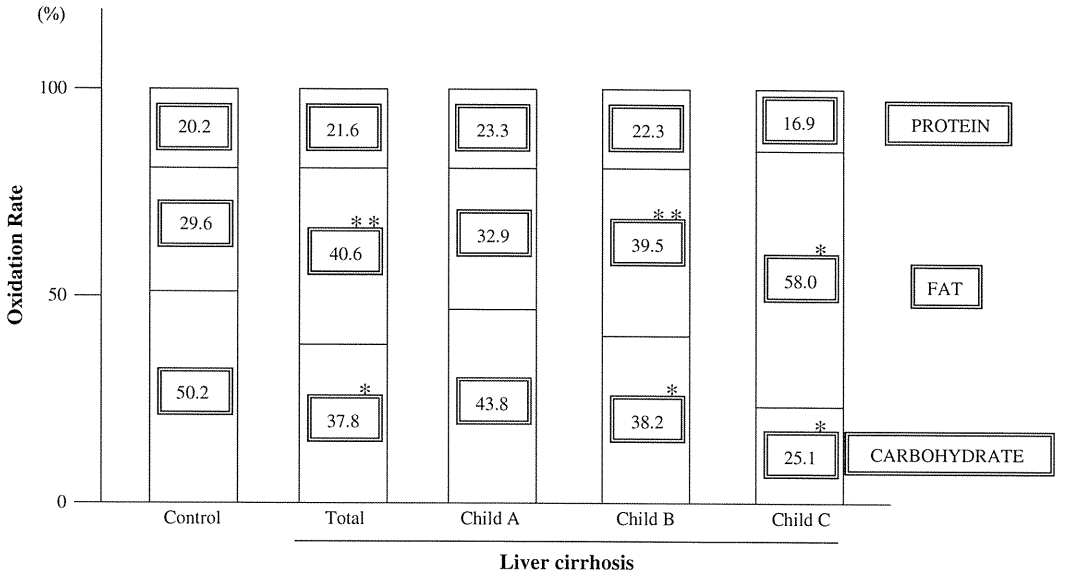

Fig. 3. Substrate oxidation rates of protein, fat, and carbohydrate in control subjects ($n = 12$) and in patients with liver cirrhosis ($n = 24$) graded by Child-Pugh classification ($n = 9$, 9, and 6 for grades A, B, and C, respectively). Values are expressed as mean. * $P < 0.05$, ** $P < 0.01$ compared with control. Child, Child-Pugh grade.

%TSF as another parameter of energy nutrition, the patients actually showed a significantly lower value than the controls with a similar BMI. However, we should point out the possibility that the patients could have had an overestimated BMI due to the presence of excess fluid before the appearance of detectable ascites or moderate to severe peripheral edema.

Tumor necrosis factor-α is a proinflammatory cytokine and postulated to regulate energy metabolism [9,10,33]. In previous reports, TNF-α increased glucose (~10%) and free fatty acid (~126%) turnover and raised REE (~34%) [9,10,33]. The actions of TNF-α are mediated by two distinct cell-surface receptors. We measured not only serum TNF-α concentration but also sTNF-R55 and sTNF-R75 concentrations for a couple of reasons. First, it is known that sTNF-R shedding is induced by the same stimuli that activate TNF-α production. Second, it has been shown that sTNF-R levels remain elevated for a longer time than TNF-α itself. Taken together, sTNF-R levels could confirm the stimulated state of the TNF-α system and could reflect the state until later after stimuli.

In this study, serum TNF-α, sTNF-R55, and sTNF-R75 concentrations were significantly higher in cirrhotic patients

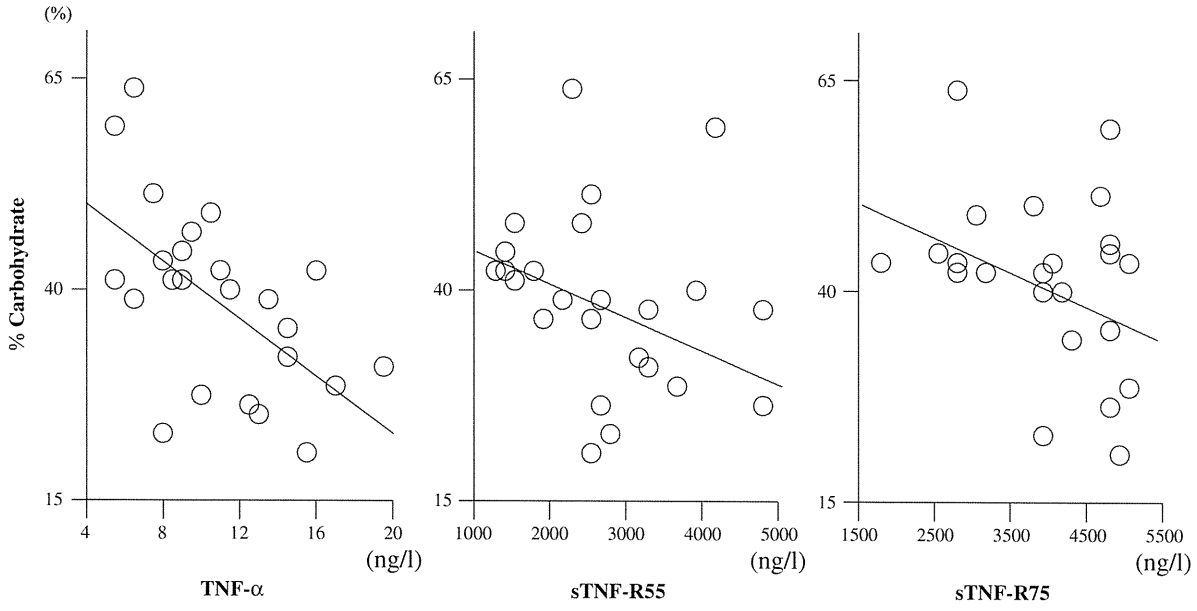

Fig. 4. Correlation between the oxidation rate of carbohydrate and serum TNF-α, sTNF-R55, or sTNF-R75 in patients with liver cirrhosis ($n = 24$). For correlation coefficients and statistical significance, see Table 2. sTNF-R55, soluble 55-kDa tumor necrosis factor receptor; sTNF-R75, soluble 75-kDa tumor necrosis factor receptor receptor; TNF-α, tumor necrosis factor-α.

Table 2
Intercorrelation coefficients among cytokines, leptin, substrate oxidation rates, and Child-Pugh score in patients with liver cirrhosis

	TNF-α	sTNF-R55	sTNF-R75	%CHO	%FAT	Leptin
sTNF-R55	0.32					
sTNF-R75	0.43*	0.85†				
%CHO	−0.56†	−0.44*	−0.41*			
%FAT	0.72†	0.61†	0.61†	−0.82†		
Leptin	−0.15	−0.01	0.06	0.08	−0.02	
Child-Pugh score	0.63†	0.61†	0.61†	−0.64†	0.78†	−0.36

%CHO, substrate oxidation rate of carbohydrate; %FAT, substrate oxidation rate of fat; TNF-α, tumor necrosis factor-α; sTNF-R55, soluble 55-kDa tumor necrosis factor receptor; sTNF-R75, soluble 75-kDa tumor necrosis factor receptor.

* $P < 0.05$.

† $P < 0.01$.

than in controls, and they correlated with the increasing grade of disease severity as defined by the Child-Pugh classification in patients with liver cirrhosis. Among damaged liver functions in cirrhosis, the high activity of the TNF-α system may particularly be related to a decrease in hepatic clearance of endotoxins, the presence of portosystemic shunts, and the systemic spillover of intestinal endotoxins by portal hypertension [15,34]. Another explanation is that the presence of chronic viral inflammation could be the reason for the elevated proinflammatory cytokines including TNF-α [35,36].

Moreover, inverse correlations were found between %CHO and serum concentrations of TNF-α, sTNF-R55, and sTNF-R75 in this study. Significant correlations were observed between %FAT and serum concentrations of TNF-α, sTNF-R55, and sTNF-R75. As described previously, TNF-α induces free fatty acid oxidation more efficiently than glucose oxidation [6,9,10]. In addition, the production of adenosine triphosphate by β-oxidation of free fatty acids is much greater than that by glucose oxidation. Hence, TNF-α seems to raise %FAT more directly and to contribute to whole aberrant energy metabolism subsequently in liver cirrhosis by altering the proportion of fat oxidation to carbohydrate oxidation.

Serum leptin is another candidate factor to alter energy balance by its wasting action and is reported to be actually high in patients with liver cirrhosis [11,20–22]. However, in this study, serum leptin concentration did not differ between cirrhotic patients and controls. Moreover, serum leptin level showed no correlation with energy metabolism parameters measured by indirect calorimetry. Most previous studies regarding leptin recruited patients with alcoholic cirrhosis [20,22], whereas all patients in our investigation had cirrhosis caused by hepatitis C virus. This difference in the cause of liver damage may account for the disagreement between previous reports and the present one.

Regarding the correlation between TNF and aberrant energy metabolism, we should point out a possibility that the correlation may merely arise from an association between cytokine levels and Child-Pugh grade, as shown in Figure 1, and that between substrate oxidation rates and the grade, as presented in Table 2. To support our hypothesis that the correlation between TNF and energy metabolism is independent of the degree of liver damage, we statistically tested the relation by confining subjects to those with each Child-Pugh grade. In each subgroup that was controlled for grade, TNF and %FAT retained the significant correlation (e.g., $r = 0.81, P < 0.01$ in Child-Pugh grade A). These results strengthen our statement

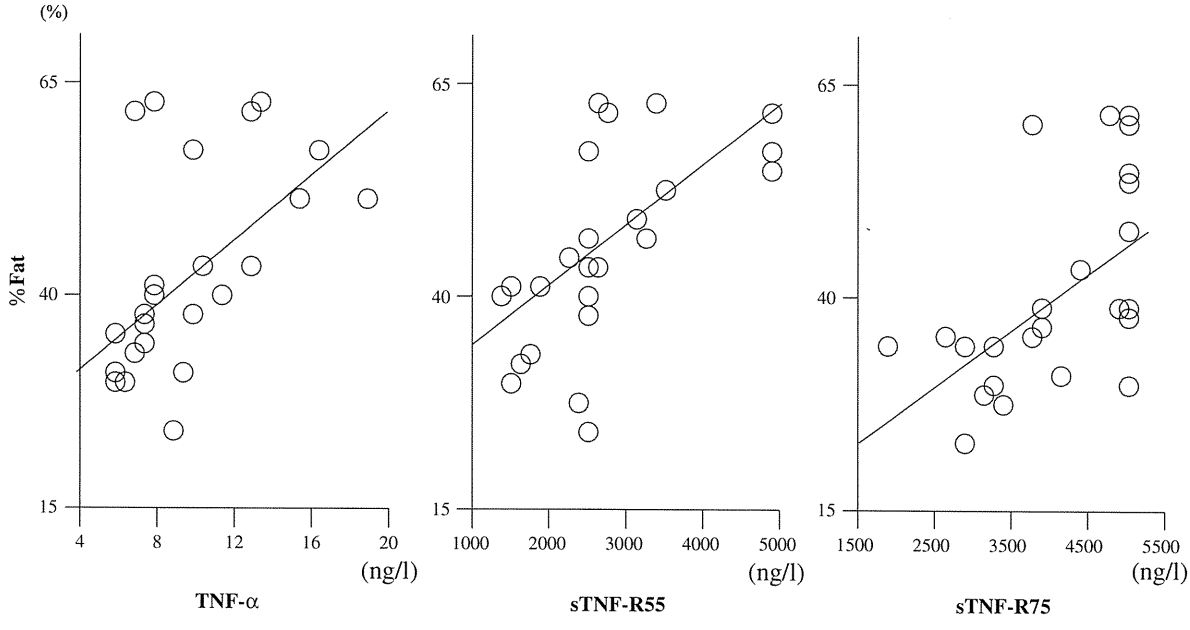

Fig. 5. Correlation between the oxidation rate of fat and serum TNF-α, sTNF-R55, or sTNF-R75 in patients with liver cirrhosis ($n = 24$). For correlation coefficients and statistical significance, see Table 2. sTNF-R55, soluble 55-kDa tumor necrosis factor receptor; sTNF-R75, soluble 75-kDa tumor necrosis factor receptor receptor; TNF-α, tumor necrosis factor-α.

that TNF-α might be associated with aberrant energy metabolism in patients with liver cirrhosis.

Taken together, it seems more likely that the high activity of the TNF-α system mediates the aberrant energy metabolism in patients with liver cirrhosis. To directly demonstrate this possibility, future clinical trials would be required to test whether or not the therapy to decrease the high TNF-α activity might correct the aberrant energy metabolism in patients with liver cirrhosis, by using such modalities as antibiotics and lactulose that control the intestinal bacterial flora or anti–TNF-α antibody.

In summary, the TNF-α system in patients with liver cirrhosis was activated and correlated with disease activity. The significant correlation of serum TNF-α, sTNF-R55, and sTNF-R75 with the aberrant energy metabolism suggested that activation of the TNF-α system contributed to cirrhosis-associated PEM.

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