Pathological roles of bone marrow-derived stellate cells in a mouse model of alcohol-induced fatty liver

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Fujimiya T, Liu J, Kojima H, Shirafuji S, Kimura H, Fujimiya M. Pathological roles of bone marrow-derived stellate cells in a mouse model of alcohol-induced fatty liver. Am J Physiol Gastrointest Liver Physiol 297: G451–G460, 2009. First published July 16, 2009; doi:10.1152/ajpgi.00055.2009.—Chronic alcohol consumption activates hepatic stellate cells (HSCs) and causes fatty degeneration in the liver. However, the origin of HSCs and the mechanism of fatty changes of the liver have not been fully elucidated. Here, we examined the roles of bone marrow-derived cells (BMDCs) in a mouse model with chronic alcohol consumption. We performed bone marrow transplantation from transgenic mice expressing green fluorescence protein (GFP) to female wild-type and ROSA mice (B6.129S7-Gt(26Sor)J, transgenic mice expressing β-galactosidase, β-gal) and treated them with ethanol (EtOH) for 8 or 16 wk. GFP-expressing BMDCs increased in the liver with EtOH treatment in a time-dependent manner. In response to excess alcohol consumption, ~68% of the BMDCs became activated HSCs in that they expressed α-smooth muscle actin. Meanwhile, ~67% and ~66% of these BMDCs expressed Tnf-α and transforming growth factor (Tgf)-β1, respectively, and the activities were further supported by the excessive mRNA expression of Tnf-α and Tgf-β1 in RT-PCR, respectively. Cell fusion occurs between BMDCs and nonparenchymal cells but scarcely occurs between BMDCs and hepatocytes, demonstrated by double staining of β-gal/GFP and further supported by the Y-chromosome staining. The EtOH withdrawal normalized most of the abnormalities produced by chronic alcohol consumption. These results indicate that excess alcohol consumption stimulates both the homing of HSCs from the bone marrow and their profibrogenic cytokine production in a mouse model of alcohol-induced fatty liver disease.

bone marrow transplantation; ethanol; liver fibrosis; hepatic stellate cell

EXCESS ALCOHOL CONSUMPTION accounts for more than half of the cases of liver fibrosis and cirrhosis (26, 28–29). Ethanol and/or its toxic metabolites possess fibrogenic properties (24), and the liver fibrosis is a consequence of the hepatic wound-healing response to the repeated injury and the loss of parenchymal tissue (4, 11). The central event leading to liver fibrosis is an activation of hepatic stellate cells (HSCs). Upon activation, the HSCs assume a myofibroblast-like conformation and, under the influence of cytokines such as tumor necrosis factor (TNF-α) and/or transforming growth factor-β (TGF-β1), produce fibril-forming type I collagen (4, 10). Before the onset of well-established alcoholic liver fibrosis, the liver becomes steatotic (alcoholic fatty liver disease, AFLD) and then undergoes steatohepatitis (alcoholic steatohepatitis) (29). Even in the steatotic stage caused by ethanol, the biochemical reactions toward the activation of HSCs and hepatic fibrogenesis are ongoing (23).

Bone marrow-derived cells (BMDCs) are known to play important roles in parenchymal regeneration and liver fibrosis (14, 21). Carbon tetrachloride or thioacetamide is reported to stimulate the direct differentiation of BMDCs to HSCs (68%) and myofibroblasts (70%) and to induce liver fibrosis (25). In bile duct ligation-induced liver fibrosis models, collagen-producing fibrocytes were also recruited from the bone marrow (15, 17). Kupffer cells have been implicated as mediators of alcoholic liver injury through their release of Tnf-α, free radicals, and other inflammatory cytokines in response to alcohol and lipopolysaccharide (31). Tnf-α produced by activated Kupffer cells may contribute to HSC activation by inducing apoptosis of hepatocytes (2), thereby forming apoptotic bodies that have been implicated in fibrogenesis (5). In addition, the HSCs activated by Kupffer cells showed an increase of mRNA expressions of α-smooth muscle actin (α-SMA) and type I collagen by a coculture of Kupffer cells and HSCs in vitro (19). Although both HSCs and Kupffer cells might be recruited from the bone marrow in an AFLD model, their roles have seldom been investigated.

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Fig. 1. Chronic ethanol (EtOH) exposure protocol.

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In this experiment, we hypothesized that the BMDCs might play important pathogenic roles as key players in the progression of alcohol-induced liver disease. Thus we examined the roles of bone marrow-derived HSCs and Kupffer cells using a mouse model of AFLD.

MATERIALS AND METHODS

Animals and bone marrow transplantation. Seven-week-old female wild-type (C57BL/6Jcl; CLEA Japan, Tokyo, Japan) mice, female ROSA mice [B6.129S7-Gt(ROSA)26Sortm1/J, transgenic mice expressing β-galactosidase, β-gal; Jackson Laboratory, Bar Harbor, ME], and male green fluorescence protein (GFP)-Tg mice [C57BL/6-Tg (UBC-GFP) 30 Scha, Jackson Laboratory] were used. For bone marrow transplantation (BMT), 20 ROSA and 30 wild-type mice were irradiated (9 Gy) and injected with 4 × 10^6 bone marrow cells isolated from GFP-Tg mice (13, 18). Engraftment was measured 6 wk after transplant by fluorescence-activated cell sorting analysis of peripheral blood using an antibody against Ly-5.2 (clone 104; Pharmingen, San Diego, CA). Peripheral blood engraftment of bone marrow cells ranged from 61 to 81%. Two ROSA and eight wild-type mice died of BMT, and the mortality rate was 20%.

Chronic exposure to ethanol. Four weeks after BMT, the mice were divided into six groups (Fig. 1). The groups were as follows: 1) 8-wk ethanol treatment mice (EtOH-8), 2) control for the EtOH-8 mice (Control), 3) 16-wk ethanol treatment mice (EtOH-16), 4) control for the EtOH-16 mice (Control), 5) 8-wk ethanol treatment and 8-wk recovery mice (Recovery), and 6) control for the recovery mice (Control). The mice were housed individually and fed standard mouse pellet chow (3.6 kcal/1 g pellet). In the first week of the experiment, all mice were given water. In the second week, the ethanol-fed group was given 5 g/dl ethanol to become accustomed to the treatment and then given 10 g/dl ethanol for 7 or 15 wk (see Fig. 1). To maintain a similar caloric intake among the groups, the groups were pair fed. The control group was offered an amount of diet equal to the previous week’s mean diet consumed by the ethanol-fed group.

All animal experiments were carried out under the procedural and ethical guidelines of and were approved by the Yamaguchi University School of Medicine; experiments were controlled by the committee’s guidelines for animal experiments (#13-010). The research conformed to the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85–23, revised 1996).

Fluorescence immunohistochemistry and confocal laser scanning microscopy. The mice were systemically perfused with 4% paraformaldehyde in 0.1 M PBS via the left ventricle of the heart, and their livers were then taken out. Three-micron-thick sections were embedded on matusnami adhesive silane (MAS)-coated glass slides for the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23, revised 1996).

For immunofluorescence staining, the following antibodies were used: glial fibrillary acidic protein (GFAP, goat polyclonal; Santa Cruz Biotechnology), and Tgf-β1 (goat polyclonal; Santa Cruz Biotechnology) as markers of cytokine. For double staining of Tnf-α/β-

Table 1. Primers and RT-PCR conditions used for detection of TNF-α, TGF-β1, and GAPDH gene expression

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5'→3')</th>
<th>GenBank Accession No.</th>
<th>Annealing T°, °C</th>
<th>Cycle</th>
<th>Product, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNF-α</td>
<td>Forward: 5'–CACGTCGGTACCAACCCACTCCG-3'</td>
<td>NM_013693</td>
<td>57</td>
<td>28</td>
<td>210</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5’–CATACGAAATGCGCTGACGGT-3’</td>
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<tr>
<td>TGF-β1</td>
<td>Forward: 5'–TGGAGGCTGAGTTTGGGCT-3’</td>
<td>NM_011577</td>
<td>55</td>
<td>25</td>
<td>170</td>
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<tr>
<td></td>
<td>Reverse: 5’–GGTCGAGTTTGGGCTGAGTC-3’</td>
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<tr>
<td>GAPDH</td>
<td>Forward: 5'–AAGCGACGCCCTATTCACTGAC-3’</td>
<td>NM_008084</td>
<td>55</td>
<td>25</td>
<td>190</td>
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<tr>
<td></td>
<td>Reverse: 5’–TCAAGAGACATCAAGGAC-3’</td>
<td></td>
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TNF, tumor necrosis factor; TGF, transforming growth factor.
SMa, Tnf-α/CD68, Tgf-β1/α-SMA, or Tgf-β1/CD68, the sections were incubated with a mixture of first antibodies diluted at 1:1,000 in PBST for 48 h at 4°C and further incubated with a mixture of FITC-labeled anti-rabbit IgG (Chemicon, Temecula, CA) and Cy3-labeled anti-goat IgG (Chemicon) diluted at 1:1,000 in PBST for 2 h at room temperature. To obtain overlap images of bone marrow-derived GFP-positive cells and β-gal, GFAP, α-SMA, Tnf-α, and Tgf-β1, the sections were incubated with the first antibody and further incubated with the second antibody of the corresponding animal source. The nuclei were stained with TO-PRO-3 iodide (Molecular Probes, Carlsbad, CA). Finally, the sections were observed under confocal laser scanning microscopy. To further define the specificity of the immune reactions, the sections were stained with fluorescein-labeled second antibodies without first antibodies.

The images of HE, Sudan III, and Azan stainings were obtained with a DXM 1200F digital camera with a Nikon Eclipse E600 microscope (Nikon, Tokyo, Japan). Images of immunofluorescence staining were obtained by confocal laser scanning microscopy (LSM5 Pascal / Version 3.2; Carl Zeiss Microimaging, Zeiss, Germany).

Quantitative morphometry. The density of GFP-positive cells in the liver was obtained as follows. Images under confocal microscopy were transmitted to the image-analyzing system MetaMorph (Ver.5.1; Nikon Roper, Chiba, Japan). A mean value of the area percentage of the liver was obtained as follows. Images under a confocal microscope at 20 objective. Values represent the means ± SD of data obtained from four animals. The total number of liver cells (number of total nuclei, Fig. 2), total number of GFP-positive cells (GFP-expressing cells, which include nuclei, Fig. 2B), total number of immunopositive cells (immunofluorescence-expressing cells, which include nuclei, Fig. 2A), and total number of overlapped cells between GFP-positive cells and immunopositive cells (overlapped cells, which include nuclei, Fig. 2C) were each counted in 30 consecutive regions under a confocal microscope at ×100 objective. The ratio of each value was calculated as a percentage of immune-reaction-positive cells, and the values are given as means ± SD of four animals.

Analysis of tissue mRNA expression. Total RNA was isolated from liver tissue from the control group, the EtOH-8 group, and the recovery group, respectively, and the complimentary DNA was synthesized by reverse transcription. The conditions for the PCR reaction to detect Tnf-α, Tgf-β1, and the GAPDH gene expression are shown in Table 1. The mRNA expression of each protein was semiquantified by real-time PCR within a linear range. The identity of the PCR products was confirmed by direct sequencing.

Fluorescence in situ hybridization (FISH) for Y-chromosome staining. We used the same protocol for chromosome staining as that of our previous study (13). Briefly, liver sample quick frozen in liquid nitrogen was cut into 3-μm-thick sections and mounted on MAS-coated glass slides. Sections were fixed with 1% PFA in sterile saline for 15 min at 4°C, washed with deionized water, and incubated with 0.5 mg/ml pepsin solution for 10 min at 37°C. Sections were washed with SSC, dehydrated with series of ethanol, and air dried. They were
denatured by 70% formamide solution for 10 s at 65°C and dehydrated by series of ethanol. The sections were covered with denatured hybridization probe (FITC-labeled mouse chromosome Y paint probe; ID Laboratories, London, ON, Canada) and incubated overnight at 37°C. The sections were further incubated with 60% formamide solution for 15 min at 37°C, washed with 0.05% Tween-20 solution, and air dried. Then, the sections were stained with TO-PRO-3 iodide for nuclear staining and observed under confocal laser scanning microscopy.

Statistical analysis. All values were expressed as means ± SD. Continuous variables among the groups were tested using one-way ANOVA. When an F value was found to be significant by ANOVA, the Bonferroni/Dunn post hoc test was used for multiple comparisons. A value of \( P < 0.05 \) was considered statistically significant. Statistical analysis was performed using StatView for Windows version 5.0 (SAS Institute, 1998, Cary, North Carolina).

RESULTS

Animal characteristics. The energy consumption, body weight, and wet weight of the liver are shown in Table 2. Total ethanol consumption in the EtOH-16 group was twice as high as in the EtOH-8 and recovery groups. No difference was found in total food intake and body weight between the control and EtOH groups in both EtOH-8 and EtOH-16. The wet weight of the liver was increased in the EtOH-16 group compared with the control group. The wet weight of the liver also showed an upward trend in the EtOH-8 group and returned to normal after EtOH withdrawal in the recovery group; however, the statistical analysis found no difference.

Histological changes in the liver in EtOH-treated mice. Liver histology after chronic ethanol consumption was performed using HE, Sudan III, and Azan staining (Fig. 3). The accumulation of small lipid droplets throughout the hepatocytes was observed in the sections stained with HE and Sudan III. This change was more pronounced in the EtOH-16 group than in the EtOH-8 group and totally disappeared in the recovery group. Azan staining showed that fibrosis was only observed around the terminal hepatic venule in both the...
EtOH-8 and EtOH-16 groups, and this change disappeared in the recovery group. Inflammatory cell infiltration was not found in the liver in either the EtOH-8 or the EtOH-16 group (Fig. 3).

Immunohistochemical analysis showed that the number of α-SMA+ (a marker for activated HSCs), Tnf-α−, and Tgf-β1+ cells was significantly increased in the EtOH-8 group compared with the controls (Fig. 4, A–C). However, the number of CD68+ (a marker of Kupffer cells) cells was not changed (Fig. 4, A–C). Immunofluorescence overlap staining showed that the Tnf-α− cells coexpressed α-SMA or CD68, and there were more α-SMA+/Tnf-α+ cells than CD68+/Tnf-α+ cells in both the EtOH-8 group and the controls (Fig. 4, A and D). The Tgf-β1+ cells also coexpressed α-SMA or CD68, and there were more Tgf-β1+/α-SMA+ cells than Tgf-β1+/CD68+ cells in both the EtOH-8 group and the controls (Fig. 4, A and E).

**BMDCs in the liver in EtOH-treated mice.** To examine the contribution of BMDCs to the EtOH-induced fatty liver, we transplanted bone marrow isolated from GFP-Tg to ROSA. In the livers of the EtOH-8 and EtOH-16 groups, the number of GFP-expressing BMDCs increased significantly compared with the controls in a time-dependent manner and returned to normal levels after recovery (Fig. 5, A and B). Immunofluorescence overlap staining was used to evaluate the cell fusion, and the results showed that the coexpression of GFP (BMDCs, a marker of donor origin) and β-gal (a marker of recipient origin) was detected in nonparenchymal cells (Fig. 5, C and E). A percentage (3.8 ± 1.1%) of nonparenchymal cells in the EtOH-8 group and 6.2 ± 0.8% in the EtOH-16 group was double positive for GFP and β-gal. The cell fusion between BMDCs and nonparenchymal cell was further supported by the chromosome staining using the FISH method (Fig. 5D). GFP and Y-chromosome were not detected in the hepatocytes after an extensive search in our model (Fig. 5, C and D), indicating that cell fusion between hepatocytes and BMDCs was very rare in our AFLD mice model.

**BMDC contribution to activated HSCs.** The contribution of BMDCs to GFAP+ cells or α-SMA+ cells was examined by immunofluorescence overlap staining (Figs. 6A and 7A). The number of GFAP+ cells or α-SMA+ cells was increased in the EtOH-8 and EtOH-16 groups in a time-dependent manner and returned to normal levels in the recovery group (Figs. 6B and
A percentage (70 ± 7%) of GFP<sup>+</sup> cells in the EtOH-8 group, 80 ± 5% of GFP<sup>+</sup> cells in the EtOH-16 group, and 17 ± 5% of GFP<sup>+</sup> cells in the recovery group was also the GFAP<sup>+</sup> cells. A percentage (55 ± 5%) of the GFP<sup>+</sup> cells in the EtOH-8 group, 68 ± 8% of GFP<sup>+</sup> cells in the EtOH-16 group, and 43 ± 11% of GFP<sup>+</sup> cells in the recovery group was also the α-SMA<sup>+</sup> cells (Fig. 7C).

The contribution of BMDCs to the Tnf-α<sup>+</sup> cells or Tgf-β1<sup>+</sup> cells was examined by immunofluorescence overlap staining (Figs. 8A and 9A). The number of Tnf-α<sup>+</sup> cells or Tgf-β1<sup>+</sup> cells was increased in the EtOH-8 and EtOH-16 groups in a time-dependent manner and returned to normal levels in the recovery group (Figs. 8C and 9C). GFP<sup>+</sup> (47 ± 5%) cells in the EtOH-8 group, 67 ± 6% of GFP<sup>+</sup> cells in the EtOH-16 group, and 21 ± 10% of GFP<sup>+</sup> cells in the recovery group were also the Tnf-α<sup>+</sup> cells (Fig. 8D). GFP<sup>+</sup> (43 ± 2%) cells in the EtOH-8 group, 66 ± 13% of GFP<sup>+</sup> cells in the EtOH-16 group, and 17 ± 3% of GFP<sup>+</sup> cells in the recovery group were also the Tgf-β1<sup>+</sup> cells (Fig. 9D). The increases in Tnf-α and Tgf-β1 mRNA expression in EtOH-treated liver were confirmed by RT-PCR (Figs. 8B and 9B), respectively.

**DISCUSSION**

In the present study, mice were voluntarily permitted to consume alcohol in liquid form and given a nutritionally replete diet. Under the treatment of EtOH, fatty degeneration of hepatocytes was predominantly observed throughout the liver. This degeneration worsened in a time-dependent manner and was reduced by the ethanol withdrawal. However, fibrosis formation in the liver tissues was mild, and inflammatory cell infiltration was not observed in the liver of animals receiving EtOH treatment. Therefore, our model in this study showed AFLD instead of alcoholic steatohepatitis or liver fibrosis. We examined the involvement of HSCs in the pathogenesis of AFLD in bone marrow-transferred mice and found that the density of cells positive for GFAP (a marker of quiescent and activated stellate cells) and α-SMA (a marker for activated HSCs) were significantly increased. Both Tnf-α and Tgf-β1...
overlapped with α-SMA-positive or CD68-positive cells, suggesting that both cytokines were produced from the activated HSCs as well as from the Kupffer cells in the AFLD model; the number of activated HSCs increased significantly in the AFLD model compared with the Kupffer cells. These results indicate that the activated HSCs seem to play more important roles in the pathogenesis of the AFLD than Kupffer cells.

Proinflammatory cytokines, especially TNF-α and TGF-β1, are important mediators in hepatic inflammation and fibrogenesis (7, 33). They act as potent profibrogenic cytokines through the activation of HSCs, the promotion of extracellular matrix, and the suppression of hepatocyte proliferation (5, 10–11, 22). TNF-α in particular are involved not only in liver fibrosis but also in the early steatotic stage of alcoholic liver disease because the mice lacking the TNF-α receptor 1 have been resistant to steatosis caused by chronic EtOH treatment (33). It is generally known that TNF-α is produced from Kupffer cells and TGF-β1 is produced from HSCs (3, 12, 16). However, in our study by immunofluorescence staining, GFP-expressing BMDCs produced Tnf-α and Tgf-β1, and the ratio of Tnf-α and Tgf-β1-producing cells was significantly increased in AFLD models. These data indicated that most, though not all, of the activated HSCs were recruited from the bone marrow and that they produce both Tnf-α and Tgf-β1 in AFLD.

In a certain form of liver disease, BMDCs play important pathological roles for normal hepatic function (17). BMDCs contribute to the regeneration of hepatic parenchymal cells through cell fusion (13) and also to the pathogenesis of liver fibrosis by differentiating into nonparenchymal cells in the liver (10, 11). Conversely, BMDCs can contribute to the regression of liver fibrosis by expressing matrix metalloproteinase or hepatocyte growth factor (1, 14). The bone marrow origin of activated HSCs has been investigated in liver fibrosis models through chronic treatment with CCl4, with the finding that most of the activated HSCs were bone marrow derived (17, 25). On the other hand, the bone marrow origin of activated HSCs has been denied in other liver fibrosis models by bile duct ligation in which collagen-producing fibroblasts
were bone marrow derived but did not express α-SMA, an activated HSC marker (25). In the present study, we performed BMT from GFP mice (transgenic mice that constitutively express GFP) into ROSA mice (transgenic mice that constitutively express β-gal) because using mice with BMT from a different genotype is helpful for examining whether cell fusion occurs (13). We observed overlapping of GFP-positive and β-gal-positive reactions in nonparenchymal cells but little overlapping in hepatocytes, indicating that BMDCs fused with nonparenchymal cells but not with hepatocytes. Cell fusion was further supported by the Y-chromosome staining by FISH methods. The number of GFP-expressing cells in the liver increased in a time-dependent manner and normalized after the ethanol withdrawal.

The activated HSCs occupied 30% of BMDCs in the controls but 76% in EtOH-16 mice by immunofluorescence overlap staining, suggesting that the increased population of activated HSCs in the liver seems to be recruited from the bone marrow in AFLD models. The present study has partly helped in gaining new insights into the mechanism of alcoholic liver disease though further experiments on the changes in the bone marrow cells and endogenous cells during the ethanol exposure are needed to better elucidate the recruitment propensity from the bone marrow during the development and/or progression of alcoholic liver disease.

There was a substantial infiltration of the liver with α-SMA-expressing cells; however, fibrosis formation in the liver tissues was mild. The exact mechanism is poorly understood. Current animal models of alcoholic liver disease have provided many valuable findings (27); however, in general, these models do not produce the type of end-stage liver failure, such as liver fibrosis or cirrhosis, observed in patients with alcoholic liver disease (27). The development of new and innovative animal models is needed to better elucidate the development and/or progression of alcoholic liver fibrosis.

In conclusion, the present study demonstrated that chronic alcohol consumption stimulated the recruitment of Tnf-α and/or Tgf-β1-producing activated HSCs from the bone mar-
row to the liver. The results indicate that excess alcohol consumption causes the migration of BMDCs to the liver in response to liver injury and reprograms gene expression in BMDCs. This leads to the increased formation of profibrogenic cytokines, which are responsible for activation of bone marrow-derived HSCs, the key cell type in liver fibrosis.

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