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Supporting Information

The following Supporting Information can be found in the online version of the article:

Supporting Information 1. Materials and Methods. ***aGalCer injection in vivo. Serum ALT activity.***

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Role of TNF-α Produced by Nonantigen-Specific Cells in a Fulminant Hepatitis Mouse Model

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In previous studies, the mechanisms of acute liver injury and virus exclusion have been examined using a model wherein HBsAg-specific CTL are injected into HBsAg transgenic (Tg) mice. The importance of the role of TNF-α in virus exclusion was shown, but its role in liver injury was unclear. We crossed the TNF-α knockout mouse and HBsAg-Tg mouse to establish the HBsAg-Tg/TNF-α KO mouse, and examined the influence of TNF-α on liver injury. The severity of liver damage, as determined by serum alanine aminotransferase activity, was ~100 times greater in HBsAg-Tg/TNF-$\alpha^{+/+}$ than in HBsAg-Tg/TNF-$\alpha^{-/-}$ mice after i.v. administration of 5×10^6 CTLs. This liver damage reached the peak of its severity within 24–48 h, and was restored 7 days later. Histopathological examination showed hepatocellular necrosis and inflammatory cell infiltrate 24 h after the CTL injection in HBsAg-Tg/TNF-$\alpha^{+/+}$ mice but not in HBsAg-Tg/TNF-$\alpha^{-/-}$ mice. The liver damage was fatal for all HBsAg-Tg/TNF-$\alpha^{+/+}$ mice that received 1.5×10^7 CTLs. In contrast, 1.5×10^7 CTLs could not kill the HBsAg-Tg/TNF-$\alpha^{-/-}$ mice. The TNF-α production level was enhanced after the CTL injection in not only intrahepatic macrophages but also other types of mononuclear cells from non-HBsAg-Tg/TNF-$\alpha^{+/+}$ mice. An adoptive transfer examination revealed that severe liver damage occurred in HBsAg-Tg/TNF-$\alpha^{-/-}$ mice that had received mononuclear cells from TNF-$\alpha^{+/+}$ mice. In conclusion, the present study provides evidence that TNF-α produced by intrahepatic non-Ag-specific inflammatory cells is critical in the development of lethal necroinflammatory liver disease. *The Journal of Immunology,* **2009, 182: 391–397.**

Hepatitis B virus (HBV)[2] is a nonlytic virus that does not directly infect cells and cause damage. Liver damage and viral clearance after an HBV infection are thought to be mediated by the host's cellular immune response to viral Ags (1). $CD8^+$ CTL play a critical role in the liver damage and viral clearance in HBV infections. These effector functions include the secretion of cytokines, such as IFN-γ and TNF-α, as well as cytolytic activity mediated by perforin and granzyme B (2–5). HBsAg transgenic (Tg) mice show no symptoms of liver disease until the adoptive transfer of HBsAg-specific CTLs, after which they develop a necroinflammatory liver disease that is histologically similar to acute viral hepatitis in man (6). The first step, which begins within 1 h of CTL administration, involves Ag recognition by the CTLs and delivery of a signal that results in the death of the hepatocyte. In the second step, which begins in 4–12 h, the CTLs recruit many host-derived inflammatory cells in their immediate vicinity, resulting in the formation of necroinflammatory foci. The third step is detectable 24–72 h after CTL administration: the livers display massive hepatocellular necrosis and an inflammatory cell infiltrate that consists principally of host-derived mononuclear cells and pronounced sinusoidal lining cell hyperplasia, which resembles the histopathological changes observed in patients dying from liver failure due to HBV-induced fulminant hepatitis. Using this murine fulminant hepatitis model, various studies have been conducted on viral clearance and liver disease in HBV infection.

TNF-α production is one of the earliest events in many types of liver injuries, and it triggers the production of other cytokines that together recruit inflammatory cells, kill hepatocytes, and initiate a hepatic healing response that includes fibrogenesis. The serum levels of TNF-α are significantly increased in patients with fulminant hepatitis (7). In viral hepatitis, elevated levels of plasma TNF-α and soluble TNFR are frequently observed (8). TNF-α is known to be released mainly by macrophages, but it also released by $CD4^+$ and $CD8^+$ T, B, NK (9), and dendritic cells (10). In particular, recent studies have shown that TNF-α is also released by CTLs (3) and contributes to CTL-mediated cytotoxicity, although its cytolytic activity is not as high as those of perforin and the Fas ligand (11–13).

The role of TNF-α is important not only in virus exclusion, but also in liver injury. However, in this model, it is considered that many host-derived inflammatory cells recruited by CTLs are critical in massive hepatocellular necrosis. In the present study, we crossed the TNF-α knockout (TNF-$\alpha^{-/-}$) mouse with the HBsAg-Tg mouse, to establish the HBsAg-Tg/TNF-$\alpha^{-/-}$ mouse, and examined the influence of TNF-α produced by non-Ag-specific inflammatory cells on liver injury in a murine viral fulminant hepatitis model.

Materials and Methods

Mice

Male B10.D2 (H-2^d) mice (age, 6–8 wk; weight, 25–30 g) were obtained from Japan SLC. The HBsAg-Tg mice lineage 107-5D (official designation

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[2] Abbreviations used in this paper: HBV, hepatitis B virus; Tg, transgenic; sALT, serum alanine aminotransferase; MNC, mononuclear cell.

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Tg (Alb-1, HBV) Bri66; inbred B10.D2, H-2^d), in which the HBV envelope coding region is under the control of the mouse albumin promoter, was provided by Dr. F. V. Chisari (Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA). TNF-$\alpha^{-/-}$ mice were produced by gene targeting as described previously (14) and backcrossed onto B10.D2 (H-2^d). HBsAg-Tg/TNF-$\alpha^{-/-}$ mice were produced by backcrossing TNF-$\alpha^{-/-}$ mice with 107-5D.

Cell lines and reagents

P815 cells expressing HBV-preS1, 2, and S (P815preS1), and HBsAg-specific, $CD8^+$ CTL clones (6C2) were provided by Dr. F. V. Chisari (Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA). The clones are H-2d restricted, and can recognize an epitope (IPQSLDSWWTSL) located between residues 28 and 39 of HBsAg. Five days after the last stimulation with irradiated P815preS1, the cells were washed and injected i.v. into HBsAg-Tg mice. (However, the phenotype of the 6C2, which we used in the present study, was changed in cytokines production. Therefore, we termed it 6C2-08.) Recombinant murine IFN-γ was obtained from R&D Systems.

Disease model

Five days after the last stimulation, the 6C2-08 cells were washed three times, suspended in PBS, and injected i.v. into HBsAg-Tg, non-Tg and HBsAg-Tg/TNF-$\alpha^{-/-}$ recipients. Hepatocellular injury was monitored biochemically by measuring serum alanine aminotransferase (sALT) activity. At appropriate time points, mice were killed by cervical dislocation and necropsy was performed. Tissue samples were fixed in 10% formalin, embedded in paraffin, and sectioned; the sections were then stained with H&E. The intrahepatic distribution of HBV Ags was assessed by the indirect immunoperoxidase method using 3-amino-9-ethyl carbazole.

Preparation of hepatic mononuclear cells and hepatocytes

Hepatic mononuclear cells (MNCs) (15) and hepatocytes (16) were isolated using the described simplified technique. The liver was perfused with liver perfusion and digestion medium (Life Technologies). Hepatocytes were separated from the MNCs by centrifugation at 50 × *g* for 2 min and were washed twice in complete RPMI 1640 (Nikken Biomedical Laboratory). MNC populations were purified using M-SMF (JINRO).

Real-time PCR

Total RNA was isolated and transcribed into cDNA using an RNeasy Mini Kit and an Omniscript Reverse Transcriptase Kit (Qiagen GmbH). The resulting cDNA was used as a template for real-time PCR along with primer/probe sets for TNF-α, TNFR1, and TNFR2 (TaqMan Gene Expression Assays; Applied Biosystems) and 2× TaqMan Universal PCR Master Mix (Applied Biosystems), according to the manufacturer's recommendations. The primer/probe sets for 18S were used as an internal control in each reaction (Applied Biosystems). Real-time PCR data were analyzed using the sequence detector software (Applied Biosystems).

TNF-α detection by ELISA

Macrophages, NK cells, CD4 T cells, and CD8 T cells were obtained from intrahepatic MNCs of HBsAg-Tg/TNF-$\alpha^{+/+}$ mice treated with 6C2 cells with the help of an immunomagnetic separation system (MACS System), and cultured for 24 h. The concentrations of TNF-α in the culture supernatant were determined by an enzyme-linked absorbent assay kit for TNF-α (R&D Systems), according to the manufacturer's instruction. The experimental results are expressed as the mean of triplicates ± SD of three independent experiments.

Intracellular cytokine staining

For intracellular staining, the hepatic MNCs from the mice that were administered 6C2 cells were incubated for 4 h with brefeldin A (10 μg/ml). Then, these cells were fixed, permeabilized the Cytofix/Cytoperm buffer (BD Pharmingen), and stained with FITC-conjugated anti-mouse TNF-α (clone MP6-XT22; eBioscience). Samples were acquired on a FACStar flow cytometer and data analysis was conducted using the CellQuest software (BD Pharmingen).

Adoptive cell transfer

HBsAg-Tg/TNF-$\alpha^{-/-}$ mice received CD4-positive splenocytes, CD8-positive splenocytes, DX5-positive splenocytes, and CD11b-positive splenocytes as well as non-CD4, non-CD8, non-DX5, and non-CD11b splenocytes (8×10^6/mouse), which were isolated by MACS from the non-HBsAg-Tg/TNF-$\alpha^{+/+}$ mice 7 days before the CTL injection. To confirm the existence of the transferred cells, we labeled the isolated cells with CFSE and performed flow cytometric analysis. Approximately 4% of IHLs were CFSE positive at 7 days after the injection. Hepatocellular injury was monitored biochemically by measuring the sALT activity.

Statistics

Values are expressed as means ± SEM. Differences between the experimental and control groups were analyzed by the Kruskal-Wallis test followed by Scheffe's F test. Significance was established at $p < 0.05$.

Results

Induction of fulminant hepatitis by HBsAg-specific CTLs in HBsAg-Tg/TNF-$\alpha^{+/+}$ mice, but not in HBsAg-Tg/TNF-$\alpha^{-/-}$ mice

CTL clones 6C2-08 (1×10^6) were incubated with 1×10^6 irradiated stimulator cells (P815-preS1) in complete medium without EL-4 supernatant. 6C2-08 were collected 12 h after stimulation and analyzed for IFN-γ and TNF-α mRNA expression by 6C2-08 (Fig. 1*A*). The IFN-γ and TNF-α mRNA expression was enhanced in 6C2-08 after stimulation. HBsAg-Tg/TNF-$\alpha^{+/+}$ and HBsAg-Tg/TNF-$\alpha^{-/-}$ mice received a single i.v. injection of CTLs (5×10^6 cells/mouse). As shown in Fig. 1*B*, severe liver damage (as determined by the sALT levels) was observed in HBsAg-Tg/TNF-$\alpha^{+/+}$ mice that were administered CTLs, but not in HBsAg-Tg/TNF-$\alpha^{-/-}$ or non-HBsAg-Tg/TNF-$\alpha^{+/+}$ mice. Histological changes in the livers of HBsAg-Tg/TNF-$\alpha^{+/+}$, HBsAg-Tg/TNF-$\alpha^{-/-}$, and non-HBsAg-Tg/TNF-$\alpha^{+/+}$ mice were examined on days 2 and 5 after the CTL injection. A histological analysis revealed widely scattered necroinflammatory foci, containing mostly mononuclear cells and apoptotic hepatocytes in the livers of HBsAg-Tg/TNF-$\alpha^{+/+}$ mice after the CTL injection (Fig. 1*C*). In contrast, in HBsAg-Tg/TNF-$\alpha^{-/-}$ mice, invasion of a large amount of inflammatory cells and hepatocellular necrosis were not observed. Next, to confirm the HBsAg expression in hepatocytes after the CTL injection, we stained the liver tissues with anti-HBsAg mAb 5 days after the CTL injection. Immunohistopathological examination revealed that HBsAg expression in the hepatocytes of HBsAg-Tg/TNF-$\alpha^{+/+}$ mice was down-regulated compared with that of HBsAg-Tg/TNF-$\alpha^{-/-}$ mice.

CTL dose-dependent induction of liver disease and mortality in HBsAg-Tg/TNF-$\alpha^{+/+}$ and HBsAg-Tg/TNF-$\alpha^{-/-}$ mice

Disease severity was also strictly dependent on the CTL dose in the HBsAg-Tg/TNF-$\alpha^{-/-}$ recipient mice (Fig. 2*A*). Liver damage was detectable biochemically at CTL doses above 1.5×10^6 CTLs per mouse in the HBsAg-Tg/TNF-$\alpha^{-/-}$ mice. The resultant liver damage (as determined by the sALT levels) at a high dose (1.5×10^7 CTLs per mouse) was not severe enough to be fatal (1900 ± 187 IU/L). In contrast, in HBsAg-Tg/TNF-$\alpha^{+/+}$ mice, the liver damage was severe enough to be fatal within 96 h in two of six recipients (33.3%) who were administered 0.8×10^7 HBsAg-specific CTLs, and it was fatal for all seven mice (100%) that received 1.5×10^7 CTLs (Fig. 2*B*).

TNF-α production by hepatic macrophages, NK cells, CD4 T cells, and CD8 T cells in HBsAg-Tg/TNF-$\alpha^{+/+}$ mice after the CTL injection

As shown in from several publications (7, 17), patients with fulminant hepatitis have increased serum TNF-α levels. Therefore, we next isolated macrophages, NK cells, CD4 T cells, and CD8 T cells from the intrahepatic MNCs of HBsAg-Tg/TNF-$\alpha^{+/+}$ mice that had received HBsAg-specific CTLs by immunomagnetic separation and examined which cell fractions produced TNF-α. Twenty-four hours after the CTL injection, the TNF-α mRNA expression was enhanced in all the cell fractions, and it was decreased

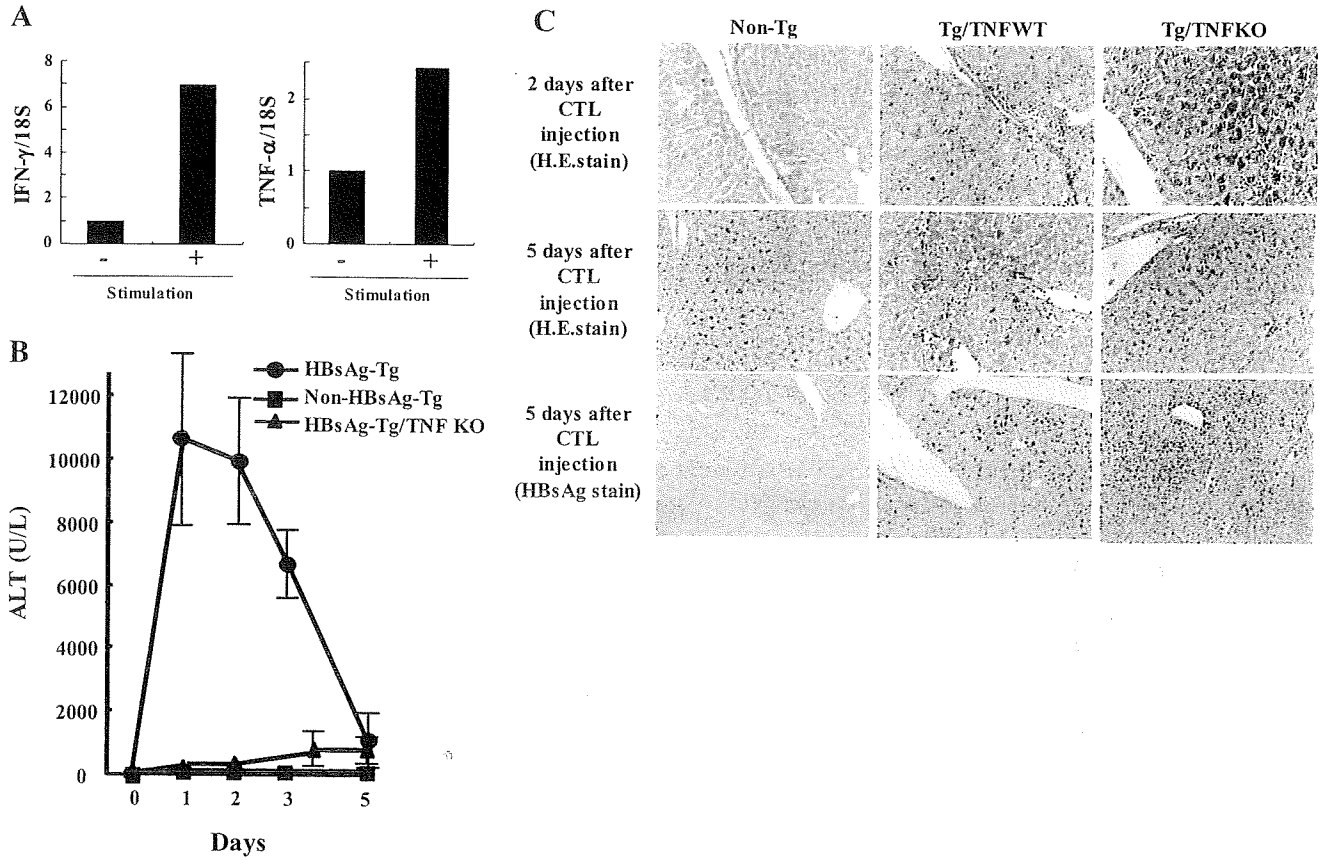

FIGURE 1. CTL-induced liver disease in HBsAg-Tg/TNF-$\alpha^{+/+}$ and HBsAg-Tg/TNF-$\alpha^{-/-}$ mice. *A*, CTL clones 6C2-08 (1×10^6) were incubated with 1×10^6 irradiated stimulator cells (P815-preS1) in complete medium without EL-4 supernatant. 6C2-08 were collected 12 h after stimulation and analyzed for IFN-γ and TNF-α mRNA expression by 6C2-08. *B*, Serum ALT activity was analyzed at varying time points relative to the injection of 5×10^6 HBsAg-specific CTLs into groups of three HBsAg-Tg/TNF-$\alpha^{+/+}$ mice, HBsAg-Tg/TNF-$\alpha^{-/-}$ mice, and non-HBsAg-Tg/TNF-$\alpha^{+/+}$ mice. *C*, Histopathological characteristics of HBsAg-Tg/TNF-$\alpha^{+/+}$ mice and HBsAg-Tg/TNF-$\alpha^{-/-}$ mice livers observed at 2 and 5 days (hematoxylin and eosin, ×200) after CTL administration in these mice. The intrahepatic HBsAg content was demonstrated by immunohistochemical staining for HBsAg in the lower panel.

48 h after the CTL injection (Fig. 3*A*). Next, we measured the TNF-α protein production levels in hepatic MNCs after the administration of HBsAg-specific CTLs by ELISA and intracellular cytokine staining. As shown in Fig. 3*B*, the level of TNF-α secreted by CD11b-positive cells and DX5-positive cells from HBsAg-Tg/TNF-$\alpha^{+/+}$ mice treated with CTLs was increased

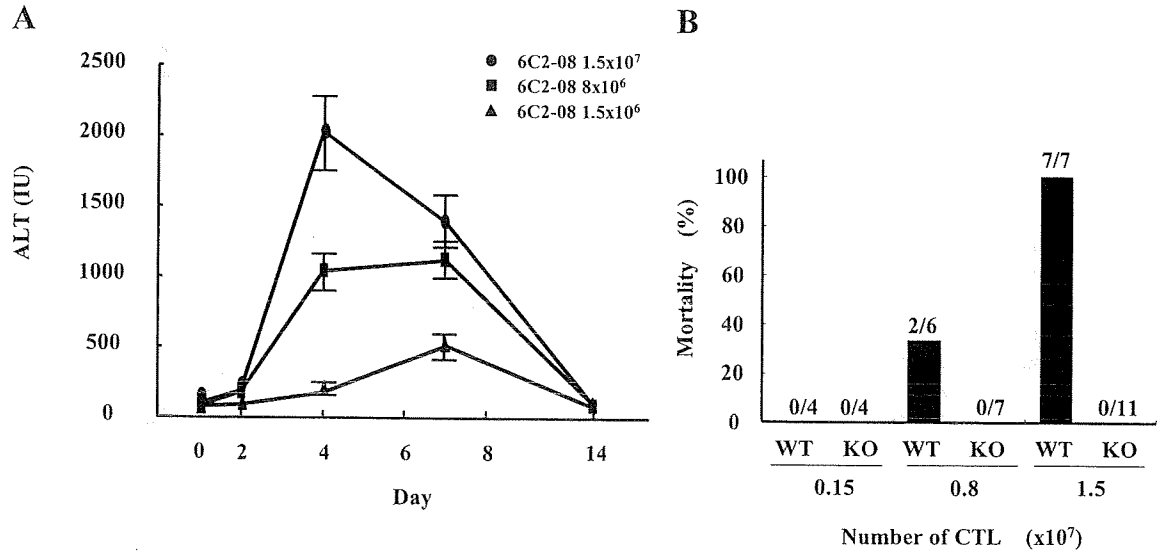

FIGURE 2. CTL-induced liver disease and mortality depended on the number of CTLs administered in HBsAg-Tg/TNF-$\alpha^{+/+}$ and HBsAg-Tg/TNF-$\alpha^{-/-}$ mice. *A*, Serum ALT activity was monitored at 0, 2, 4, 6, 8, and 14 days after the injection of varying doses of HBsAg-specific CTLs into HBsAg-Tg/TNF-$\alpha^{-/-}$ mice. *B*, The results reflect the mortality after i.v. injection of 1.5×10^6, 8×10^6, and 1.5×10^7 CTLs into HBsAg-Tg/TNF-$\alpha^{+/+}$ and HBsAg-Tg/TNF-$\alpha^{-/-}$ mice.

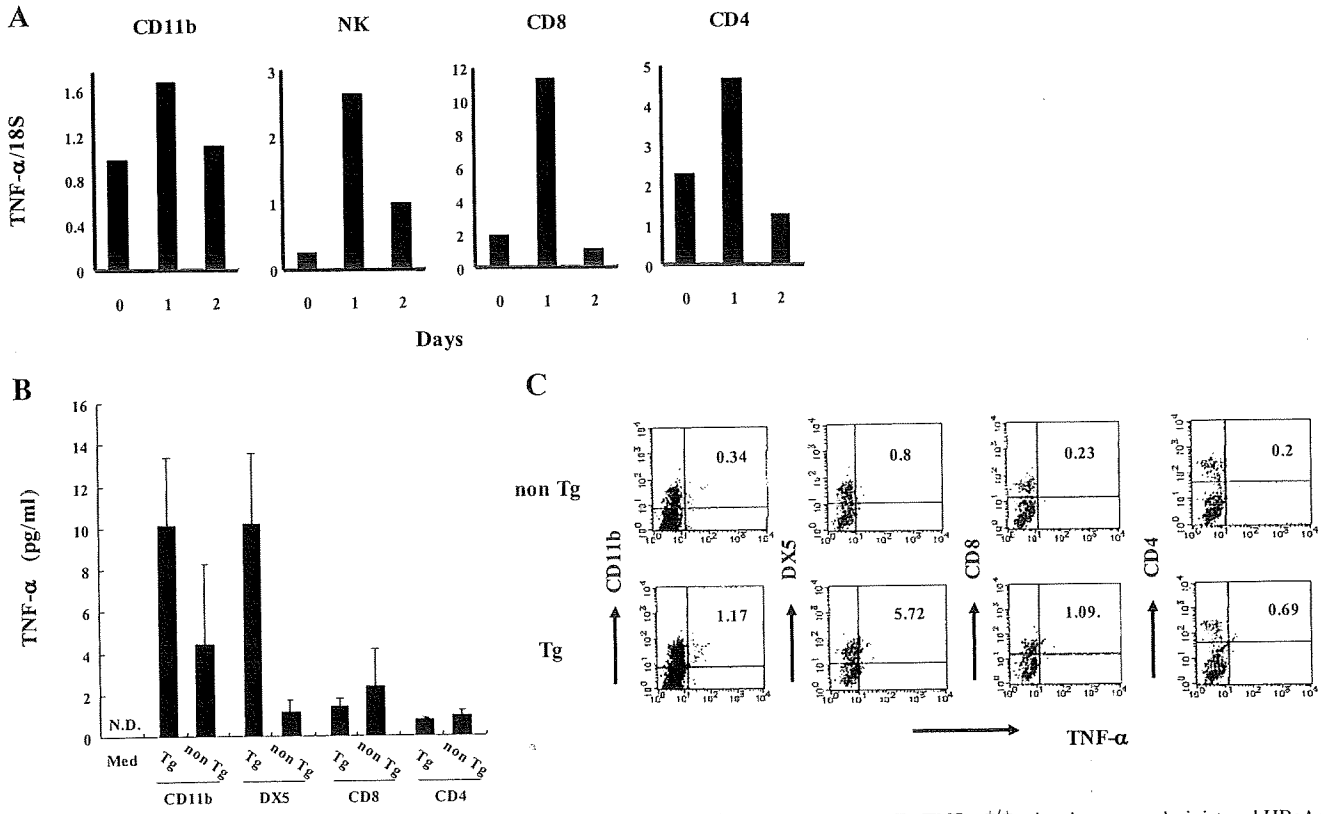

FIGURE 3. TNF-α production by intrahepatic CD11b$^+$, NK$^+$, CD8$^+$, and CD4$^+$ cells from HBsAg-Tg/TNF-α$^{+/+}$ mice that were administered HBsAg-specific CTLs. *A*, TNF-α mRNA expression by intrahepatic CD11b$^+$, NK$^+$, CD8$^+$, and CD4$^+$ cells from HBsAg-Tg/TNF-α$^{+/+}$ mice that were administered CTLs. Hepatic CD11b$^+$, NK$^+$, CD8$^+$, and CD4$^+$ cells were purified with the MACS system (purity of each cell fraction, >95%). The mRNA levels for TNF-α were normalized to that of 18S mRNA. Representative charts derived from the analyses of three mice per group. *B*, Hepatic CD11b$^+$, NK$^+$, CD8$^+$, and CD4$^+$ cells from HBsAg-Tg/TNF-α$^{+/+}$ mice that were administered HBsAg-specific CTLs were purified by the MACS system. These cells were cultured for 24 h. TNF-α concentrations in the culture supernatants were measured by ELISA. *C*, Flow cytometric analysis for intracellular TNF-α produced by hepatic CD11b$^+$, NK$^+$, CD8$^+$, and CD4$^+$ cells obtained from mice 24 h after the injection of CTLs and cultured for 4 h in brefeldin A.

compared with those from non HBsAg-Tg mice. However, CD8-positive cells and CD4-positive cells from HBsAg-Tg/TNF-α$^{+/+}$ mice treated with CTLs did not produce TNF-α. The intracellular staining results indicated that TNF-α production in CD11b-positive, DX5-positive, CD8-positive cells, and CD4-positive cells from HBsAg-Tg/TNF-α$^{+/+}$ mice increased after the CTL injection (Fig. 3*C*).

Role of TNF-α-producing non-Ag-specific cells

The requirement of TNF-α-producing cells for the development of hepatitis was further evaluated with the help of experiments involving the adoptive transfer of macrophages, NK cells, CD4 T cells, and CD8 T cells from non-HBsAg-Tg/TNF-α$^{+/+}$ mice into HBsAg-Tg/TNF-α$^{-/-}$ mice. HBsAg-Tg/TNF-α$^{-/-}$ recipient mice were administered 1.5 × 10^7 HBsAg-specific CTLs 5 days after the i.v. injection of freshly isolated macrophages, NK cells, CD4 T cells, and CD8 T cells from TNF-α$^{+/+}$ mice. We confirmed the existence of the CFSE-labeled transferred cells by flow cytometric analysis (data not shown). As shown in Fig. 4, the sALT levels several days after the CTL injection were significantly increased in HBsAg-Tg/TNF-α$^{-/-}$ mice pretreated with of isolated macrophages, NK cells, CD4 T cells, and CD8 T cells from TNF-α$^{+/+}$ mice compared with the sALT levels in non-pretreated HBsAg-Tg/TNF-α$^{-/-}$ mice.

Effect of IFN-γ on HBsAg-Tg/TNF-α$^{+/+}$ and HBsAg-Tg/TNF-α$^{-/-}$ mice

IFN-γ and TNF-α were thought to be critical components in this murine fulminant hepatitis model (6). We examined the direct effect of IFN-γ on HBsAg-Tg/TNF-α$^{+/+}$ and HBsAg-Tg/TNF-α$^{-/-}$ mice. As shown in Fig. 5*A*, recombinant murine IFN-γ administration caused marked elevations in sALT activity in the HBsAg-Tg/TNF-α$^{+/+}$ compared with in the HBsAg-Tg/TNF-α$^{-/-}$

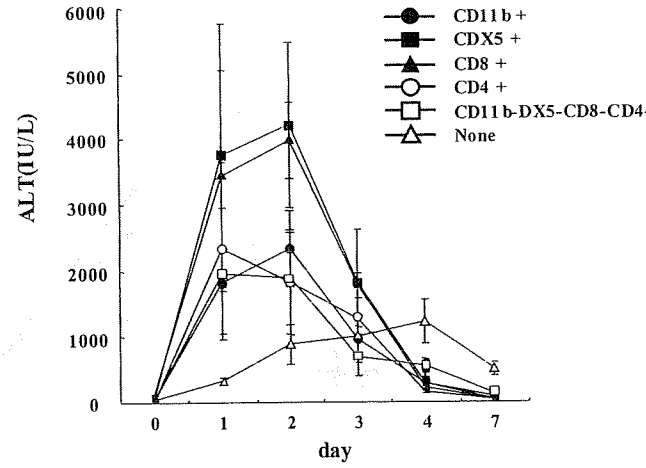

FIGURE 4. MACS-sorted CD11b$^+$, NK$^+$, CD8$^+$, and CD4$^+$ cells from TNF-α$^{+/+}$ mice were transferred i.v. into HBsAg-Tg/TNF-α$^{-/-}$ mice 5 days before injection of the HBsAg-specific CTLs (1.5 × 10^7/mouse). The number of transferred cells was 8 × 10^6/mouse. Serum ALT activities were assessed at the indicated days after the CTL injection. Each value is represented by the mean ± SEM of three mice.

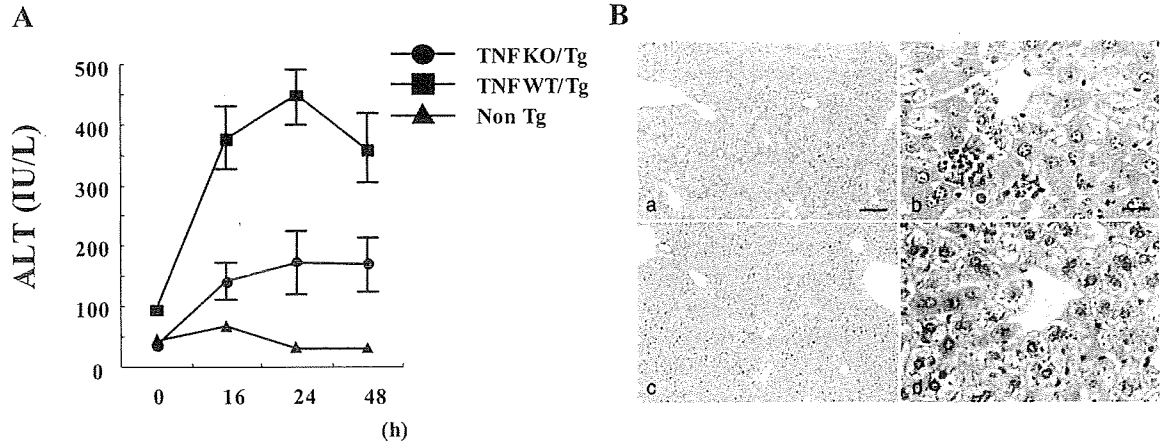

FIGURE 5. Kinetics of recombinant murine IFN-γ-induced liver injury in HBsAg-Tg/TNF-$\alpha^{+/+}$ and HBsAg-Tg/TNF-$\alpha^{-/-}$ mice. Groups of five HBsAg-Tg/TNF-$\alpha^{+/+}$ mice, matched HBsAg-Tg/TNF-$\alpha^{-/-}$ mice, and non-HBsAg-Tg/TNF-$\alpha^{+/+}$ mice were i.v. administered recombinant IFN-γ (8000 U/mouse). *A*, The serum ALT levels were measured at 0, 16, 24, and 48 h after the recombinant IFN-γ injection. Each value is represented by the mean ± SEM of three mice. *B*, Histopathological examination of HBsAg-Tg/TNF-$\alpha^{+/+}$ mice and matched HBsAg-Tg/TNF-$\alpha^{-/-}$ mice livers observed at 24 h after the recombinant murine IFN-γ administration in these mice. *a* and *b*, HBsAg-Tg/TNF-$\alpha^{+/+}$ mice. *c* and *d*, HBsAg-Tg/TNF-$\alpha^{-/-}$ mice. *a* and *c*, Low-power field of liver tissue. *b* and *d*, High-power field of liver tissue. Scale bars: 100 μm (*a*) and 25 μm (*b*). Yellow arrowheads, small necroinflammatory foci; blue arrows, apoptotic hepatocytes.

mice. In contrast, recombinant murine TNF-α administration caused a similar elevation in sALT activity in the HBsAg-Tg/TNF-$\alpha^{+/+}$ and HBsAg-Tg/TNF-$\alpha^{-/-}$ mice, but it did not enhance the sALT levels in non-HBsAg-Tg mice (data not shown). Histopathological examination revealed the presence of small necroinflammatory foci and apoptotic hepatocytes in the liver of HBsAg-Tg/TNF-$\alpha^{+/+}$ mice after the administration of recombinant IFN-γ; however, these changes were not observed in the liver of HBsAg-Tg/TNF-$\alpha^{-/-}$ mice (Fig. 5*B*).

Kinetics of hepatocyte TNFR mRNA expression in HBsAg-Tg/TNF-$\alpha^{+/+}$ mice after the CTL injection

Next, we tested the TNFR 1 and 2 mRNA expressions in hepatocytes after the CTL injection. The expression of TNFR 1 mRNA in hepatocytes in the HBsAg-Tg/TNF-$\alpha^{+/+}$ mice was obviously enhanced (more than 40-fold) after the CTL injection and peaked 24 h thereafter (Fig. 6). In contrast, the expression of TNFR 2 mRNA in hepatocytes in the HBsAg-Tg/TNF-$\alpha^{+/+}$ mice was unchanged after the CTL injection.

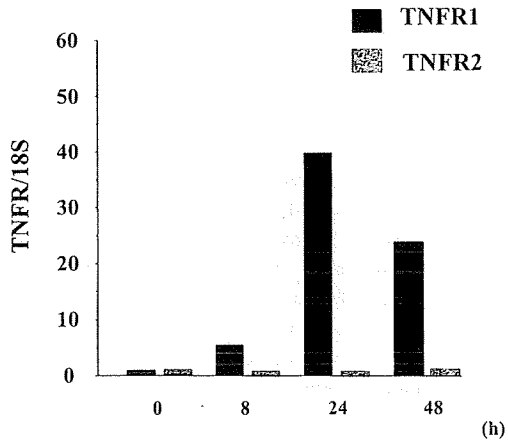

FIGURE 6. Kinetics of TNFR mRNA expression on hepatocytes in HBsAg-Tg/TNF-$\alpha^{+/+}$ mice that were administered HBsAg-specific CTLs. Hepatocytes were collected by perfusion from HBsAg-Tg/TNF-$\alpha^{+/+}$ mice at the indicated times after the CTL injection. Levels of TNFR1 and TNFR2 mRNA were normalized to that of 18S mRNA. The data expressed are relative to the findings from untreated HBsAg-Tg/TNF-$\alpha^{+/+}$ mice. The representative charts are derived from the analyses of three mice per group.

Discussion

Fulminant hepatitis is a clinical syndrome consisting of sudden and severe liver injury that results in hepatic encephalopathy and acute liver failure (17, 18). The rate of mortality from fulminant hepatitis remains very high, although intensive medical care and implementation of the latest therapies, including liver transplantation, are progressing. The HBsAg-Tg mouse model contributed to the study on the mechanisms of fulminant hepatitis (4, 6). Previously, various reports provided HBV transgenic mice. The HBsAg transgenic mouse lineage 107-5D, lineage pFC80-219, or HBV transgenic mouse lineage 1.3.32 were suitable for examining CTL-induced hepatitis (19). Lethal fulminant hepatitis was only observed when the HBsAg transgenic mouse lineage 107-5D received HbsAg specific CTL clones. In lineage pFC80–219 and lineage 1.3.32 HBV Tg mice, lethal hepatitis was not caused by the high-dose CTL injection. Recently, many reports have discussed the influence of the genotypes on outcome of HBV infections. It was reported that the intracellular accumulation of HBV DNA and Ags may play a role in inducing liver damage (20). Moreover, HBV/genotype Bj was an independent risk factor for the development of fulminant hepatitis, and the expression of intracellular Ags in HBV/genotype Bj was the highest (20, 21). Therefore, the HBsAg transgenic mouse lineage 107-5D may be suitable for the analysis of human fulminant hepatitis because the HBsAg particles were retained within the endoplasmic reticulum of the hepatocyte in this lineage. However, it is thought that the HBsAg transgenic mouse lineage 107-5D should be used only for the analysis of the fulminant hepatitis and not acute hepatitis or chronic hepatitis. Previous studies have described the relative hierarchy of the perforin-granzyme, FasL-Fas, IFN-γ, and TNF-α death pathways in the pathogenesis of the necroinflammatory liver disease induced by HBsAg-specific CTL clones in HBsAg transgenic mice (3, 6). Kondo et al. (22) demonstrated that liver injury was attenuated by blocking the FasL-Fas and TNF-α-TNFR pathway in the fulminant hepatitis model. In contrast, it was reported that IFN-γ-dependent signals are primarily responsible for killing HBsAg-positive hepatocytes irrespective of the presence or absence of FasL and Fas. Moreover,

hepatocytes are much less sensitive to destruction by TNF-α than by the other death pathways in the fulminant hepatitis model (3). This discrepancy remains unsolved. However, the difference might be due to the difference in the methods used to block the FasL and Fas signaling. Nakamoto et al. (3) used Fas and FasL knockout mice for blocking the signaling. In contrast, the use of the soluble form of Fas has also been reported. We previously reported that anti-TNF-α Abs were only modestly protective against CTL-induced liver disease. In contrast, Kondo et al. (22) appropriated soluble TNFRβ-Fc for the TNF-TNFR blocking experiment. TNFRβ-Fc greatly inhibited the increase in the ALT level in the fulminant hepatitis model. Thus, in the present study, we examined the role of TNF-α using TNF-α knockout mice. The proinflammatory cytokine TNF-α is thought to play a critical role in acute viral hepatitis (7, 8, 23).We established the HBsAg-Tg (lineage 107-5D)/TNF-$\alpha^{-/-}$ mouse strain and have shown that TNF-α secreted by intrahepatic non-Ag-specific inflammatory cells plays a critical role in the development of acute and lethal necroinflammatory liver disease.

TNF-α is a pleiotropic cytokine that induces cellular responses such as proliferation, production of inflammatory mediators, and cell death, and plays a major role in the pathogenesis of septic shock and wasting syndrome. In the liver, TNF-α is involved in the pathophysiology of viral hepatitis, alcoholic liver disease, nonalcoholic fatty liver disease, and ischemia-reperfusion injury. TNF-α plays a dichotomous role in the liver, where it not only acts as a mediator of cell death but also induces hepatocyte proliferation and liver regeneration. In particular, in HBV-related acute hepatitis, TNF-α is thought to contribute to the viral clearance and development of the necroinflammatory liver disease. It was reported that mice that express HBV envelope proteins in their hepatocytes develop acute vial hepatitis after adoptive transfer of CD8-positive, HBsAg-specific CTL lines and clones (6, 24). Various established HBsAg-specific CTL clones produce TNF-α following HBsAg stimulation (2). Moreover, it was reported that anti-TNF-α Abs (the soluble form of TNFR) markedly blocked the development of hepatitis induced by HBsAg-specific CTLs in HBsAg-Tg mice (22). These results indicated that TNF-α is deeply involved in the progress of liver injury in acute viral hepatitis. Although activated HBsAg-specific CTLs produce TNF-α in fulminant hepatitis, it is considered that TNF-α secreted from only HBsAg-specific CTLs is not enough to cause necroinflammatory liver disease because only 22 CTLs infiltrate in the liver tissue per mm^2 (25). Therefore, TNF-α secreted by non-Ag-specific cells seems to play a critical role in the fulminant hepatitis, but this issue remains to be elucidated.

In this study, we first found that necroinflammatory liver disease did not occur in HBsAg-Tg/TNF-$\alpha^{-/-}$ mice that had received even a large number of HBsAg-specific CTLs. Furthermore, there were no deaths among HBsAg-Tg/TNF-$\alpha^{-/-}$ mice that had received 1.5×10^7 HBsAg-specific CTLs, which caused lethal liver disease in all HBsAg-Tg/TNF-$\alpha^{+/+}$ mice (Fig. 2*B*). Although the peak of severe liver injury was observed within 24–48 h after the CTL injection in this murine hepatitis model, similar injury was not observed in HBsAg-Tg/TNF-$\alpha^{-/-}$ mice at the same time (Fig. 1*B*). When HBsAg-specific CTLs were administered at an extremely high dose into HBsAg-Tg/TNF-$\alpha^{-/-}$ mice, the sALT level slightly increased at 96 h after the CTL injection (Fig. 2*A*). These data suggest that TNF-α secreted by host cells other than Ag-specific CTLs plays a critical role in the acute progression of necroinflammatory liver disease, and it may cause acute hepatitis to develop into lethal liver disease.

In previous studies, it was thought that intrahepatic macrophages mainly produced TNF-α and had the potential to damage hepatocytes in the murine hepatitis model (26). In this model, it was clear that cells of the monocyte/macrophage lineage dominated the inflammatory infiltrate (6). Because the irradiation slightly attenuated the liver injury in this model but did not prevent the massive destruction of most hepatocytes, it can be said that the macrophage lineage plays the major role in this murine fulminant hepatitis (6). However, NK-positive cells (including NKT cells) are also resistant to irradiation (27) and are the second largest mononuclear cell population in the liver after macrophages (Kupffer cells). Therefore, it is possible that the TNF-α secreted by NK-positive cells plays an important role in this model. In contrast, T cells are sensitive to irradiation, and non-Ag-specific T cells in the liver were killed by the irradiation. Thus, the mild decrease in the sALT levels with irradiation as described above can be accounted for by the depletion of the non-Ag-specific T cells in the liver. Taken together, TNF-α secreted by NK-positive cells and non-Ag-specific T cells including CD8-positive cells may play a partially important role in this model.

In vivo, IFN-γ is released by activated HBsAg-specific CTLs when they recognize Ags. Pretreatment with anti-IFN-γ Abs in this murine fulminant hepatitis model completely inhibited the CTL-induced liver injury (6). IFN-γ administration caused marked elevations in the sALT levels in HBsAg-positive Tg mice, whereas it was entirely nontoxic to HBsAg-negative Tg controls (28). In contrast, the Tg and control mice were similarly sensitive to the hepatocytotoxic effects of TNF-α (28). Therefore, we next examined the direct effect of IFN-γ and TNF-α in HBsAg-Tg/TNF-$\alpha^{+/+}$ and HBsAg-Tg/TNF-$\alpha^{-/-}$ mice. IFN-γ administration enhanced the sALT levels in the HBsAg-Tg/TNF-$\alpha^{+/+}$ mice compared with those in the HBsAg-Tg/TNF-$\alpha^{-/-}$ and non-Tg mice (Fig. 5). To confirm the hepatotoxic ability of TNF-α in HBsAg-Tg/TNF-$\alpha^{+/+}$ and HBsAg-Tg/TNF-$\alpha^{-/-}$ mice, we measured the serum ALT level in HBsAg-Tg/TNF-$\alpha^{+/+}$ and HBsAg-Tg/TNF-$\alpha^{-/-}$ mice after the administration of recombinant murine TNF-α. Recombinant murine TNF-α similarly induced liver injury in HBsAg-Tg/TNF-$\alpha^{+/+}$ and HBsAg-Tg/TNF-$\alpha^{-/-}$ mice (data not shown). It is possible that the hepatotoxic response to TNF-α in the HBsAg-Tg/TNF-$\alpha^{+/+}$ mice was similar to that in the HBsAg-Tg/TNF-$\alpha^{-/-}$ mice. IFN-γ secreted by activated CTLs may trigger the enhancement of TNF-α production by non-Ag-specific host cells. The results demonstrated the occurrence of necroinflammatory liver disease in the HBsAg-Tg/TNF-$\alpha^{+/+}$ mice that had received HBsAg-specific CTLs. In the primary culture, the hepatocytes from HBsAg-Tg mice were not destroyed by the addition of high-dose recombinant TNF-α to the culture medium (data not shown). However, it is possible that TNF-α directly exerts its effects to destroy the hepatocytes of HBsAg-Tg mice in this fulminant hepatitis model, because mRNA expression of TNFR1 was up-regulated after the CTL injection. Moreover, the kinetics of sALT levels and the mRNA expression of TNFR1 were quite similar after the CTL injection. These data indicate that TNF-α produced by non-Ag-specific cells may directly destroy the hepatocytes of HBsAg-Tg mice because of CTL-induced enhancement of TNFR1 expression on hepatocytes. However, our data suggests that TNF-α secreted by non-Ag-specific cells plays a critical role in the progression of fulminant hepatitis. In severe viral necroinflammatory liver disease, anti-TNF therapy is thought to be one of the effective treatments.

In summary, we established HBsAg-Tg/TNF-$\alpha^{-/-}$ mice to investigate the role of TNF-α in liver injury caused by CTLs, and demonstrated that TNF-α produced by intrahepatic non-Ag-specific inflammatory cells is critical in the pathogenesis of acute and lethal necroinflammatory liver disease. Anti-TNF therapy may

provide therapeutic benefit to acute viral fulminant hepatitis patients. In contrast, a recent study reported hepatitis B reactivation in a chronic hepatitis B surface Ag carrier after therapy with anti-TNF Ab. Therefore, in severe viral necroinflammatory liver disease, anti-TNF therapy is thought to be an effective treatment. However, anti-TNF therapy should not be administered to patients with persistent HBV infection without careful consideration.

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Disclosures

The authors have no financial conflict of interest.

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(−)-Epigallocatechin gallate prevents carbon tetrachloride-induced rat hepatic fibrosis by inhibiting the expression of the PDGFRβ and IGF-1R☆

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ABSTRACT

Hepatic fibrosis is a major complication of various chronic liver diseases. Activated hepatic stellate cells (HSCs) play a critical role in the development of liver fibrosis and the axis of platelet-derived growth factor (PDGF)/PDGF receptor (PDGFR), a member of receptor tyrosine kinases (RTKs), is closely associated with the activation of HSC. Insulin-like growth factor (IGF)-1 receptor (IGF-1R), which also belongs to RTKs, interacts with the PDGF/PDGFR axis, thereby cooperatively promoting hepatic fibrosis. We herein examined the effects of (−)-epigallocatechin gallate (EGCG), which inhibits the activation of several types of RTKs, on the development of rat liver fibrosis induced by carbon tetrachloride (CCl_4). Drinking water with 0.1% EGCG significantly decreased the serum levels of both aspartate aminotransferase and alanine aminotransferase raised by CCl_4, thus indicating an improvement of liver injury. In CCl_4-injected rats, EGCG markedly attenuated hepatic fibrosis and decreased the amount of hydroxyproline in the experimental liver. The expression of PDGFRβ and IGF-1R mRNAs in the liver was significantly lowered by the treatment with EGCG. EGCG also decreased the expression of PDGFRβ and α-smooth muscle actin proteins, thus indicating the inhibition of HSC activation. These findings suggest that EGCG can exert, at least in part, an anti-fibrotic effect on the liver by targeting PDGFRβ and IGF-1R. EGCG might therefore be useful in both the prevention and treatment of hepatic fibrosis.

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1. Introduction

Hepatic fibrosis is a common response to chronic liver injury from a variety of causes, including infection with hepatic viruses, drug-related, alcohol and metabolic disorders [1]. Progressive fibrosis eventually leads to cirrhosis which is often associated with a high risk of liver failure and hepatocellular carcinoma (HCC) [1,2]. Therefore, the inhibition and prevention of the development of fibrosis might be an effective strategy to improve the prognosis of patients with chronic liver disease. Indeed, recent clinical trials have revealed that treatment with interferon prevents or delays the development of liver cirrhosis and HCC in patients with chronic viral hepatitis [3,4].

The activation of hepatic stellate cells (HSCs) plays a key role in the development of liver fibrosis because activated HSCs are major cellular source of collagen in the injured liver [1]. Following liver injury of any etiology, quiescent HSCs transform to activated cells, which are proliferative and fibrogenic [1]. Several types of growth factors, cytokines, chemokines and their cognate receptors are associated with this transition. Among these factors, autocrine signaling by platelet-derived growth factor (PDGF), which binds to and activates PDGF receptor (PDGFR), is regarded as one of the most potent mitogens and chemotactics for HSCs [5]. The expressions of PDGF and the beta isoform of its receptor (PDGFRβ) have been shown to increase in both experimental rat and human models of liver fibrosis [6,7]. These findings suggest that the activated PDGF/PDGFR signaling pathway may therefore be a candidate therapeutic target for antifibrogenic therapy in liver disease.

Numerous *in vivo* and *in vitro* studies suggest that green tea catechins can exert both cancer therapeutic and cancer preventive

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; α-SMA, α-smooth muscle actin; EGCG, (−)-epigallocatechin gallate; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; HCC, hepatocellular carcinoma; HSC, hepatic stellate cell; IGF-1R, insulin-like growth factor-1 receptor; PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; RTK, receptor tyrosine kinase.

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properties at various organ sites [8]. One of the anticancer mechanisms of green tea or its constituents is explained by their inhibitory effect on the expression and activation of specific receptor tyrosine kinases (RTKs), such as epidermal growth factor receptor (EGFR), insulin-like growth factor (IGF)-1 receptor (IGF-1R) and PDGFRβ, and related downstream signaling pathways [9–12]. In the present study we investigated the effects of (−)-epigallocatechin gallate (EGCG), the major biologically active component of green tea, on liver fibrosis and on the expression of PDGFRβ using a rat model of carbon tetrachloride (CCl_4)-induced hepatic fibrosis. We also examined whether EGCG alters the expression of IGF-1R in the fibrotic liver because this RTK is closely associated with the PDGF/PDGFR axis and thus plays an important role in liver fibrosis [13].

2. Materials and methods

2.1. Animals and chemicals

Four-week-old male Wistar rats were obtained from Japan SLC, Inc. (Shizuoka, Japan). CCl_4 was purchased from Sigma Chemical Co. (St. Louis, MO). EGCG was provided by the Mitusi Norin Co., Ltd. (Tokyo, Japan).

2.2. Animal protocol

All rats were maintained at Gifu University Life Science Research Center, according to the Institutional Animal Care Guidelines, and were housed in plastic cages with free access to drinking water (tap water supplemented with or without EGCG) and a pelleted basal diet, CRF-1 (Oriental Yeast Co., Ltd., Tokyo, Japan). After 1 week of acclimatization, a total of 26 rats were randomly divided into 4 groups. Groups 1 and 2 (5 rats per group) received an intraperitoneal injection of olive oil (0.5 ml/kg body weight, twice a week) for 8 weeks. Groups 3 and 4 (8 rats per group) received an intraperitoneal injection of CCl_4 (0.5 ml/kg body weight, twice a week) for the same period of time. At the start of the intraperitoneal injections, the rats in Groups 2 and 4 were given tap water containing 0.1% EGCG. The rats in Groups 1 and 3 were given only tap water throughout the experiments. A freshly prepared solution of EGCG in tap water was supplied to the experimental rats three times a week. At the termination of the experiment (13 weeks of age), all rats were sacrificed by CO_2 asphyxiation to determine the development of hepatic fibrosis.

2.3. Histopathological and immunohistochemical examinations

In all experimental groups, 3–4 μm thick sections of 10% buffered formaldehyde-fixed and paraffin-embedded livers were stained with either hematoxylin and eosin (H&E) for histopathology or Azan stain to observe liver fibrosis. Immunohistochemistry of α-smooth muscle actin (α-SMA) was performed using a primary anti-α-SMA antibody (DAKO, Glostrup, Denmark) with paraffin-embedded sections, as previously described [14]. Computer-assisted quantitative analyses of fibrosis development were carried out using the WinROOF image-processing software program (Mitani Corp., Tokyo, Japan) in three low power (×40) fields per specimen, as previously described [14].

2.4. Hepatic hydroxyproline analysis

The hepatic hydroxyproline content (μmol/g wet liver) was quantified colorimetrically in duplicate samples from approximately 200 mg wet-weight of liver tissues, as previously described [15].

2.5. Clinical chemistry

At sacrifice, blood samples were collected from the inferior vena cava and the serum activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured using a standard clinical automatic analyzer (type 726, Hitachi, Tokyo, Japan).

2.6. Protein extraction and Western blot analysis

Equivalent amounts of protein lysates (30 μg/lane) from the liver of experimental rats were subjected to a Western blot analysis, as described previously [16]. Anti-PDGFRβ antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-α-smooth muscle actin (α-SMA) antibody was from DAKO. An antibody to GAPDH (Chemicon International, Temecula, CA) served as a loading control.

2.7. RNA extraction and quantitative real-time reverse transcription-PCR analysis

A quantitative real-time reverse transcription-PCR (RT-PCR) analysis was performed, as described previously [17]. Total RNA was isolated from the liver of the experimental rats using the RNAqueous-4PCR kit (Ambion Applied Biosystems, Austin, TX), according to the manufacturer's protocol. The cDNA was synthesized from 0.2 μg of total RNA using SuperScript III First-Strand Synthesis System (Invitrogen, San Diego, CA). The primers used for the amplification of *PDGFRβ*, *IGF-1R* and *GAPDH* specific genes are described previously [18,19]. Real-time PCR was done in a LightCycler (Roche Diagnostics Co., Indianapolis, IN) with SYBR Premix Ex Taq (TaKaRa Bio Inc., Shiga, Japan). The expression level of both the *PDGFRβ* and *IGF-1R* genes was normalized to the *GAPDH* gene expression level. Each experiment was done in triplicate and the average was then calculated.

2.8. Statistical analysis

The data are expressed as the mean ± SD. The statistical significance of the difference in the mean values was evaluated using one-way analysis of variance (ANOVA) and the unpaired *t*-test. Significance was defined as a p value of less than 0.05. All analyses were performed using the StatView ver. 5.0 software (SAS Institute, Cary, NC).

3. Results

3.1. Effects of EGCG on the serum levels of AST and ALT in CCl_4-injected rats

As shown in Fig. 1, the serum AST and ALT levels significantly increased in the CCl_4-injected group (Group 3, $p < 0.01$) in comparison to the control group (Group 1, olive oil-injected group), but they did not increase in the treatment with EGCG alone (Group 2). When compared to the CCl_4-treated group, drinking water with 0.1% EGCG (Group 4) gave lower serum levels of both AST ($p < 0.01$) and ALT ($p < 0.01$), thus indicating suppression of the liver injury (Fig. 1).

3.2. Effects of EGCG on the liver fibrosis in CCl_4-injected rats

Examinations of the Azan-stained sections indicated that treatment with CCl_4 resulted in the development of marked liver fibrosis (Fig. 2C and G). On the other hand, drinking water with 0.1% EGCG significantly prevented the liver fibrosis in comparison to the CCl_4-injected group (Fig. 2D and H). No evidence of fibrosis was observed

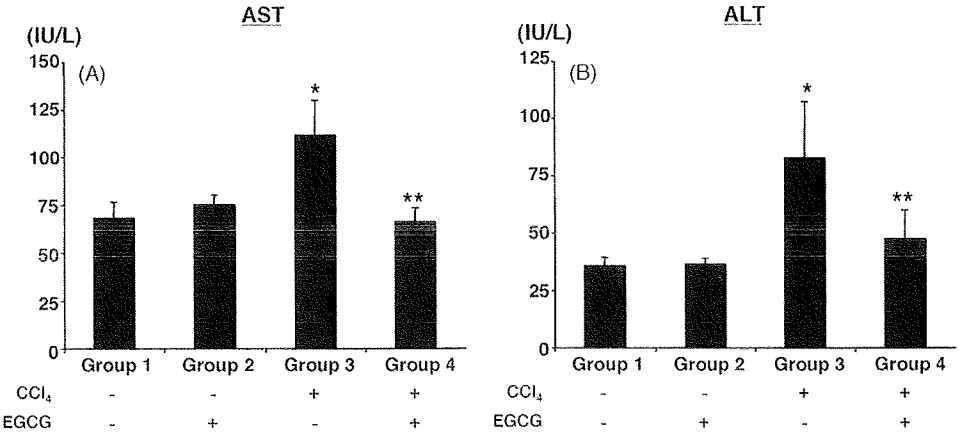

Fig. 1. Effects of EGCG on the serum levels of AST and ALT in the experimental rats. At sacrifice, blood samples were collected and the serum levels of AST (A) and ALT (B) were then assayed. Values are the means ± SE ($n = 5$). $^{*}p < 0.01$, compared with control group (Group 1, olive oil-injected group); $^{**}p < 0.01$, compared with CCl_4-injected group (Group 3).

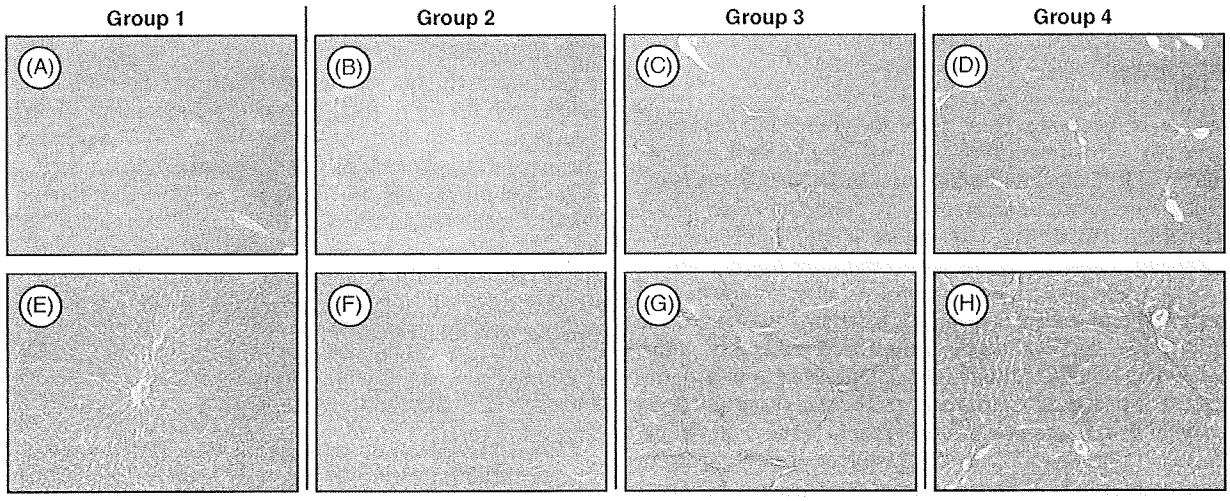

Fig. 2. Photomicrographs of liver sections from the rats in control group (Group 1, A and E), olive oil-injected and EGCG drinking group (Group 2, B and F), CCl_4-injected group (Group 3, C and G) and CCl_4-injected and EGCG drinking group (Group 4, D and H). Paraffin-embedded sections were stained with Azan stain to show fibrosis. Original magnification: ×40 (A–D) and ×100 (E–H).

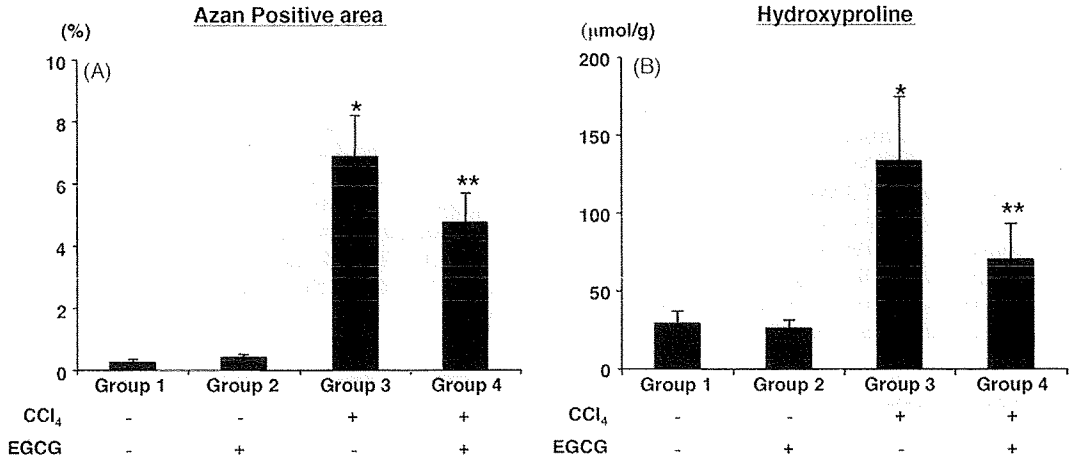

Fig. 3. Effects of EGCG on hepatic fibrosis area and hydroxyproline content in the experimental rats. (A) The fibrosis area was evaluated by Azan stain (Fig. 2) using an image analyzer. (B) The hepatic hydroxyproline contents were quantified colorimetrically, as described in Section 2. Values are the means ± SE ($n = 5$). $^{*}p < 0.01$, compared with control group (Group 1); $^{**}p < 0.01$, compared with CCl_4-injected group (Group 3).

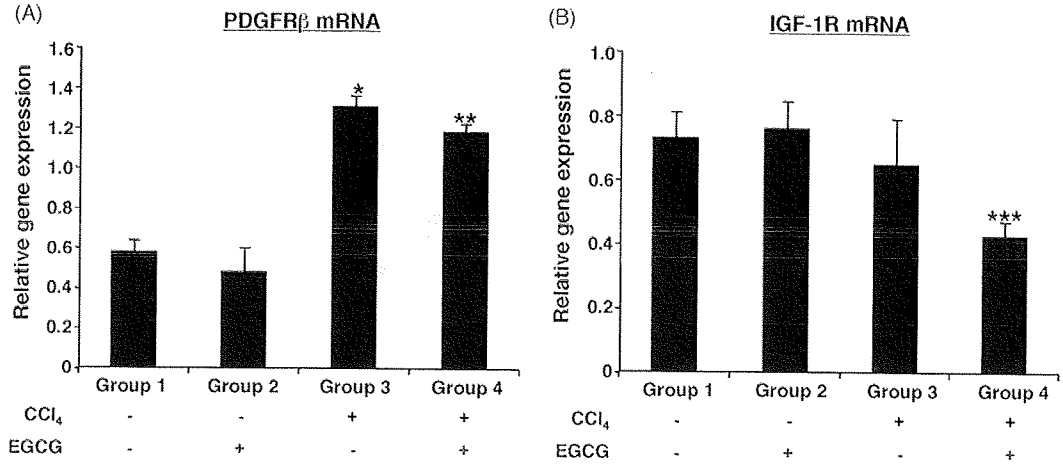

Fig. 4. Effects of EGCG on the expression levels of PDGFRβ and IGF-1R mRNAs in the experimental rats. cDNA was synthesized from the livers of experimental rats and real-time PCR was performed using PDGFRβ (A) and IGF-1R (B) specific primers. The expression levels of these genes were normalized to the level of *GAPDH* gene. Values are the means ± SE ($n = 5$). $^{*}p < 0.01$, compared with control group (Group 1); $^{**}p < 0.05$, compared with CCl_4-injected group (Group 3); $^{***}p < 0.01$, compared with CCl_4-injected group (Group 3).

in the olive oil-injected group either with (Fig. 2B and F) or without EGCG (Fig. 2A and E). A densitometric analysis showed the fibrosis areas to be markedly suppressed in the EGCG-treated rats (Fig. 3A, $p < 0.01$). Similar findings were also observed in measurements of the liver hydroxyproline contents; in the CCl_4-injected rats, drinking water with 0.1% EGCG caused a significant decrease in the amounts of hydroxyproline observed in the liver (Fig. 3B).

3.3. Effects of EGCG on the expression of PDGFRβ and IGF-1R mRNAs in the liver of the CCl_4-injected rats

To elucidate the possible mechanisms in regard to how EGCG attenuates liver fibrosis (Figs. 2 and 3), the effects of this agent on the expression levels of PDGFRβ and IGF-1R mRNAs in the experimental liver were then examined because these RTKs play a critical role in the development of liver fibrosis [1,5,13]. The expression level of PDGFRβ mRNA was elevated in the liver of CCl_4-injected rats and drinking EGCG significantly lowered the level of this mRNA raised by CCl_4 (Fig. 4A). No significant increase was observed in the level of IGF-1R mRNA by CCl_4 injection, whereas treatment with EGCG remarkably decreased the expression of this mRNA (Fig. 4B).

3.4. Effects of EGCG on the expression of PDGFRβ and α-SMA proteins in the liver of the CCl_4-injected rats

Next, the effects of EGCG on the expression levels of PDGFRβ and α-SMA, an indicator of HSC activation, in the rat liver were examined using a Western blot analysis. As shown in Fig. 5A, the intraperitoneal injection of CCl_4 markedly increased the levels of both PDGFRβ and α-SMA proteins in the experimental rat liver. On the other hand, drinking EGCG significantly decreased the expression of PDGFRβ as well as α-SMA proteins raised by CCl_4 (Fig. 5A). Immunohistochemical analysis also indicated that the α-SMA-immunoreactive areas remarkably increased in the liver of the CCl_4-injected group when compared to the olive oil-injected group. In addition, drinking EGCG significantly reduced the expression area of this protein, thus indicating the inhibition of HSC activation (Fig. 5B).

4. Discussion

The activation of HSCs, which is induced by PDGF/PDGFR interaction, plays a pivotal role in the development of liver fibrosis [1,5]. Therefore, targeting the PDGF/PDGFR axis is considered to be an effective strategy to inhibit the progress of hepatic fibrosis. Yoshiji et al. [14] reported that, imatinib mesylate, a clinically used PDGFR tyrosine kinase inhibitor, markedly attenuated liver fibrosis in rats by inhibiting the PDGF-induced proliferation and migration of activated HSCs. The present study demonstrates that drinking EGCG significantly suppressed the liver injury caused by CCl_4 (Fig. 1). Moreover, the results of the present study clearly indicate that EGCG effectively prevented the development of liver fibrosis (Figs. 2 and 3) and this finding was associated with the inhibition of the expression of PDGFRβ and α-SMA (Figs. 4A and 5). These findings are consistent with those of a previous *in vitro* report which showed EGCG to inhibit both HSC proliferation and *PDGFRβ* gene expression by blocking the activation of AP-1 and NF-κB [20]. This report seems to be interesting because these transcription factors are regarded as effective targets of EGCG to exert its anticancer properties [9,21].

Several studies have pointed the interactions between the IGF-1R and PDGF/PDGFR axis in the development of liver fibrosis. For instance, PDGF stimulated the IGF-1R mRNA expression through the activation of the *IGF-1R* gene promoter [22]. Functional IGF-1R was required for the mitogenic activity of the PDGFR in liver myofibroblasts [13]. The cooperative activation of the intracellular signaling pathways, including extracellular signal-regulated kinase (ERK) and Akt, by PDGF and IGF-1 played an important role in perpetuating the activated state of HSC during liver fibrogenesis [23]. Recent studies have revealed that EGCG in drinking water suppressed obesity-related colonic carcinogenesis by inhibiting the activation of the IGF/IGF-1R axis in the colonic mucosa [10]. Treatment of HepG2 human HCC cells with EGCG also decreased the production of IGF-1 from these cancer cells, thus inhibiting the phosphorylation of IGF-1R and its downstream ERK and Akt proteins [11]. These reports, together with our present finding that drinking EGCG significantly reduced the expression of IGF-1R mRNA in the fibrotic liver (Fig. 4B), may suggest that EGCG, which targets both the PDGF/PDGFR and IGF/IGF-1R axes, might also be useful for inhibiting liver fibrosis.

Moreover, in addition to the effects of EGCG on specific RTKs, recent studies have indicated that green tea polyphenols may also possess other anti-fibrotic properties, such as antioxidant properties. EGCG has also been shown to arrest the progression of hepatic fibrosis in the rat model by inhibiting oxidative damage [24]. Supplementation with green tea extract inhibited a progression of cirrhosis in a rat model of steatohepatitis and this was associated with its antioxidant and radical scavenging activities [25]. These

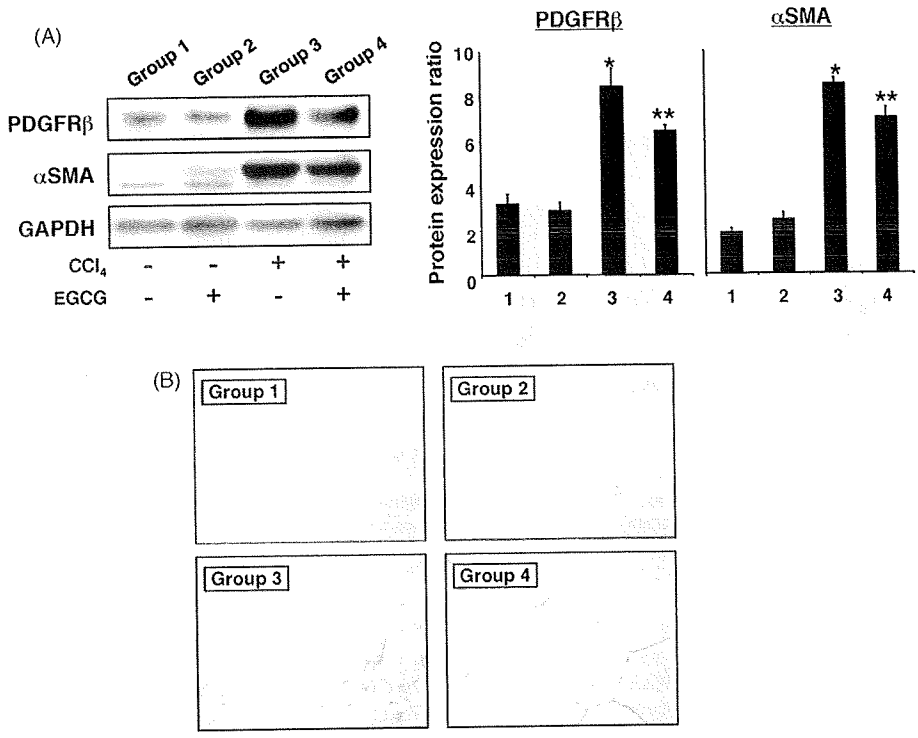

Fig. 5. Effects of EGCG on the expression levels of PDGFRβ and α-SMA in the experimental rats. (A) Total protein was extracted from the liver of experimental rats and equivalent amounts of protein were examined by a Western blot analysis using the respective antibodies. An antibody to GAPDH served as a loading control. Repeat Western blots gave similar results. The results obtained from Western blot analysis were quantitated by densitometry and are displayed in the right panels. Values are the means ± SE ($n = 5$). $^{*}p < 0.01$, compared with control group (Group 1); $^{**}p < 0.01$, compared with CCl_4-injected group (Group 3). (B) Immunohistochemical expression of α-SMA in the liver of control group (Group 1), olive oil-injected and EGCG drinking group (Group 2), CCl_4-injected group (Group 3) and CCl_4-injected and EGCG drinking group (Group 4). Original magnification: ×40.

reports also support the possibility that the administration of EGCG is useful for preventing the progression of hepatic fibrosis. In conclusion, the ability of EGCG to target PDGFR and IGF-1R, both of which play critical roles in the progression of liver fibrosis, is considered to provide evidence that this naturally occurring agent may be effective in both the prevention and therapy of liver fibrosis.

Conflict of interest

The authors declare no conflicts of interest.

Acknowledgments

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Extracellular matrix is required for the survival and differentiation of transplanted hepatic progenitor cells

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ABSTRACT

Engelbreth–Holm–Swarm (EHS) gel has been reported to maintain the mature hepatocyte phenotypes in primary cultured hepatocytes. We investigated the effect of EHS gel on the differentiation of fetal liver cells, which contain stem/progenitor cells. The isolated fetal liver cells cultured on EHS gel formed a spherical shape and increased liver-specific gene expressions compared with cells cultured on collagen. The hepatic progenitor cells that were transplanted subcutaneously to BALB/c nude mice could survive and express hepatocyte marker alpha-fetoprotein when the cells were suspended with EHS gel. These findings demonstrate that EHS gel supports cytodifferentiation from immature progenitor cells to hepatocytes and maintain its differentiated phenotypes *in vitro* and *in vivo*.

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Extracellular matrix (ECM) plays an important role in cell survival, proliferation, and differentiation [1]. Adhesive interactions between hepatocyte and ECM retain its differentiated phenotypes and maintain liver-specific functions, accompanied with up-regulation of the liver-enriched transcription factors including hepatocyte nuclear factor (HNF)s [2,3]. Lamining-rich ECM, Engelbreth–Holm–Swarm sarcoma (EHS) gel has been reported to keep a high expression of liver-specific products such as albumin, and normal cell polarity and structure for prolonged periods in primary cultured hepatocytes [4]. On the other hand, culture on dried type 1 collagen leads the cells to dedifferentiated phenotypes such as flattened monolayer and low expression of liver-specific proteins [2,5].

Liver transplantation is the primary treatment for severe liver diseases. However, the therapy is limited because of insufficient organ availability, and cell transplantation is believed to become alternative therapy. Various types of cells are reported as candidates for cell transplantation for liver diseases. THLE-5b cells, an immortalized non-tumorigenic human cell line, were well localized in the peritoneal cavity of BALB/c nude mice for 3 weeks after cell transplantation [6]. Immortalized human hepatocytes provide life saving metabolic supports in rats when they are transplanted into spleen [7].

Recently, stem cells have been thought to be suitable source of cell transplantation for liver diseases. Bone marrow derived mesenchymal stem cells rescued experimental mouse liver failure when they were engrafted to the liver [8]. Human embryonic stem cells [9], oval cells [10], and cord blood cells [11] are also reported to have the potential to develop into viable hepatocytes. However, these cells might be too immature to be directed to mature differentiated hepatocytes. Fetal liver cells contain stem/progenitor cells, which are able to differentiate bipotentially into hepatocytes and cholangiocytes, and represent differentiated property of hepatocyte by transduction of HNF-4 gene [12] or culture on ECM [13]. However, it is still not known if the cells cultured on EHS gel survive and function as the mature hepatocytes. In this study, we investigated if the hepatic stem/progenitor cells obtain and retain the differentiated hepatocyte phenotypes under such condition *in vitro* and *in vivo*.

Materials and methods

Fetal liver cell isolation and culture. The beta-actin promoter-driven GFP-transgenic mice (GFP mice) were bred for studies. Pregnant female C57BL/6 J mice were purchased from Nippon SLC (Sizuoka, Japan). Fetal mouse liver parenchymal cells were harvested as previously reported [12]. Briefly, the cells were isolated from the embryonic day 14.5 livers by mechanical pipetting. Hematopoietic cells were removed by magnetically activated cell sorter using a Lineage cell depletion kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Then the separated fetal liver cells were plated on either normal plastic, EHS gel coated (BD Bioscience,

Abbreviations: EHS gel, Engelbreth–Holm–Swarm gel; AFP, alpha-fetoprotein; ECM, extracellular matrix; HNF, hepatocyte nuclear factor; GFP, green fluorescent protein; CK-19, cytokeratin-19; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; C/EBP, CCAAT/enhancer binding protein.

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Bedford, MA, USA), or type 1 collagen coated dishes (Iwaki, Tokyo, Japan) (5×10^6 cells/100 mm dish) in DMEM/F12 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10^{-6} M insulin (Wako, Osaka, Japan), 10^{-7} M dexamethasone (Wako), 10% fetal bovine serum (ThermoTrace, Melbourne, Australia), 10 mM nicotinamide (Wako), 50 μM β-mercaptoethanol (Sigma Chemical Co., St. Louis, MO, USA), 5 mM HEPES (Wako), and 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco). The medium was replaced every other day. Animal use and experimentation was performed under the strict guidelines of the Institutional Animal Use and Care Committee at the Gifu University Graduate School of Medicine.

Immunohistochemistry. The cells were fixed by methanol at −20 °C for 10 min, and washed in PBS including 0.05% polyoxyethylene sorbitan monolaurate (Tween 20) (Wako). Nonspecific binding was blocked with 10% nonimmune rabbit serum. Fixed cells were incubated with the rabbit primary antibodies, fluorescein isothiocyanate-conjugated anti-mouse albumin (BETHYL, Montgomery, TX, USA) and anti-mouse cytokeratin 19 (CK19, gift from Dr. N. Tanimizu, Kanagawa Academy of Science and Technology, Japan), in a moist chamber at 4 °C for 16 h. After washing in PBS-Tween 20, cells were incubated with Texas Red-conjugated goat anti-rabbit IgG (Invitrogen, CA, USA) at 4 °C for 3 h to detect CK19. Nuclear counterstain was performed with 4′,6-diamidino-2-phenylindole. The signal was detected using a fluorescence microscope (Olympus, Tokyo, Japan).

Quantitative real-time RT-PCR. Fetal liver cells were cultured for 4 days and detached by cell scraper. Total RNA was isolated from the cells using ISOGEN (Wako). For each sample, 2.0 μg of total RNA was reverse-transcribed using a high-capacity complementary DNA reverse transcription kit as described by the manufacturer (Applied Biosystems, Foster City, CA, USA). The mRNA quantification was performed using a 2-step real-time RT-PCR (Light Cycler, Roche Diagnostics, IN, USA) with Light cycler TaqMan Master and Universal ProbeLibrary Probes (Roche). The PCR primers and probes were as follows; albumin (sense, 5′-CCAAAGT CAACAAGGAGTGCT-3′; antisense 5′-TCGCCTGGTTTTCACACAT-3′; probe #62), HNF-4 (sense, 5′-CCGAGGGACGATGTAGTCAT-3′; antisense 5′-CAAGAGGTCCATGGTGTTCA-3′; probe #68), CK19 (sense, 5′-CCTCAGGGCAGTAATTTCCTC-3′; antisense 5′-TGACCTGGAGATG CAGATTG-3′; probe #17). Target complementary DNAs were normalized to the endogenous mRNA levels of 18S ribosomal RNA (sense, 5′-GCTCTTAGAATTACCACAGTTATCC-3′; antisense, 5′-AATCAGTTATGGTTCCTTTGTCG-3′; probe #55).

Western blot analysis. For the preparation of total cell proteins, the cells cultured for 4 days on the dishes were homogenized in the radioimmunoprecipitation lysis buffer (Santa Cruz, CA, USA). The protein concentration was measured using a DC-protein assay (Bio-Rad). The extracted proteins (10 μg) were separated by SDS–PAGE and then transferred onto a nitrocellulose membrane (Bio-Rad). The membranes were first incubated with the primary antibodies against albumin (Santa Cruz, sc-46293), HNF-4 (Santa Cruz, sc-6556), HNF-1 (Santa Cruz, sc-8986), CK19 (Santa Cruz, sc-33119), alpha-fetoprotein (AFP) (Santa Cruz, sc-8108) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Cell Signaling, #2118) and then incubated with the HRP-coupled secondary antibodies (Santa Cruz). Detection was performed using ECL system (Amersham Biosciences, NJ, USA).

Cell transplantation to nude mice. To determine the effect of EHS gel on cell survival and cytodifferentiation *in vivo*, the fetal liver cells (1×10^5 cells) isolated from GFP mice were suspended in EHS gel, collagen gel (0.3% Cellmatrix type 1-A, Nitta-Gelatin), or DMEM/F12 medium, and then injected subcutaneously to 6-week-old male BALB/c nude mice (Nippon SLC). The grafts were removed with skin tissue at 3 weeks after transplantation, and observed with fluorescent microscope to detect GFP. The sections were stained with hematoxylin and eosin (HE). Immunohistochemical staining for AFP were performed with anti-AFP antibody (Santa Cruz, sc-8108) using avidin–biotin–peroxidase complex technique (Vector, Burlingame, CA, USA).

Results

Morphology and hepatic gene expression of fetal liver cells on different culture substrate

We have previously shown that the fetal liver contains hepatic stem/progenitor cells [12]. When the fetal liver cells were cultured on EHS gel coated dishes, cells formed clusters like a spherical shape 4 days after inoculation. In contrast, the cells showed a flattened and extended shape on type 1 collagen coated or normal plastic dish (Fig. 1). The spherical cells on EHS gel and flattened, extended cells on collagen or plastic dishes expressed albumin, a hepatocyte marker, and CK19, a cholangiocyte marker. In cells on collagen and plastic, cells located in the center of colonies were stained by albumin antibody, whereas the peripheral cells were mainly stained by CK19 antibody. These results indicate that fetal liver cells have bipotent differentiation ability.

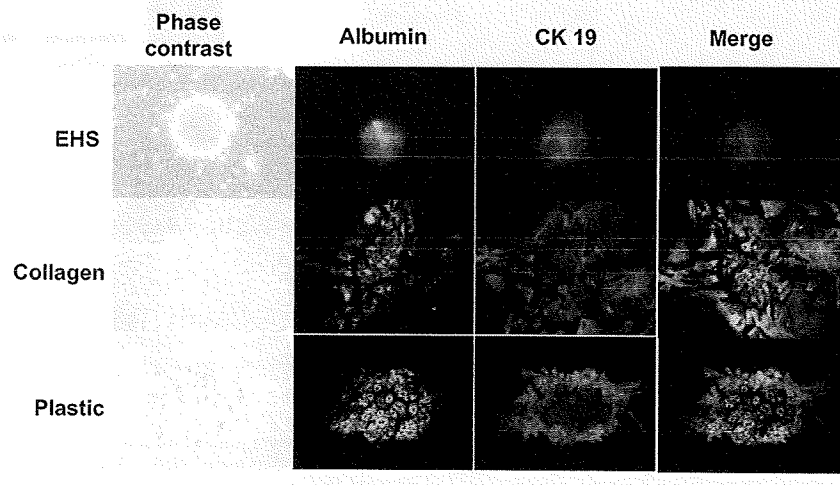

Fig. 1. Morphological changes of the fetal liver cells cultured on ECMs. The isolated fetal liver cells were cultured on each indicated ECMs for 4 days. Expression of albumin (green) and CK19 (red) were examined by double immunofluorescent staining with anti-albumin and CK 19 antibodies. The fluorescence was visualized with fluorescent conforcal microscope. (Original magnification ×200.)

Effects of ECMs on the liver-specific genes and proteins

As observed in immunohistochemistry, Western blot analysis also showed that the protein level of albumin was higher in the cells cultured on EHS gel than in the cells on type 1 collagen or plastic dish 4 days after culture. Reversely, CK19 was higher in the cells on type 1 collagen or plastic dish (Fig. 2A). Another hepatocyte maker AFP was also higher in the cells on EHS gel (A). Moreover, HNF-4 and -1 proteins were also higher in the cells on EHS gel. In parallel with the protein expressions, mRNA of albumin was higher in the cells cultured on EHS gel and CK19 mRNA was higher in the cells on type 1 collagen (Fig. 2B). These results demonstrate that EHS gel supports cytodifferentiation of hepatic stem/progenitor cells to hepatocytes.

Cell transplantation to nude mice

On the basis of these findings, we performed a further experiment to examine the cytodifferentiation ability of EHS gel *in vivo*. The fetal liver cells were isolated from GFP mice, were suspended with EHS gel, collagen gel, or DMEM medium, and were transplanted into subcutaneous tissues of BALB/c nude mice. The

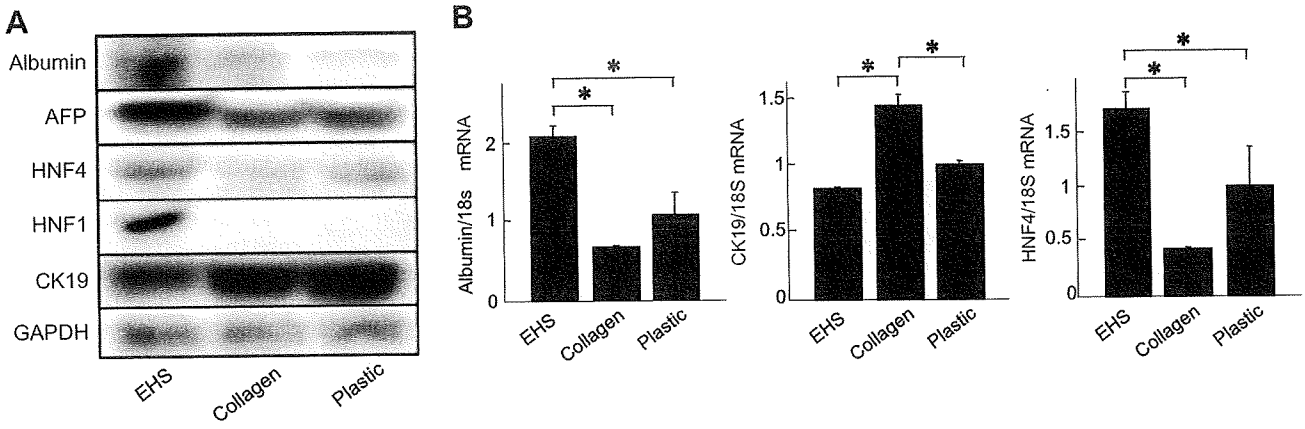

Fig. 2. Liver-specific protein and gene expressions of the fetal liver cells cultured on ECMs. The isolated fetal liver cells were cultured on each indicated ECMs for 4 days. (A) Extracted proteins were subjected to SDS–PAGE, and immunoblotting was performed with anti-albumin, AFP, HNF-4, HNF-1, CK19, and GAPDH antibodies. (B) mRNA levels of albumin, CK19, and HNF-4 in the cultured cells were determined by quantitative real-time RT-PCR. Data are means ± SD from at least three independent experiments. *, $P < 0.05$ using Student's *t*-test.

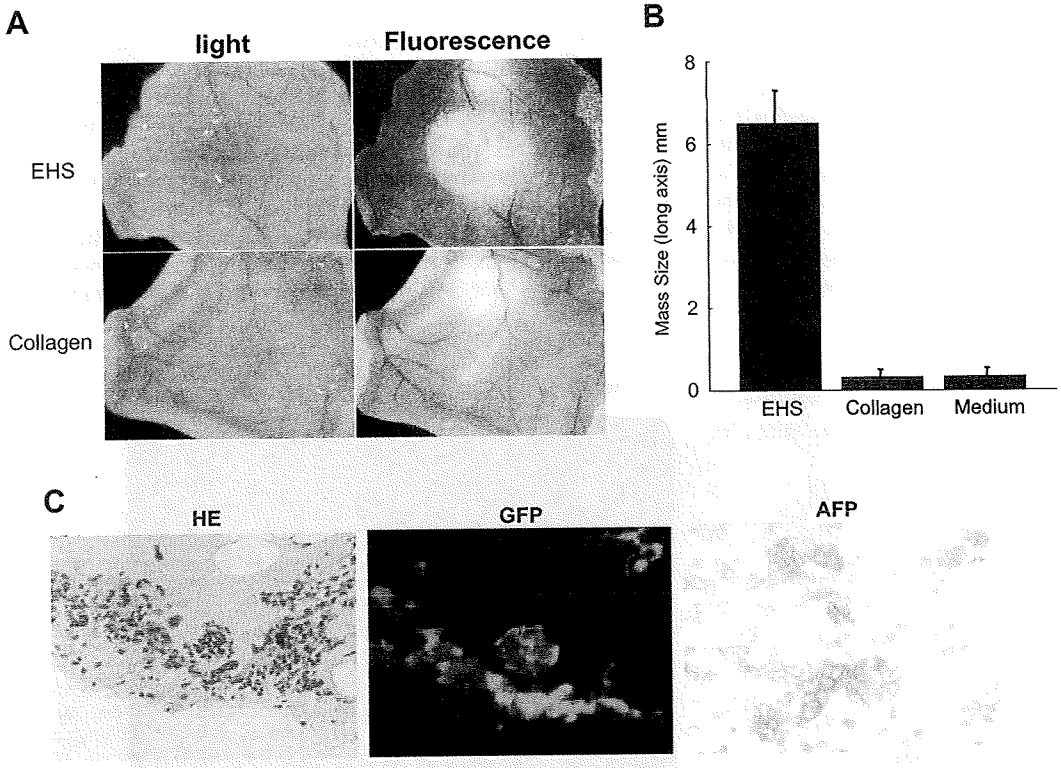

Fig. 3. Transplantation of the fetal liver cells with ECMs into BALB/c nude mice. (A) The fetal liver cells were isolated from GFP mice. The cells were suspended with EHS gel, collagen gel, or DMEM medium, and then transplanted subcutaneously into BALB/c nude mice. The animals were killed at 3 weeks after transplantation, and subcutaneous tissue was excised and photographed with light (left panels) or fluorescence (right panels). (B) The mass size (long axis) of the GFP positive area was measured. Data are means ± SD from at least three independent experiments. (C) The grafts of fetal liver cells with EHS gel were excised and stained with HE (left panel). GFP fluorescence was detected with fluorescence confocal microscope (middle panel). Expression of AFP was examined 12 days after transplantation by immunohistochemistry with anti-AFP antibody (right panel). (Original magnification ×200.)

grafted fetal liver cells with EHS gel remained even 3 weeks after transplantation, whereas cells with collagen gel or DMEM medium showed faint GFP signals (Fig. 3A and B). Moreover, the transplanted cells with EHS gel expressed AFP (Fig. 3C). These results suggest that EHS gel is able to maintain the cell survival and supports cytodifferentiation to hepatocyte *in vivo*.

Discussion

Cell transplantation has been investigated for an alternative therapy of liver organ transplantation. Differentiated hepatoma cell lines or primary cultured hepatocytes from adult liver have been thought as potential candidates [9]. However, hepatoma cells provide low levels of liver functions, and primary cultured hepatocytes rapidly lose their differentiated phenotypes and show a reduction of liver-specific gene expression once they are plated on plastic dishes [14]. Several ECMs are reported to maintain differentiated hepatocyte phenotypes. EHS gel modulates the shapes of cultured rat hepatocytes [14] and rat small hepatocytes [15]. EHS gel keeps hepatocyte-phenotypic gene expression through high expression of liver-enriched transcription factors, such as HNF-4 [16], and enhances many liver-specific functions [17]. EHS gel or sandwich culture with collagen gel maintains features of mature hepatocytes such as albumin expression, and they are attempted to use for development of bioartificial liver system [3]. However, insufficient availability of donor organ is still a problem because primary cultured hepatocytes have less regenerative ability. Thus, it would be greatly beneficial for cell transplantation if functional hepatocytes could be generated from other sources. Recently, stem cells, which have abilities of self-renewal and multilineage differentiation, are highlighted as candidates for cell transplantation. Liver stem cells can be isolated from the fetal liver, and these cells have extensive replication ability and maintain the expression of liver-specific genes, such as albumin and CK19, for prolonged cultured period [12].

In this study, we demonstrated that EHS gel promoted cytodifferentiation of hepatic progenitor cells to hepatocytes and maintained their efficiency as hepatocytes consistent with primary cultured adult hepatocytes. Interaction with ECM influenced the cell morphology and maturation of fetal liver cells. In matured hepatocytes, cells in spheroids retain the *in vivo* levels of albumin and glucokinase, whereas cells in monolayer lose the differentiated phenotypes [18]. Organization of the cytoskeleton by cell–cell and cell–ECM interaction associates with liver-specific gene expression [19]. Indeed, actin depolymerization in hepatocytes by EHS gel increases HNF-4 and albumin expression [20]. In small hepatocytes, morphological changes of the cells induce specific liver-enriched transcription factors such as HNF-4α, HNF-6, C/EBPα, and C/EBPβ [21]. HNF-4 is a key molecule for fetal liver development [22]. Overexpression of HNF-4 in hepatic progenitor cells increases liver-specific gene expression such as albumin and ApoA1 [12]. Reversely, blockage of HNF-4 expression by siRNA inhibits the up-regulation of the liver-specific gene expression in primary cultured hepatocytes cultured on EHS gel [20]. These findings and our results indicate that EHS gel induces the morphological change of hepatic progenitor cells to a spherical shape, and that HNF-4 expression via the organization of the cytoskeleton might be required for the cytodifferentiation of hepatic progenitor cells.

The differentiation potential of fetal liver cells to hepatocytes was also supported by EHS gel *in vivo*. The fetal liver cells suspended with EHS gels kept their viability and AFP expression, suggesting that cell–ECM interaction is also required for cell survival and cytodifferentiation to the hepatocyte *in vivo*. Hepatocyte transplantation into liver has been performed via portal vain for various liver diseases [23,24]. The transplanted cells might interact with ECM of the liver. Consistent with our results, it has been reported that ECM components are the predominant factor to maintain hepatocytes at heterotopic sites [25]. Ectopic subcutaneous hepatocyte transplantation may have advantages as follows; (i) surgical operation is not required, (ii) risk of embolisms is low, and (iii) the transplanted cells are removable or replaceable. Of note, the ectopic cells require additional ECMs such as EHS gel to maintain hepatocyte functions.

In conclusions, our results indicate that EHS gel promotes the differentiation of hepatic progenitor cells to functional hepatocytes. We also demonstrated that EHS gel supports cytodifferentiation and maintains the cell survival and functions *in vivo*. Our findings may therefore have relevance to the clinical application of ectopic liver stem/progenitor cell transplantation as an option to treat liver diseases.

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Interaction between LPS-induced NO production and IDO activity in mouse peritoneal cells in the presence of activated Vα14 NKT cells

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ABSTRACT

In this study, we demonstrated that lipopolysaccharide (LPS) markedly increased nitric oxide (NO) production and indoleamine 2,3-dioxygenase (IDO) activity in mouse peritoneal cells in the presence of activated Vα14 natural killer T cells. Moreover, LPS-induced NO production in peritoneal cells from IDO-knockout (KO) mice was more increased than that from wild-type mice. However, there was no significant difference in the expression of inducible nitric oxide synthase (iNOS) mRNA and protein between the wild-type and IDO-KO mice. No significant difference was also observed in the ratio of CD3- and DX5-positive cells and F4/80- and TLR4-positive cells in peritoneal cells between the wild-type and IDO-KO mice. Since the IDO activity was enhanced by an NO inhibitor, NO may be post-translationally consumed by inhibiting the IDO activity. IDO is well known to play an important role in immunosuppression during inflammatory disease. Therefore, the inhibition of IDO by NO may exacerbate inflammation in the peritoneal cavity.

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Introduction

Vα14 natural killer T (NKT) cells produce large amounts of both Th1 and Th2 cytokines on stimulation with the ligand, α-galactosylceramide (α-GalCer), and they play a crucial role in various immune responses [1,2]. In particular, interferon (IFN)-γ is known to promote macrophage activation and enhance inducible nitric oxide synthase (iNOS) expression [3]. Bacterial infection strongly induces iNOS, and considerable nitric oxide (NO) is produced to attack bacteria [4]. On the other hand, NO is also known to cause severe cell and tissue injury [5–8]. Previously, we reported that NO production by α-GalCer-treated peritoneal cells and intra-hepatic lymphocytes was highly sensitive to lipopolysaccharide (LPS) stimulation [9,10]. These findings suggest that even mild LPS stimulation is dangerous in the presence of activated NKT cells.

Indoleamine 2,3-dioxygenase (IDO) catalyzes the rate-limiting step in the kynurenine pathway of tryptophan degradation [11]. In many cell types, IDO is strongly induced by IFN-γ and tumor necrosis factor (TNF)-α during an inflammatory response. IDO plays a important role in the suppression of acute inflammatory responses and regulates both innate and acquired immunity [12,13]. IDO and some downstream metabolites also contribute to the generation of regulatory T (Treg) cells, which are involved in immunotolerance and anti-inflammation [14–16]. Thus, IDO maintains the immune balance in inflammatory diseases by curbing the response.

Recently, experiments have shown that IDO is inhibited by NO and peroxynitrite, a reactant in the reaction between NO and superoxide [17,18]. However, few studies have addressed the significance of this interaction between NO and IDO in the acute inflammatory model under conditions of excessive NO production. Gram-negative bacterial infection with primary disease involving NKT cells activation is predicted that both LPS and IFN-γ derived from activated NKT cells increase NO production and IDO activity in macrophages. Peritoneal cells are well known to have a large proportion of macrophages, which may contribute to acute inflammation after bacterial infection. If we find that NKT cells activated by α-GalCer enhance both LPS-induced NO production and IDO activity in peritoneal cells, it may provide a clue to understanding the interaction between NO and IDO in the peritoneal cavity under inflammatory conditions in the presence of activated NKT cells. Therefore, the aim of our study was to investigate the effect of α-GalCer-activated NKT cells on LPS-induced NO production and IDO activity and the subsequent interaction between NO and IDO.

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