guidelines set forth by the Animal Care Committee of Yamaguchi University.

BMSC and ASC

Human (h)BMSC and hASC derived from six different donors were purchased from Lonza (Basel, Switzerland): BMSC1 (22-year-old man; 1F4287), BMSC2 (21-year-old man; 7F3915), BMSC3 (20-year-old man; 8F3520), ASC1 (26-year-old woman; 1F3651), ASC2 (52-year-old woman; 0F4505), and ASC3 (38-year-old woman; 1F3737). Cells were seeded in 10-cm culture dishes and incubated overnight in a humidified incubator at 37°C and 5% CO_2. The following day, the medium was replaced to remove floating cells. Adherent cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal blood serum (FBS) and penicillin/streptomycin (both from Gibco, Grand Island, NY, USA) on non-coated dishes (Becton Dickinson, Franklin Lakes, NJ, USA) until they reached 80% confluence, and were then detached with 0.05% trypsin-ethylenediaminetetraacetic acid (tripsin-EDTA; Life Technologies) and replated. Mouse (m)BMSC derived from C57BL/6 wild-type mice were purchased from Cyagen Biosciences (Santa Clara, CA, USA), and mASC were obtained from the inguinal subcutaneous adipose tissue of C57BL/6 wild-type mice. Tissue was placed in DMEM with 0.03% collagenase (Wako Pure Chemical Industries, Osaka, Japan), resuspended and centrifuged at 500 *g*. The pellet was resuspended and cultured, and cells from passage 2 or 3 were used for assays. The potential of hBMSC, hASC, mBMSC and mASC to differentiate into adipogenic, chondrogenic and osteogenic lineages was confirmed by oil red O, alkaline phosphatase and Alcian blue staining, respectively (Figs S1,3).

DNA-chip analysis

We compared DNA expression between hBMSC and hASC using the DNA-chip system (Agilent Technology, Santa Clara, CA, USA) and analyzed the expression pattern using the IPA software system (Ingenuity Systems, Redwood City, CA, USA).

Polymerase chain reaction (PCR) and quantitative real-time (qRT)–PCR

Total RNA was extracted from hBMSC and hASC with ISOGEN reagent (NipponGene, Tokyo, Japan) according to the manufacturer's instructions. Contaminating genomic DNA was removed using a DNase kit and RNA was purified with the RNeasy MinElute Cleanup kit (both from Qiagen, Tokyo, Japan), in accordance with the manufacturer's protocols. First-strand cDNA was synthesized using the ReverTra Ace-α system (Toyobo, Osaka, Japan) from 500 ng of total RNA, in accordance with the manufacturer's instructions. Gene expression was evaluated by qRT–PCR using Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA) and TaKaRa ExTaq DNA polymerase (Takara Bio, Shiga, Japan) on a StepOnePlus Real-Time PCR system (Applied Biosystems). Thermal cycling conditions for PCR were 94°C for 1 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s and 72°C for 30 s; the conditions for qRT–PCR were 95°C for 3 s and 60°C for 30 s, until the fluorescence signal exceeded the threshold. The following primer sequences were used: human tissue factor (TF), 5′-CAG ACA GCC CGG TAG AGT GT-3′ (forward) and 5′-CCA CAG CTC CAA TGA TGT AGA A-3′ (reverse); mouse TF, 5′-TCC AGG AAA ACT AAC CAA AAT AGC-3′ (forward) and 5′-CCC ACA ATG ATG AGT GTT TCT C-3′ (reverse); human β-actin, 5′-CCA ACC GCG AGA AGA TGA-3′ (forward) and 5′-CCA GAG GCG TAC AGG GAT AG-3′ (reverse); and mouse β-actin, 5′-TGA CAG GAT GCA GAA GGA GA-3′ (forward) and 5′-GCT GGA AGG TGG ACA GTG AG-3′ (reverse). The identity of MSC was confirmed by analyzing the expression of cluster of differentiation (CD) cell surface markers using previously described primer sequences.[5]

Western blotting and enzyme-linked immunosorbent assay (ELISA)

The hBMSC, hASC, mBMSC and mASC samples were homogenized in 400 μL of cell lysis buffer consisting of *n*-octyl-β-D-glucopyranoside dissolved in radioimmunoprecipitation buffer to a final concentration of 20 mM. Protein samples were mixed with an equal volume of loading buffer consisting of 5% 2-mercaptoethanol and 95% Laemmli sample buffer, heated at 100°C for 3 min, and separated on a 12% polyacrylamide gel. Proteins were transferred to an Immobilon-P transfer membrane (Millipore, Billerica, MA, USA), which was blocked for 30 min in blocking buffer (0.1% I-Block in phosphate-buffered saline with Tween [PBST]) and was then washed with PBST before incubation for 12–16 h at room temperature with primary antibodies against TF (Abcam, Tokyo, Japan) and β-actin (Becton Dickinson) in blocking buffer. Membranes were incubated for 30 min at room temperature with appropriate secondary antibodies, and protein bands were detected by enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA) and autoradiography according to the manufacturer's instructions. TF protein expression in hBMSC and hASC was quantified using the Tissue Factor Human ELISA Kit (Abcam) according to the manufacturer's protocol. Samples were the same as those used for western blot analysis.

Fluorescence-activated cell sorting analysis

Adherent cells were dissociated with 0.05% trypsin-EDTA and resuspended in DMEM containing 10% FBS. Cells were washed with PBS (Life Technologies) and incubated in PBS containing 10% Fc receptor blocking reagent (MACS Miltenyi Biotec, Cologne, Germany) for 20 min on ice, followed by labeling for 20 min on ice with a monoclonal antibody against TF (R&D Systems, Minneapolis, MN, USA); phycoerythrin-conjugated antibodies against CD11b, CD44, CD45, CD90 (Beckman Coulter, Brea, CA, USA), CD105 (eBioscience, San Diego, CA, USA) or CD73 (R&D Systems); or an fluorescein isothiocyanate-conjugated antibody against CD34 (BD Biosciences, San Jose, CA, USA). Isotype-matched immunoglobulin G was used as controls. Flow cytometry was performed using a Gallios system (Beckman Coulter). Cells were also stained with propidium iodide (Sigma-Aldrich, St Louis, MO, USA) to exclude dead cells from the analyses. Each sample was evaluated at least three times. Data were analyzed using Kaluza (Beckman Coulter) or FloJo (Tree Star, Ashland, OR, USA) software.

Clotting assay

Adherent cells were dissociated with 0.01% trypsin-EDTA diluted in PBS. After resuspension in DMEM, cells were washed with ice-cold PBS and HBSA (20 mM HEPES-NaOH, pH 7.5; 100 mM NaCl; 0.02% NaN_3; and 1 mg/mL bovine serum albumin). Cell suspensions were frozen at −80 °C and thawed at 37 °C. This lysis step was repeated over three cycles, and 100 μL of cell lysates were transferred to a coagulometer cuvette and mixed with 100 μL of pooled normal human plasma (George King Bio-Medical, Overland Park, KS, USA), to which 100 μL of 25 mM $CaCl_2$ was added. Clot formation time was measured from the moment of $CaCl_2$ addition using a KCl Delta semiautomatic coagulation analyzer (Tcoag, Bray, Ireland).

Prothrombin time

Blood was obtained from cell-infused mice; 3.2% Na citrate was immediately added and the mixture was centrifuged at 2000 g for 15 min. Serum was stored at −80 °C. The procedure was performed using silicone-coated tubes (Sarstedt, Tokyo, Japan), and prothrombin time was measured (Monolis, Tokyo, Japan) using the manufacturer's protocol.

Cell transplantation

Cultured mBMSC and mASC were dissociated into single cell suspensions by trypsinization. Cells were resuspended in PBS and 200 μL (3.0×10^4–3.0×10^6 cells) were carefully infused into the tail vein of syngenic C57BL/6 mice using a 27-G needle.

Statistical analysis

Significant differences were tested with Student's *t*-test. Differences were considered statistically significant at $P < 0.05$. Data are presented as means ± standard deviation for triplicate experiments.

RESULTS

THERE WERE NO phenotypic differences between hBMSC and hASC, namely, with respect to cell surface marker and differentiation potential (Figs S1–S3), and the similar morphology of the two types of cell can be seen on differential interference contrast microscopy (Fig. 1a).

Expression of TF, which can trigger pro-coagulation and induce thromboembolism,[6] was analyzed by DNA-Chip in hBMSC and hASC. TF was expressed at higher levels in hASC than in hBMSC in three donors (Fig. 1b); when the change in expression over multiple cell passages was evaluated by qRT–PCR, the upregulation of TF transcript levels in hASC persisted in cells from passages 2, 3 and 4 in all donors (Fig. 1c). TF protein levels were also higher in hASC than in hBMSC (Fig. 2a–c). In addition, coagulation time, which varied as a function of the number of cells, was shorter in hASC than in hBMSC (Fig. 3).

The safety of mBMSC versus mASC infusion was compared in a mouse model. We previously reported that not only MSC but also macrophages can be easily expanded from murine bone marrow cells in culture dishes.[7] Here, we found that mASC were able to differentiate into adipocytes and osteocytes (Fig. S3). Consistent with the findings in human cells, mBMSC and mASC had similar expression profiles (Fig. S4), but TF mRNA was expressed at higher levels in mASC than in mBMSC (Fig. S5).

Cell infusion analysis using mBMSC and mASC showed that the survival rate at 24 h after mASC infusion was 46.4% (Table 1), with most of the mice dying immediately (i.e. within the first 10 min). The survival rate decreased with increasing cell concentrations. In contrast, the 24-h survival rate after mBMSC infusion was 95.5%, and there was no effect of cell number on survival in this group (Fig. 4).

The prothrombin time, a measure of blood clotting, was assessed in blood obtained from mice that died immediately after mASC injection, those that survived beyond 10 min after mBMSC injection and untreated mice. There were no differences in the prothrombin times of mBMSC-infused and untreated mice (6.7 ± 0.8 s vs 6.5 ± 1.7 s; $P = 0.79$);

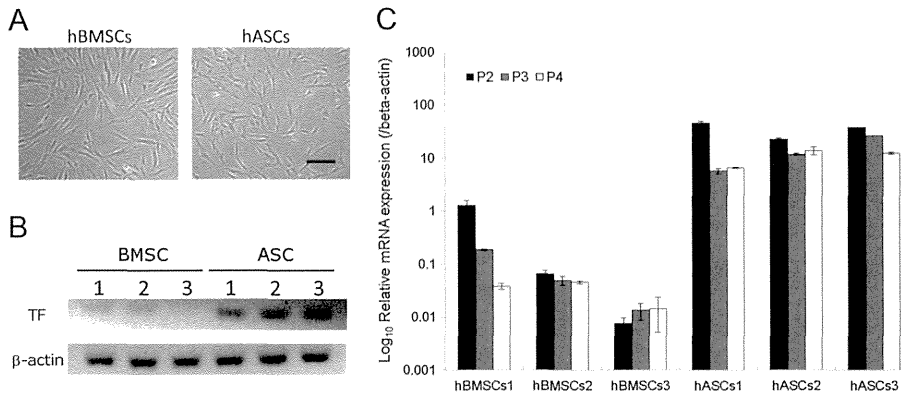

Figure 1 Microscopy of human bone marrow-derived mesenchymal stem cells (hBMSC) and human adipose-derived mesenchymal stem cells (hASC), and tissue factor (TF) mRNA expression in hBMSC and hASC. (a) Similar morphology of the two types of cell can be seen on differential interference contrast microscopy. Scale bar = 200 μm. (b) TF mRNA expression relative to β-actin levels in hBMSC and hASC from three donors for each cell type, as determined by quantitative reverse transcription polymerase chain reaction. TF was expressed at higher levels in hASC than in hBMSC in the three donors. (c) Quantitative analysis of results from (b) (mean ± standard deviation, $n = 3$) in cells from passages 2, 3 and 4 (P2, P3 and P4, respectively). TF transcription in hASC was maintained at high levels even after passage.

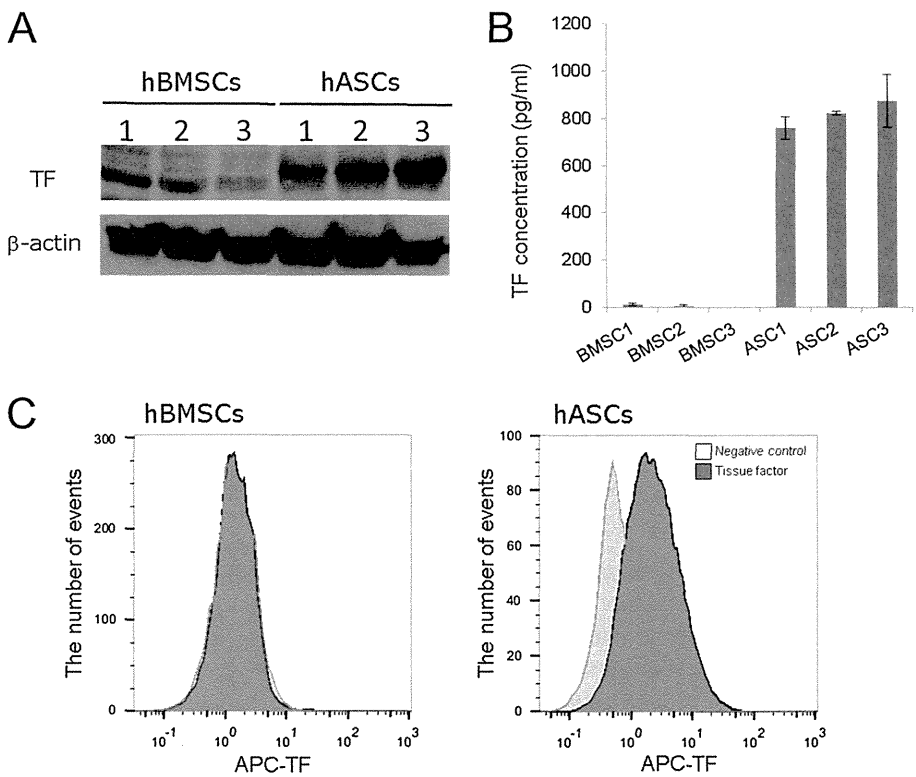

Figure 2 Tissue factor (TF) protein expression in human bone marrow-derived mesenchymal stem cells (hBMSC) and human adipose-derived mesenchymal stem cells (hASC). The 47-kDa TF protein was detected by (a) western blotting and (b) enzyme-linked immunoassay in cells from three donors each. Data represent means ± standard deviation. (c) TF protein expression on the cell surface was analyzed by flow cytometry.

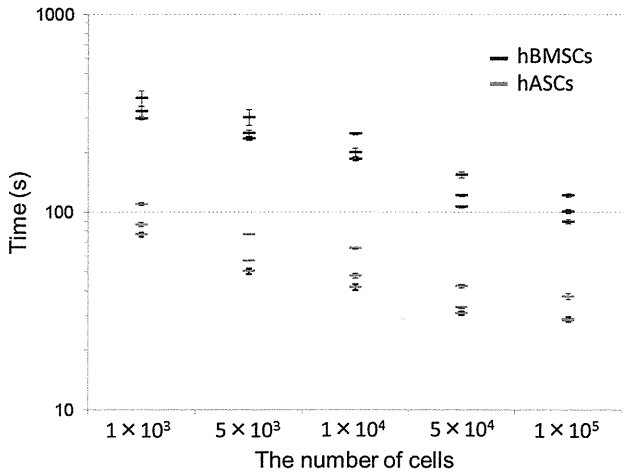

Figure 3 Coagulation time in human bone marrow-derived mesenchymal stem cells (hBMSC) and human adipose-derived mesenchymal stem cells (hASC) using citrated human plasma (pooled). Concentrations ranging 1×10^3–1×10^5 cells were tested. Clotting assay was performed three times for each donor ($n = 3$ for each cell type); data represent means ± standard deviation.

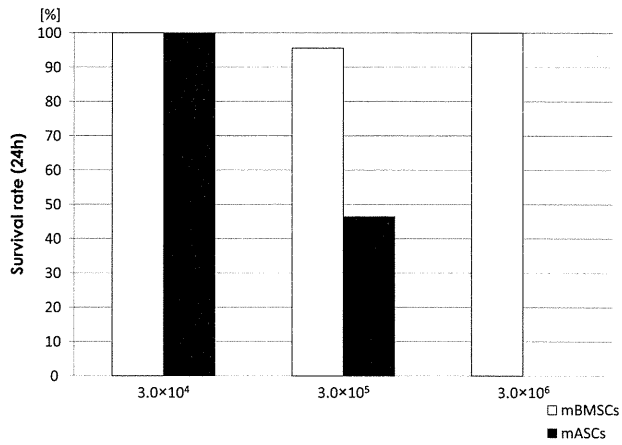

Figure 4 Survival rate in mice infused with mouse bone marrow-derived mesenchymal stem cells (mBMSC) or mouse adipose-derived mesenchymal stem cells (mASC). Survival rate in mASC-infused mice declined markedly as a function of the number of cells infused. On the other hand, mBMSC-infused mice did not show changes in survival rate, regardless of the number of cells infused.

Table 1 Survival rate 24 h after mBMSC or mASC infusion

No. of injected cells	Total mouse number	No. of surviving mice (24 h)	Survival rate (%)
3.0×10^5 mBMSC/mouse	22	21	95.5%
3.0×10^5 mASC/mouse	28	13	46.4%
PBS (same volume)	8	8	100%

ASC, adipose-derived mesenchymal stem cell; BMSC, bone marrow-derived mesenchymal stem cell; PBS, phosphate-buffered saline.

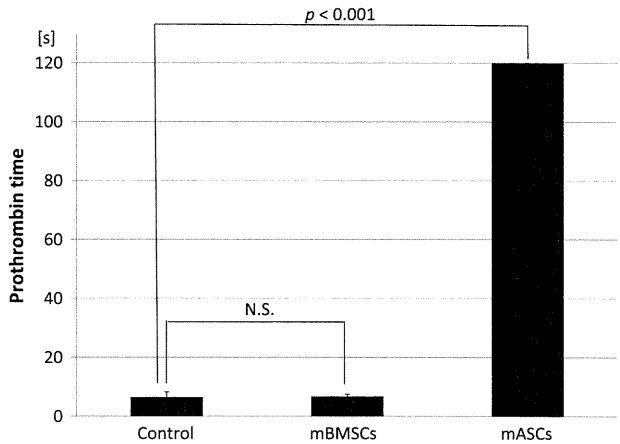

Figure 5 Prothrombin time after mouse bone marrow-derived mesenchymal stem cell (mBMSC) and mouse adipose-derived mesenchymal stem cell (mASC) infusion. Mice were infused through the tail vein and blood clotting was measured in mice that died immediately after mASC injection, those that survived beyond 10 min after mBMSC injection and untreated mice. Prothrombin time in the mASC group was markedly extended, as compared with the other two groups. Data represent means ± standard deviation. N.S., not significant.

however, the prothrombin time was markedly higher for mASC-infused mice than for untreated mice (>120 s vs 6.5 ± 1.7 s; $P < 0.001$), indicating that pro-coagulation activity was enhanced by mASC infusion (Fig. 5). Histopathological analysis of mice that died immediately following mASC infusion revealed multiple thrombi in the blood vessels of the lungs and liver, which were not observed in the lungs and liver of mBMSC-infused mice (Fig. 6). These results suggest that infusions carry greater risk with mASC than with mBMSC.

DISCUSSION

THIS STUDY COMPARED MSC derived from bone marrow and adipose tissue in terms of their general characteristics and relative safety for use in infusions. Cell surface marker expression and differentiation capacity were similar in BMSC and ASC; however, TF was differentially expressed and coagulation capacity also varied between the two cell types. Despite the absence of any obvious phenotypic differences between hBMSC and

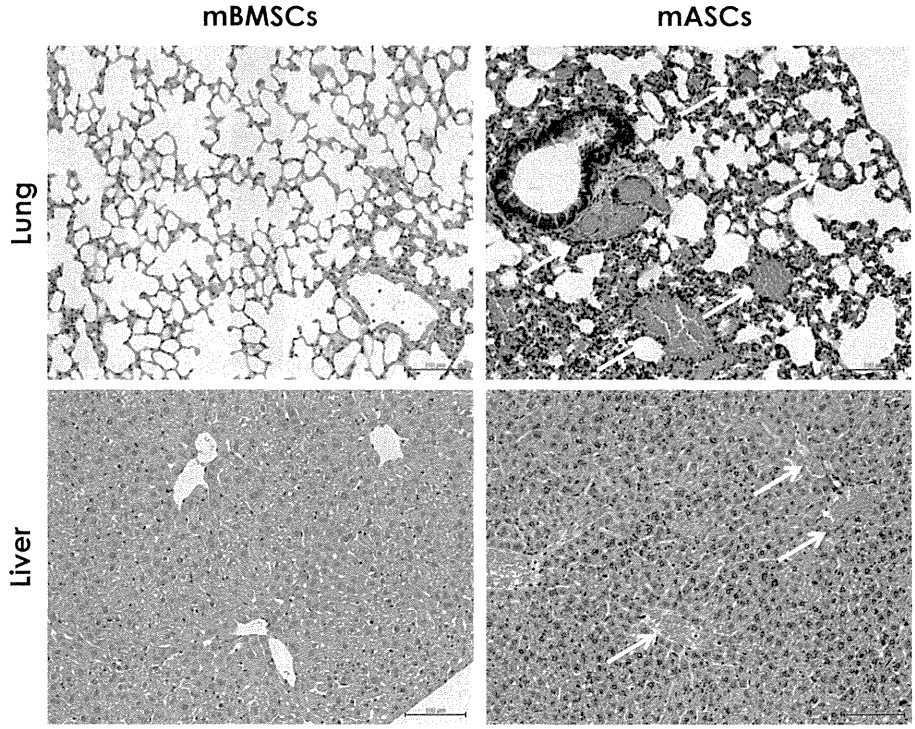

Figure 6 Histopathological analysis of lung and liver tissues after mouse bone marrow-derived mesenchymal stem cell (mBMSC) and mouse adipose-derived mesenchymal stem cell (mASC) infusion. Mice infused with mASC had thrombi in blood vessels (arrows), as seen by hematoxylin–eosin staining of lung and liver tissue sections. In contrast, thrombus was not observed in mice infused with mBMSC. Scale bar = 100 μm.

hASC, TF gene expression was consistently observed in hASC. In addition, TF protein levels were higher in ASC, and were accompanied by a shorter coagulation time than that of BMSC. These results indicate that ASC are distinguished by TF expression, which promotes clotting activity. The survival rate at 24 h was lower and prothrombin time was extended in mASC-infused mice, which also died at a higher rate after infusion. TF is known to stimulate the downstream extrinsic coagulation pathway by forming a compound with factor VII that activates factor VII.[6] Therefore, enhanced pro-coagulation activity by higher expression of TF on the ASC cellular membrane induced multiple thrombi in the peripheral pulmonary and hepatic tissue, inducing deadly multiple organ thromboembolisms. Moreover, prothrombin time is thought to have increased, as extrinsic coagulation factor is consumed in the above process.

We previously demonstrated the safety and effectiveness of ABMi therapy;[2] we also showed the potential for BMSC to improve liver fibrosis and functioning by carbon tetrachloride induction in non-obese diabetic/severe combined immunodeficient mice. These results provide a basis for the development of new types of cell therapy using BMSC.[8,9] Importantly, as the number of transplanted cells may represent an improvement in the efficacy of cell-based therapy, the present data clearly show that BMSC represent a better candidate for this purpose.

In conclusion, the present findings demonstrate that BMSC are a better option than ASC for peripheral vein infusion and provide evidence supporting the use of BMSC for cell therapy in mice.

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Status and Prospects of Liver Cirrhosis Treatment by Using Bone Marrow-Derived Cells and Mesenchymal Cells

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In 2003, we started autologous bone marrow cell infusion (ABM*i*) therapy for treating liver cirrhosis. ABM*i* therapy uses 400 mL of autologous bone marrow obtained under general anesthesia and infused mononuclear cells from the peripheral vein. The clinical study expanded and we treated liver cirrhosis induced by HCV and HBV infection and alcohol consumption. We found that the ABM*i* therapy was effective for cirrhosis patients and now we are treating patients with combined HIV and HCV infection and with metabolic syndrome-induced liver cirrhosis. Currently, to substantiate our findings that liver cirrhosis can be successfully treated by the ABM*i* therapy, we are conducting randomized multicenter clinical studies designated "Advanced medical technology B" for HCV-related liver cirrhosis in Japan. On the basis of our clinical study, we developed a proof-of-concept showing that infusion of bone marrow cells (BMCs) improved liver fibrosis and sequentially activated proliferation of hepatic progenitor cells and hepatocytes, further promoting restoration of liver functions. To treat patients with severe forms of liver cirrhosis, we continued translational research to develop less invasive therapies by using mesenchymal stem cells derived from bone marrow. We obtained a small quantity of BMCs under local anesthesia and expanded them into mesenchymal stem cells that will then be used for treating cirrhosis. In this review, we present our strategy to apply the results of our laboratory research to clinical studies.

Introduction

THE USE OF SOMATIC STEM CELLS in regenerative medicine may help in the development of new treatments for currently intractable diseases. Since November 2003, we have conducted clinical studies in patients with decompensated liver cirrhosis and have supported the development of a liver regeneration therapy using bone marrow cells (BMCs) for autologous BMC infusion (ABM*i*) therapy, a new treatment for liver failure. In addition, aiming to expand the application of the treatment, we are currently conducting research and development studies of a liver regeneration therapy based on cultured mesenchymal cells. In this review, we report on the status of research studies in the field and discuss future challenges in this area.

What is liver cirrhosis?

The liver is composed of a variety of cells such as hepatocytes, cholangiocytes, stellate cells, Kupffer cells, and endothelial cells. Although the liver is an organ with a high regenerative capacity, sustained and chronic inflammation results in the onset of liver fibrosis and the development of cirrhosis when the hepatocytes surrounded by fibrous tissue

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can hardly proliferate, which markedly reduces regeneration and promotes liver dysfunction and cirrhosis. In addition, the risk of liver carcinogenesis increases with the advance of liver cirrhosis. Globally, more than half of the cirrhosis cases are attributable to hepatitis B and C virus (HBV and HBC, respectively) infections; HCV infection is the most common cause in Japan, where approximately 300,000 cirrhosis cases are currently diagnosed. There is, therefore, an urgent need to develop new therapeutic strategies aimed at replacing living donor liver transplantation, which is mostly used for cirrhosis treatment in Japan, contrary to the Western countries where livers from brain-dead donors are used.

Developing improved methods for the administration of BMCs to treat liver fibrosis

A previous study confirmed the presence of Y-chromosomes in the liver tissues of a female leukemia patient who had undergone transplantation of hematopoietic BMCs from a male donor.[1] This groundbreaking finding revealed the existence of cross talk between BMCs and liver tissue, indicating that BMCs were able to fuse with or transdifferentiate into albumin-producing hepatocytes. These results suggested that BMCs might represent a new cell source to repair liver cirrhosis. To investigate the feasibility of the ABM*i* therapy for cirrhosis patients, we developed a mouse green fluorescent protein (GFP)/carbon tetrachloride (CCl_4) model where mild cirrhosis induced by administration of CCl_4 was treated by infusion of BMCs fluorescently labeled with the GFP. Our results confirmed that in cirrhotic livers with persistent liver damage, BMCs administered through peripheral blood differentiated into Liv2-positive hepatoblasts and albumin-producing hepatocytes; in addition, the activation of the surrounding A6-positive hepatoblasts was induced.[2,3] Furthermore, we found that liver fibrosis could be improved by the activity of donor-derived BMCs that migrated into the fibrous area of the cirrhotic liver where they expressed matrix metalloproteinase (MMP)-9, an enzyme capable of degrading the fibrotic tissue. As a result, mice with BMC transplants showed liver regeneration and significant improvement in liver function and survival rate.[4] Currently, the beneficial effects of MMPs on hepatic fibrosis and liver function are important in liver repair and regeneration, and there have been several reports indicating that adenoviral delivery of MMPs into the liver ameliorated experimental liver cirrhosis.[5–7] In addition, the analysis of our GFP/CCl_4 model together with the data obtained from human patients showed that the administered BMCs represented a heterogeneous cell population.[2,4] Identification of the cell types that are involved in liver regeneration and repair will be an important step in the development of next-generation treatments for cirrhosis. By now, it is established that administration of these cells clearly improves fibrosis in the cirrhotic liver and may stimulate proliferation of liver progenitor cells and hepatocytes, ultimately leading to the induction of liver regeneration (Fig. 1).

The status of the ABMi therapy

Based on the results of our basic research, we initiated clinical studies involving liver cirrhosis patients ranging from 18 to 70 years (all the participants provided informed consent).

The participants were patients with total bilirubin levels of 3 mg/dL or lower, platelet counts of 50,000/μL or higher, and not showing hepatocellular carcinoma as detected using MRI and CT scans. Bone marrow fluid (400 mL) was collected under general anesthesia; the cells were washed, and the mononuclear cell components were harvested. The end product was administered through a peripheral vein, and follow-up observations were subsequently conducted.[8] This clinical study, initiated at Yamaguchi University in 2003, was, to our knowledge, the first in the world to show that cirrhosis and liver function in patients could be improved without severe adverse effects.[8] Where possible, the biopsy specimens were analyzed, revealing proliferative PCNA-positive hepatocytes in the liver. The results confirmed that the BMC administration induced proliferation of endogenous hepatocytes. Meanwhile, in a joint research project involving Yamaguchi and Yonsei Universities, the ABM*i* therapy was conducted in 10 patients with Child–Pugh B cirrhosis, and significant improvements in the liver function and in the Child–Pugh score were observed. The therapeutic effect was maintained for 12 months. Liver biopsies performed over time confirmed activation of the liver progenitor cell fraction. An additional 20 patients have received the ABM*i* therapy in Yonsei University.[9] In another collaborative effort between Yamagata and Yamaguchi Universities, the ABM*i* therapy was conducted in six patients with alcoholic cirrhosis and a significant improvement in the Child–Pugh score was observed, revealing the benefits of the ABM*i* therapy in treatment of alcoholic cirrhosis. This clinical study also demonstrated the activation of the bone marrow after the ABM*i* therapy confirmed by scintigraphy.[10] Furthermore, a multicenter clinical study on the ABM*i* therapy was conducted in five HIV/HCV coinfected cirrhotic patients, and in three patients with cirrhosis due to nonalcoholic steatohepatitis (Fig. 2). A comparison of laboratory and clinical research data showed that the BMC administration caused rapid changes in the blood levels of granulocyte colony stimulating factor and interleukin-1β.[11] This phenomenon may be attributable to BMCs, which are normally absent in the body, so their rapid administration can induce changes in the host cytokine dynamics. We have previously shown successful treatment based on the administration of autologous BMCs providing a proof-of-concept for the ABM*i* treatment of liver cirrhosis. This treatment aimed at activation of the hepatic progenitor cells and induction of hepatocellular growth through peripheral administration of BMCs, which should result in improvement of fibrosis in cirrhotic livers (Fig. 2).

Our analysis has shown that patients treated with a combination of splenectomy and administration of autologous BMCs showed an improved liver function because splenectomy enhanced the repopulation of peripherally administered BMCs into the cirrhotic liver.[12] Recently, a study on the dynamics of radiolabeled mesenchymal cells in humans showed that immediately after administration, the cells settled primarily in the lungs, then in the spleen, and finally in the liver. This is consistent with our findings that splenectomy promoted establishment of a large number of BMCs in the liver.[13]

A clinical study was conducted in China when 527 patients with liver failure due to HBV received the same medical treatment, and among them, a group of 53 patients received BMCs through the hepatic artery, with 105 designated as the

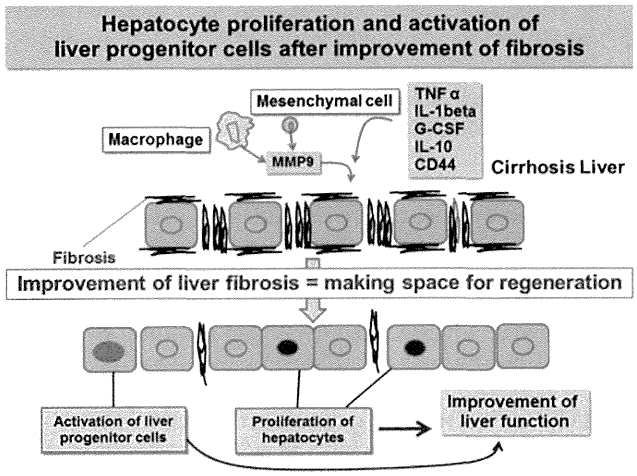

FIG. 1. Mechanism of sequential activation of liver progenitor cells and hepatocytes and improvement of liver fibrosis by BMCs (macrophages and mesenchymal cells).

control group. The analyses showed that the BMC treatment had no side effects. Furthermore, when the patients were divided into early-stage (2–3 weeks) and late-stage (192 weeks) observation groups, improvement of liver function was seen in the early-stage group. In addition, long-term observations suggest that the BMC administration did not increase the incidence of hepatocellular carcinoma.[14] These results are consistent with our findings that frequent administration of BMCs reduces liver carcinogenesis rather than increasing it.[15]

Currently, to substantiate our findings that liver cirrhosis can be successfully treated with transplantation of autologous BMCs, we are conducting randomized multicenter clinical studies designated "Advanced medical technology B" in Japan.

Extending the applications of the ABMi treatment: development of therapeutic methods using the next-generation cultured cells

The current method of the ABM*i*-based therapy consists of administering 400 mL of the bone marrow aspirate under general anesthesia. However, general anesthesia is often inadvisable for individuals with liver failure, which makes the ABM*i* therapy unsuitable for most patients. The problem can be solved when small volumes of bone marrow aspirates are collected under local anesthesia, and fractions of the cells most effective in liver repair and regeneration are expanded and administered to the patients.[16] Figure 3 is a schematic representation of the therapeutic method we are currently developing, which is based on using the cultured BMCs for regenerative treatment of liver cirrhosis. In this method, ~30 mL of bone marrow aspirate is collected under local anesthesia in an outpatient setting and is cultured for about 20 days; the safety is checked before administration to the patient. The development of this therapeutic method and the initiation of the relevant clinical studies are urgently needed, because they will provide treatment options for the patients who thus far have been limited in their choices. A clinical study conducted by Mohamadnejad *et al.* showed that autologous BMCs harvested from four patients

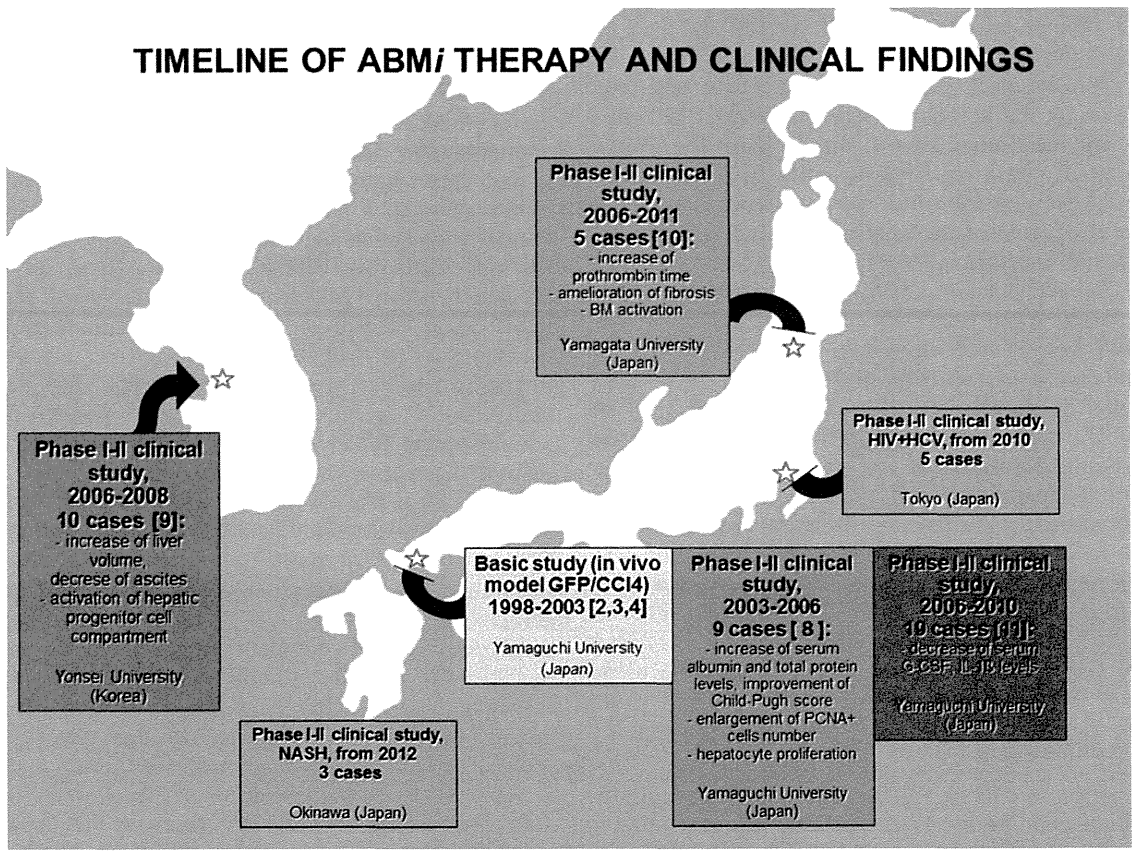

FIG. 2. Timeline of the autologous bone marrow cell infusion (ABM*i*) therapy and clinical findings.

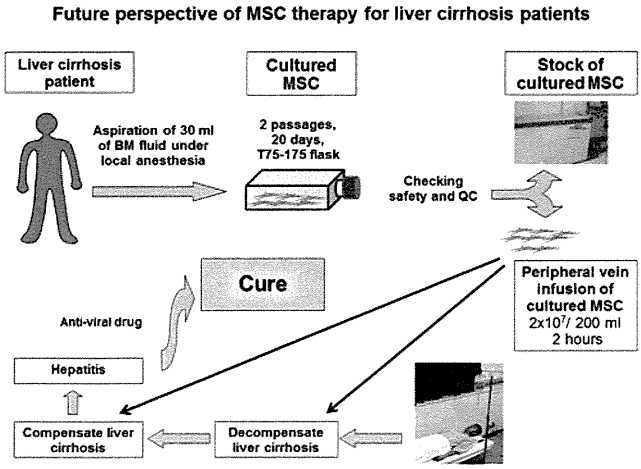

FIG. 3. Schematic representation of the therapeutic method of cultured MSC for liver cirrhosis patients.

with decompensated liver cirrhosis could be cultured and advanced into the mesenchymal stem cell stage, and then be administered through the peripheral vein. The results showed no adverse effects, and at 24 weeks, the model for end-stage liver disease score and liver volume showed improvements in two and three patients, respectively. An improvement in health-related Quality of Life parameters measured by the short form 36 [Medical Outcomes Study 36-Item Short-Form Health Survey (SF-36)] was also confirmed in this study.[17]

Status of the development of a liver-repair-and-regeneration therapy by using the next-generation BMCs

For the development of a minimally invasive treatment, we are conducting research and development investigations while taking into account the guidelines for clinical research on human stem cells.

Flow of the study

1. Establishment of proof-of-concept by using small animal models
2. Evaluation of safety and efficacy by using large- and middle-sized animal models
3. Preparation of clinical research
4. Evaluation of the safety of cultured cells by using cell processing centers (isolators)
5. Clinical research (application for human stem cells).

Establishment of proof-of-concept using small animal models. Our previous treatment regimen based on the administration of autologous BMCs should activate hepatic progenitor cells, induce the proliferation of hepatocytes, and improve the fibrosis associated with liver cirrhosis. In this case, BMCs were introduced through the peripheral blood vessels. We also assessed whether BMCs improved the symptoms of liver fibrosis. To achieve this, we purchased commercially available human bone marrow-derived mononuclear cells (Lonza, Inc., Basel, Switzerland) and evaluated their capability to improve fibrosis in the CCl_4-induced cirrhosis model in SCID mice.[18] Stable cultures of mesenchymal cells were indeed obtained, and our assessments validated that they could improve fibrosis.

Evaluation of safety and efficacy by using large- and middle-sized animal models. In our treatment strategy, we cultured mesenchymal cells from autologous bone marrow aspirated from beagle dogs. These cells were then administered to the same dogs through peripheral blood vessels in approximately three times the quantities delivered by other methods, and at 10 times the concentration used in humans. Subsequently, blood tests were conducted and the development of pulmonary embolism was monitored by computed tomography (CT) tests. No incidents of pulmonary embolism due to the administration of mesenchymal cells were observed, and the method can be used safely.

Cold run at a cell-processing center for preparation of clinical study. Once the quality had been assessed, the cell culture was established in a cell-processing center (CPC) and monitored in compliance with the standard operating procedures. In addition, if the cell culture involved fetal bovine serum (FBS), tests for the detection of residual FBS in the cell preparations were conducted before the evaluations. This stage of the study is focused on assessing whether the cell culture method established outside the CPC can be adopted and used by the CPC. Currently, we are conducting simulations of the practicality of this method for application in humans.

Quality assessment and standard criteria. The quality of the cultured mesenchymal stem cells was examined using flow cytometry with cell-specific surface markers. Cultures were tested for sterility, mycoplasma contamination, and presence of endotoxins and viruses. In addition, if the cell culture involved FBS, the cell preparations were analyzed for residual FBS before further evaluations.

Preparation and realization of a clinical research proposal. We plan to make our clinical research proposal comparable to those already implemented for treating cirrhosis patients who have already been treated with the ABM*i* therapy. In addition, we plan to conduct experiments with cultured mesenchymal cells in accordance with the image shown in Figure 3, starting with laboratory studies. We believe that sufficient amounts of cultured mesenchymal cells can be obtained from small volume aspirates taken under local anesthesia, and that those cell numbers will be higher than those in the currently obtained 30-mL bone marrow aspirates. In addition, the cells can be preserved after culturing and thus can be administered frequently (Fig. 3). This procedure may induce gradual repair and regeneration of the liver and progression from the decompensated cirrhosis to the cirrhosis state and later to the hepatitis state.

Future Prospects

The ABM*i* therapy developed for treating liver cirrhosis has been officially approved as an "Advanced medical technology B" in Japan. In future, we plan to further investigate the ABM*i* therapy by conducting randomized and multicenter clinical studies and gather more evidence. We are also preparing clinical studies on regenerative therapy by using small amounts of the next-generation BMCs (harvested through minimally invasive bone marrow aspiration) based on our previous laboratory and clinical research. A

therapeutic approach based on using somatic stem cells such as cultured BMCs for the treatment of liver cirrhosis is urgently needed. The development of such a strategy will firmly establish an effective regenerative therapy for the treatment of liver cirrhosis, potentially saving the lives of many patients.

Acknowledgments

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Disclosure Statement

No competing financial interests exist.

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Epiregulin promotes the emergence and proliferation of adult liver progenitor cells

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Tomita K, Haga H, Mizuno K, Katsumi T, Sato C, Okumoto K, Nishise Y, Watanabe H, Saito T, Ueno Y. Epiregulin promotes the emergence and proliferation of adult liver progenitor cells. *Am J Physiol Gastrointest Liver Physiol* 307: G50–G57, 2014. First published May 8, 2014; doi:10.1152/ajpgi.00434.2013.—We have previously reported that epiregulin is a growth factor that seems to act on liver progenitor cells (LPCs) during liver regeneration. However, the relationship between epiregulin and LPCs has remained unclear. The aim of the present study was to clarify the role of epiregulin during liver regeneration. The serum levels of epiregulin in patients with acute liver failure were examined. A liver injury model was developed using mice fed a diet containing 0.1% 3.5-diethoxycarbonyl-1.4-dihydrocollidine (DDC) to induce LPCs. We then evaluated the expression of epiregulin and LPCs in these mice. The proliferation of epithelial cell adhesion molecule + LPCs cultured with epiregulin was examined in vitro, and finally epiregulin was overexpressed in mouse liver. In patients with acute liver failure, serum epiregulin levels were elevated significantly. In DDC mice, LPCs emerged around the portal area. Epiregulin was also detected around the portal area during the course of DDC-induced liver injury and was partially coexpressed with Thy1. Serum epiregulin levels in DDC mice were also significantly elevated. Recombinant epiregulin augmented the proliferative capacity of the LPCs in a dose-dependent manner. In mice showing overexpression of epiregulin, the expression of PCNA on hepatocytes was increased significantly. Finally, LPCs emerged around the portal area after epiregulin gene delivery. We concluded that epiregulin promotes the proliferation of LPCs and DNA synthesis by hepatocytes and is upregulated in the serum of patients with liver injury. Furthermore, induction of epiregulin leads to the appearance of LPCs. Epiregulin would be a useful biomarker of liver regeneration.

liver regeneration; progenitor cells; epiregulin; epithelial cell adhesion molecule; Thy1; liver injury

CURRENTLY, LIVER TRANSPLANTATION is the only therapeutic option for rescue of patients with end-stage liver disease. However, the shortage of organ donors has limited its application for most patients, including those in Japan, suggesting the need for an alternative therapeutic option. One such promising option for liver regeneration is cellular transplantation, using resources such as pluripotent stem cells, embryonic stem cells, umbilical cord blood cells, bone marrow cells (BMCs), liver cells, and liver progenitor cells (LPCs). We have been focusing on BMCs and LPCs, the former having been shown to be capable of differentiating into the liver cell lineage under a variety of conditions (2, 16, 23). When liver injury occurs, the stem cells among BMCs subsequently engraft and differentiate, thus contributing to regeneration (24). Recently, autologous BMC transplantation (a technique known as autologous BMC infusion therapy) has been applied for patients with liver cirrhosis and is reported to improve the serum levels of albumin and total protein, leading to an improvement of the Child-Pugh score (22). We have also applied this autologous BMC infusion therapy for patients with alcoholic cirrhosis, resulting in successful improvement of liver function (18).

Previously, we have demonstrated the differentiation of LPCs into hepatocytes during coculture with BMCs. Moreover, fibroblast growth factor (FGF) 2 has been shown to be a critical factor for this LPC migration (8). Gene expression analysis using a cDNA microarray on BMCs under coculture with LPCs demonstrated upregulated gene expression of various other growth factors. Among these growth factors, we have focused on epiregulin, a growth factor that was upregulated along with FGF2.

Epiregulin, first isolated from a fibroblast-derived cancer cell line in 1995 (26), is a membrane-bound growth factor belonging the epidermal growth factor (EGF) family, which binds to and activates both the EGF receptor (EGFR) and erythroblastic leukemia viral oncogene homolog-4 (ErbB4) (11). A few studies have focused on the role of epiregulin during regeneration. Komurasaki et al. (12) reported that epiregulin promoted the proliferation of primary cultured hepatocytes in vitro and that the epiregulin gene was upregulated at 24 and 48 h after partial hepatectomy (PH) in a rat model. However, the precise relationship between epiregulin and its effects on LPCs has remained unclear.

In general, two mechanisms of liver regeneration operate: *1*) after PH, residual mature hepatocytes restore the liver mass volume (13); *2*) in cases of severe liver injury where the proliferation of residual mature hepatocytes is limited, the LPCs are activated and then proliferate and differentiate, thus contributing to liver regeneration (7). The latter pattern of regeneration involving LPCs is more important in patients with advanced liver failure. However, the precise mechanism of LPC-dependent regeneration, including the effect of epiregulin, remains unclear.

The aim of the present study was to clarify the role of epiregulin during liver regeneration using a mouse model. Our results suggested that epiregulin plays a role in liver regeneration by promoting cellular proliferation and the emergence of LPCs.

MATERIALS AND METHODS

Animals. C57BL/6J mice (CLEA, Tokyo, Japan) were used for all the experiments. All animals were maintained under standard

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Table 1. *Patients' characteristics*

	Acute Liver Failure, n = 14	Acute Hepatitis, n = 24	Liver Cirrhosis, n = 24	Chronic Hepatitis, n = 24	Control, n = 24	P Value
Age, yr	47 ± 14.6	48.5 ± 12.2	48.1 ± 11.1	48.6 ± 12.9	48.8 ± 15.7	0.99
Sex, m/f	10/4	14/10	13/11	14/10	14/10	0.88
Total bilirubin, mg/dl	9.8[2.4–32.4]	3.8[0.4–40]	1.7 ± 1.2	0.9 ± 0.3	0.7 ± 0.4	$P < 0.05$
AST, IU/l	1111[82–6335]	557[38–7403]	45[16–192]	32[16–122]	20.6 ± 5.6	$P < 0.05$
ALT, IU/l	1377[37–5690]	545[56–4640]	31[8–122]	38[13–129]	17.7 ± 6.5	$P < 0.05$
Plt, 1.0 × 103/μl	11 ± 6.8	23.3 ± 10.2	11.6 ± 7.5	18.2 ± 5.1	25.6 ± 8.7	$P < 0.05$
PT, %	33.7 ± 10.6	92.8 ± 28.2	68.8 ± 18.8	107.2 ± 8.9	—	$P < 0.05$
PT-INR	1.9 ± 0.3	1.08 ± 0.18	1.27 ± 0.24	0.65 ± 0.05	—	$P < 0.05$
Etiology						
HBV/HCV/AIH/PBC/	3/0/2/0/	7/1/3/0/	2/8/2/1/	7/15/0/0/		
Alcohol/Drug/NAFLD/	4/3/0/	4/3/0/	6/0/2/	1/1/0/		
Other/Unknown	0/2	4/2	3/0	0/0	—	

Applicable values are presented as means ± SD or median[range]. AST, aspartate aminotransferase; ALT, alanine aminotransferase; Plt, platelets; PT, prothrombin time; PT-INR, prothrombin time and international normalized ratio; HBV, hepatitis B virus; HCV, hepatitis C virus; AIH, autoimmune hepatitis; PBC, primary biliary cirrhosis; NAFLD; nonalcoholic fatty liver disease.

SPF conditions, and all the experiments using animals were performed under institutional guidelines. Mouse liver progenitor cells were induced by feeding 8-wk-old male mice a diet containing 0.1% 3.5-diethoxycarbonyl-1.4-dihydrocollidine (DDC) (CLEA). In a gene-delivery experiment involving hydrodynamic tail vein injection (HTVi), male mice weighing 18–20 g were used. The experiments involving both gene delivery and animals were approved by the institutional review board (animal experiment; approval number 24139, gene-splicing research; approval number 24-27).

Gene delivery. A plasmid in which mouse epiregulin cDNA was cloned into the pLIVE (Liver in Vivo Expression) vector and the empty expression pLIVE vector (Mirus Bio, Madison, WI) were purchased from Takara (Shiga, Japan). The mouse epiregulin plasmid was amplified by *Escherichia coli* DH5α competent cells (Takara) and purified using an Endofree Plasmid Mxi Kit (Qiagen, Dusseldorf, Germany). Plasmid DNA was administered to mice using a HTVi technique involving rapid injection of a large volume of DNA solution through the tail vein. Briefly, 20 μg of plasmid DNA (1 mg/ml) was diluted in 2 ml of TransIT-EE Hydrodynamic Delivery Solution (Mirus Bio) and injected into male mice weighing 18–20 g via the tail vein within 6 to 8 s.

Immunohistochemistry. For immunohistochemistry (IHC), a rabbit polyclonal antibody against mouse CK19 (Proteintech, Chicago IL) and a rabbit polyclonal antibody against mouse epiregulin (Abbiotec, San Diego, CA) were used at a dilution of 1:50. A mouse monoclonal antibody against PCNA (clone PC-10; Nichirei, Tokyo, Japan), a rat monoclonal antibody against mouse epithelial cell adhesion molecule (EpCAM) (BD Pharmingen, San Diego, CA), and a rat monoclonal antibody against mouse Thy1 (BD Pharmingen) were used at a dilution of 1:100. The formalin-fixed paraffin-embedded liver sections (3 μm) underwent antigen retrieval processing in a microwave oven after deparaffinization. After being blocked with blocking one Histo reagent (Nakarai, Kyoto, Japan), the samples were incubated with primary antibodies and subsequently with high-molecular-weight polymer secondary antibodies (Simple Stain kit, Nichirei). The samples were finally developed with a peroxidase substrate ImmPACT NovaRED kit (Vector Laboratories, Burlingame, CA). Frozen sections (6 μm) from the liver were placed on APS-coated glass slides using a cryostat (Leica CM 3050S). After being fixed with ethanol, the samples were incubated with the primary antibodies and then incubated with fluorescence-conjugated secondary antibodies before observation by fluorescence microscopy (D1; Zeiss Axio Observer, Jena, Germany).

RNA extraction and RT-PCR. Total RNA was extracted from cells and tissues using a RNeasy Plus Mini Kit (Qiagen). Total RNA (2 μg) was used to synthesize cDNA using a SuperScript VILO cDNA synthesis kit (Life Technologies, Carlsbad, CA). The samples were subjected to 40 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s. PCR primers for mouse genes were as follows (5′ to 3′): *1*) epiregulin, TTGGGTCTTGACGCTGCTTTGT and TGAGGTCACTCTCATATTC; *2*) EGFR, CTGCCAAGGCACAAGTAACA and ATTGGGACAGCTTGGATCAC; *3*) Erbb4, GCTGAGGAATATTTGGTCCCCCAG and AAACATCTCAGCCGTTGCACCCTG; *4*) β-actin, GGCTGTATTCCCCTCCATCG and CCAGTTGGTAATGCCATGT. Quantitative real-time PCR was performed using a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) with the TaqMan Gene Expression Assay (Assay ID): epiregulin, Mm00514794_m1; and GAPDH, Mm99999915_g1.

Cell culture and proliferation assay. An EpCAM-positive liver progenitor cell line from the liver of a DDC-fed adult mouse was kindly provided by Dr. Atsushi Miyajima (Laboratory of Cell Growth and Differentiation, Institute of Molecular and Cellular Biosciences, The University of Tokyo). The EpCAM+ cells were maintained in a cell culture flask (Corningware, Corning, NY) using medium supplemented with fetal bovine serum and 10 ng/ml of each human recombinant EGF and hepatocyte growth factor (HGF). The proliferative response of EpCAM+ cells was exam-

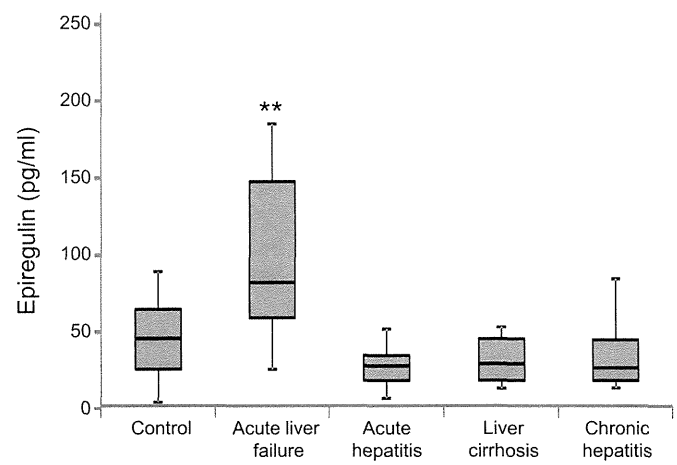

Fig. 1. ELISA for determination of human epiregulin was performed on serum samples from healthy controls (n = 25) and patients with acute liver failure (n = 14), acute hepatitis (n = 24), liver cirrhosis (n = 24), and chronic hepatitis (n = 24). Acute liver failure was defined as severe liver dysfunction, as indicated by a prothrombin time of less than 40% or prothrombin time and international normalized ratio (commonly called PT-INR) >1.5 within 8 wk after initial presentation of symptoms. Serum epiregulin levels in patients with acute liver failure were significantly higher than those in healthy controls. However, there were no significant differences among the other disease groups. Means ± SD, $^{**}P < 0.01$.

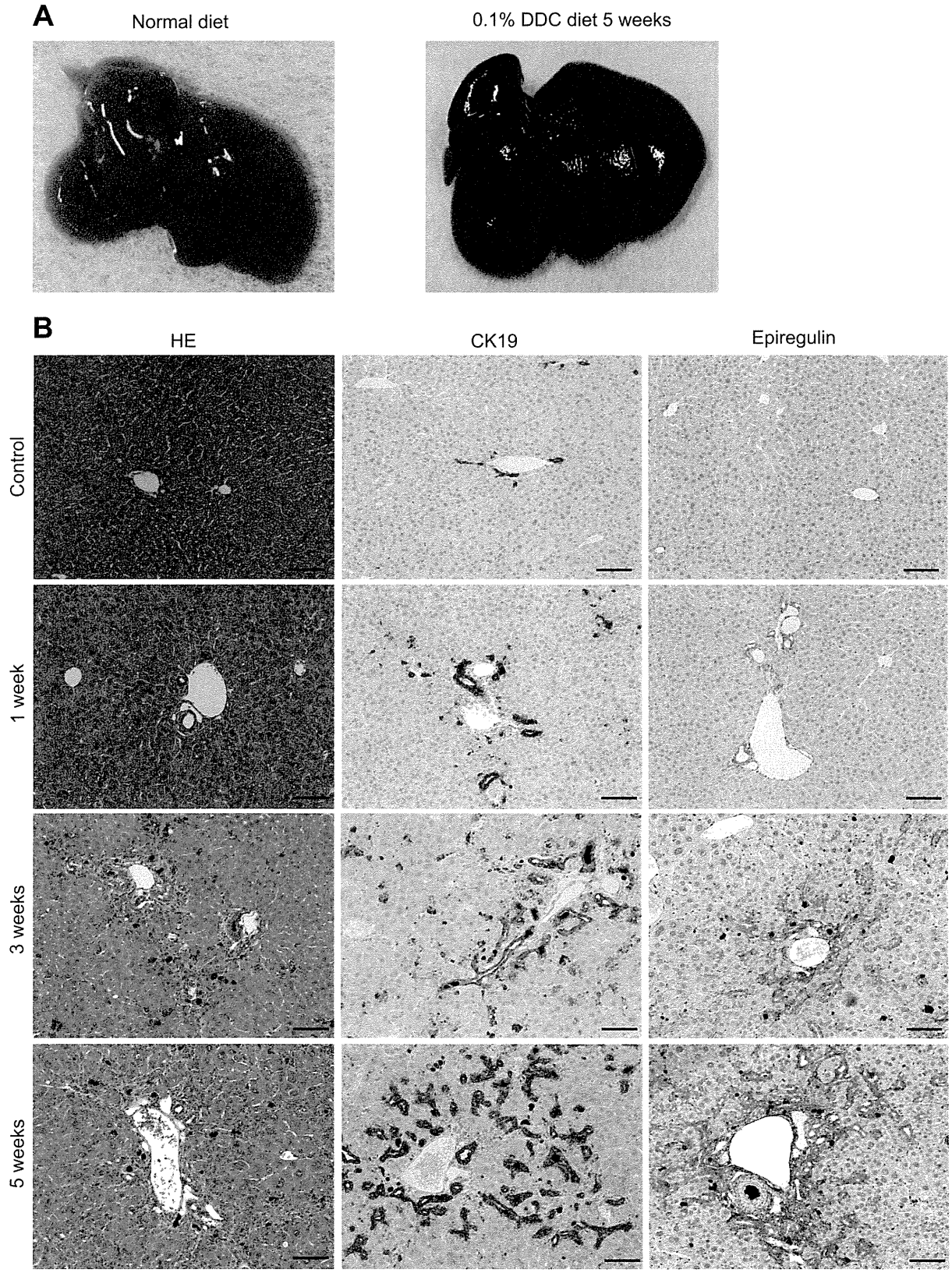

Fig. 2. Mouse liver progenitor cells (LPCs) were induced by a diet containing 0.1% 3.5-diethoxycarbonyl-1.4-dihydrocollidine (DDC) in C57BL/6J mice. *A*: liver turned black and stiff after 5 wk on the DDC diet. *B*: hematoxylin and eosin (HE) staining of livers from mice on the DDC diet demonstrated ductular proliferation consisting of small cells in the portal area from 1 to 5 wk. These small cells were positive for CK19, indicating that they were LPCs. The expression of epiregulin protein was markedly elevated around ductular structures close to the portal area during the course of DDC-induced liver injury. Bars = 100 μm.

ined in the presence of FBS, EGF, or HGF in different concentrations, using the water-soluble tetrazolium salt (WST)-1 cell proliferation reagent (Roche, Basel, Switzerland). The absorbance value (OD450-OD650) was measured using a Benchmark Plus microplate reader (Bio-Rad, Hercules, CA).

Serum epiregulin immunoassay. The human and mouse serum epiregulin values were measured using an epiregulin-specific ELISA (USCN, Hubei, China) in accordance with the manufacturer's protocol (assay range: 6.4–1,000 pg/ml). The absorbance value (450 nm) was measured using a Benchmark Plus microplate reader (Bio-Rad).

Statistical analysis. Comparison of gene expression assays, immunoassay, and patient background were done using one-way ANOVA with subsequent Steel-Dwass or Tukey-Kramer tests. Analysis of the cell proliferation assay was done using two-way ANOVA. Differences at $P < 0.05$ were considered to be statistically significant. The above statistical analyses were done using the SAS software package (SAS Institute, Cary, NC).

RESULTS

Elevation of serum epiregulin levels in patients with acute liver failure. To investigate whether epiregulin is involved in liver regeneration, we determined the serum epiregulin levels in patients with a variety of liver diseases, including acute liver failure, acute hepatitis, chronic hepatitis, and liver cirrhosis. Those in healthy subjects were also examined for comparison. Acute liver failure was defined as severe liver dysfunction with a prothrombin time of less than 40% or prothrombin time and international normalized ratio (commonly called PT-INR) >1.5 within 8 wk after initial presentation (14). There was no significant difference in background factors such as age or sex ratio between the groups. Other characteristics including the degree of liver damage are listed in Table 1. Serum epiregulin levels in patients with acute liver failure were significantly higher than in healthy controls (Fig. 1). However, there were no significant differences among the other disease groups. This observation may have reflected the promotion of epiregulin production only under conditions in which proliferation of normal hepatocytes would be limited.

Expression of epiregulin in injured adult mouse liver concomitant with LPC emergence. The 2-acetylaminofluorene (2-AAF)/PH model, in which hepatocyte proliferation is blocked by 2-AAF before PH, has been used to induce LPCs in rats (5, 6). Recently, a 0.1% DDC-containing diet has been developed to induce LPCs in mice, and this mouse model was employed for the present study (1, 17). After 4–5 wk on the DDC diet, the liver of mice appeared black and stiff (Fig. 2*A*). Hematoxylin and eosin staining was used to evaluate adult normal liver and liver with DDC-induced injury. After 1 wk on the DDC diet, the liver demonstrated ductular structures around the portal area, and these gradually increased up to 5 wk (Fig. 2*B*). These ductular structures consisted of small cells with large nuclei, with a so-called "oval cell" appearance. IHC for CK19, one of the markers of LPCs and cholangiocytes, showed that these small cells included CK19-expressing LPCs (Fig. 2*B*). Epiregulin protein expression was barely detectable in normal liver but was markedly induced around the ductular structures close to the portal area during the course of DDC-induced liver injury (Fig. 2*B*). Quantitative gene expression analysis confirmed that the expression of epiregulin mRNA was significantly increased in the DDC-injured liver after 4–5 wk on the DDC diet (Fig. 3, *A* and *B*). In addition, the serum epiregulin level was significantly increased from 2 wk on the DDC diet and gradually increased up to 4 wk (Fig. 3*C*).

Coexpression of epiregulin with Thy1-positive cells around ductular structures in injured mouse liver. To investigate the site of epiregulin expression, we examined injured mouse liver using IHC. As briefly mentioned above, IHC for CK19 and epiregulin confirmed that epiregulin was expressed around the LPCs forming ductular structures. Recently, as it has been reported that Thy1-positive cells express growth factors nec-

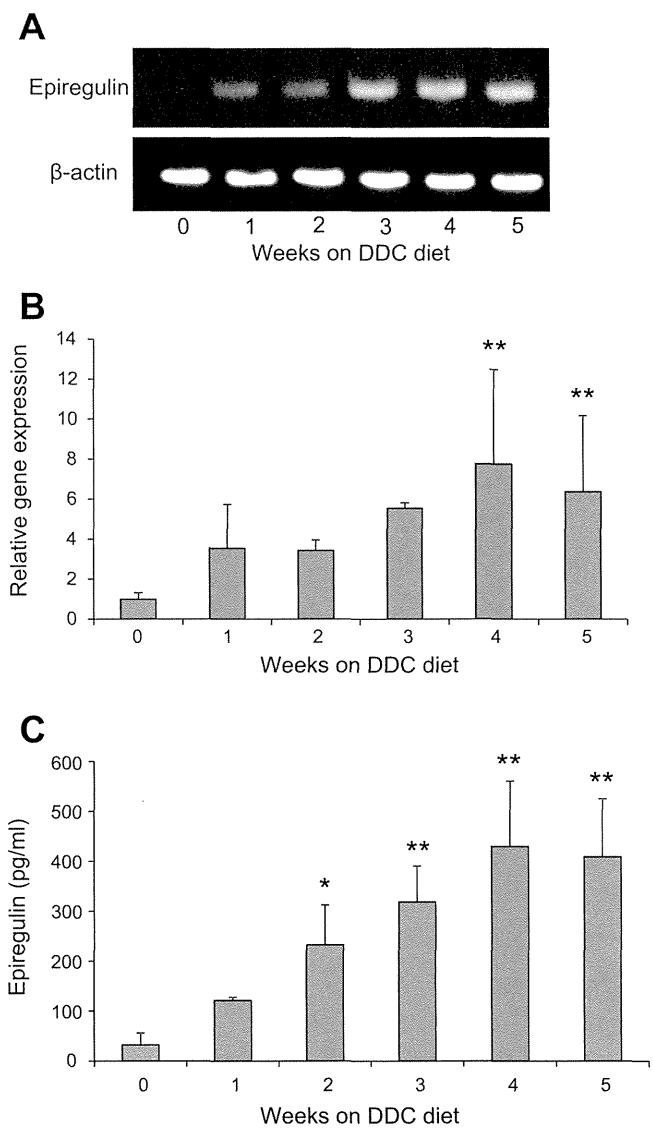

Fig. 3. Serum and hepatic epiregulin expression in DDC mice. *A*: RT-PCR for epiregulin mRNA in mouse liver after DDC diet feeding. The epiregulin gene was expressed from 1 to 5 wk after the start of the DDC diet. *B*: quantitative changes in the level of epiregulin mRNA in mouse liver after DDC diet feeding, determined by real-time PCR analysis. Epiregulin expression was normalized against that of GAPDH. Epiregulin mRNA was increased from 1 wk after the start of the DDC diet and was significantly increased at 4–5 wk relative to the value at 0 wk. Mean ± SD ($n = 3$). *C*: ELISA for serum epiregulin showed a significant increase from 2 wk after the start of DDC diet feeding and reached a peak at 4 wk relative to the value at 0 wk. Means ± SD ($n = 4$). $^{**}P < 0.01$, $^{*}P < 0.05$.

essary for the maintenance of LPCs (15), we performed IHC for epiregulin and Thy1, which are markers of mesenchymal cells or LPCs. First, we performed double immunostaining for CK19 and EpCAM as LPC markers and confirmed that both were coexpressed around ductular structures in the portal area at 5 wk. Double immunostaining for epiregulin and EpCAM revealed that epiregulin was expressed adjacent to the LPCs forming ductular structures. Further double immunostaining for Thy1 and epiregulin revealed partial coexpression of these two markers (Fig. 4).

Epiregulin promotes the proliferation of EpCAM+ LPCs in vitro. Because epiregulin was expressed close to LPCs in injured mouse liver, we performed an in vitro study to investigate whether epiregulin affects LPCs. First, to determine whether epiregulin acts on LPCs directly, we examined the expression of the epiregulin receptors EGFR and ErrbB4 in EpCAM+ LPCs. Although epiregulin is known to bind to and activate both EGFR and Erbb4 (11), only EGFR was expressed in EpCAM+ LPCs (Fig. 4*A*). Subsequently, we examined the proliferation of LPCs under different dosages of either recombinant epiregulin or EGF, another EGFR ligand. EpCAM+ LPCs were exposed to 0, 20, or 100 ng/ml epiregulin or EGF in culture, and their proliferations were measured after 3 days of incubation in 96-well plates using the standard WST-1 assay. This revealed that recombinant epiregulin promoted the proliferation of EpCAM+ LPCs in a concentration-dependent manner, and the proliferation efficacy was significantly higher in epiregulin than EGF (Fig. 4*B*).

Epiregulin overexpression in adult mouse liver induces LPCs and promotes the synthesis of DNA in hepatocytes. To investigate the effect of epiregulin on hepatocytes, the epiregulin gene was overexpressed in mice using the HTVi method, which involves injection of plasmid DNA via a tail vein and facilitates overexpression of the target gene in organs including the liver. Since Zhang et al. (19, 31) first reported the HTVi method in 1997, it has been used in various gene overexpression studies because of its *1*) high transfer efficiency and reproducibility and *2*) easier preparation of plasmid DNA (30). The epiregulin-overexpressing mice were killed at 3, 7, 14, and 21 days after gene delivery. The control mice were injected with empty expression plasmid vector. IHC for epiregulin confirmed its expression in the entire liver up to 21 days after gene delivery, reaching a peak during the initial 3–7 days (Fig. 6*A*). Empty vector-injected mice livers demonstrated no detectable levels of epiregulin expression. To evaluate DNA synthesis, IHC for PCNA was performed on epiregulin-overexpressing mouse liver. IHC for CK19 confirmed that LPC induction was evident from 3 days after epiregulin gene delivery (Fig. 5*A*). Furthermore, the ratio of PCNA-positive cells was increased significantly at 3, 7, and 14 days after epiregulin gene delivery (Fig. 6, *A* and *B*).

DISCUSSION

In the present study, we have shown that expression of epiregulin promoted the induction of LPCs and DNA synthesis by hepatocytes and that epiregulin was expressed by the stem cell niche including mesenchymal cells located around ductular structures in the portal area of adult mice. Furthermore, the serum epiregulin level was elevated in both patients and mice with liver injury. Taken together, these data suggested that

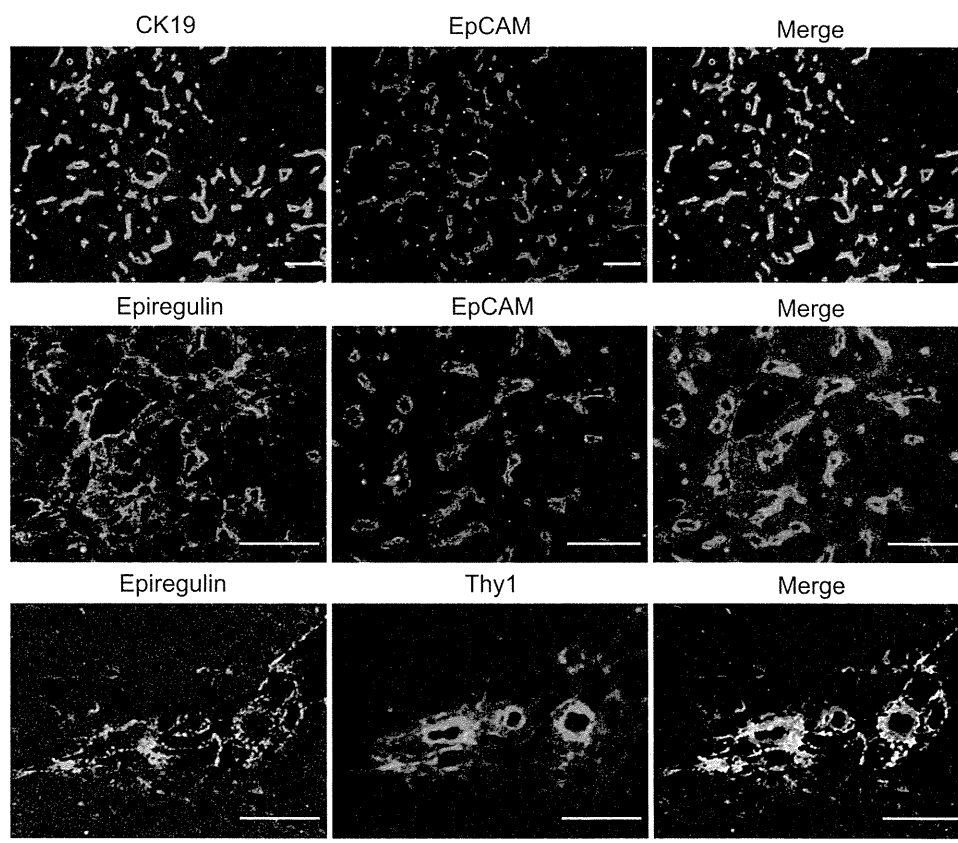

Fig. 4. Double immunostaining study of mouse liver after DDC diet feeding. First, we performed double immunostaining for CK19 and epithelial cell adhesion molecule (EpCAM) as LPC markers and confirmed that both markers were coexpressed and associated with ductular structures in the portal area of mice at 5 wk. Double immunostaining for epiregulin and EpCAM revealed that epiregulin was expressed adjacent to LPCs forming ductular structures. Further double immunostaining for Thy1 and epiregulin revealed partial coexpression of these 2 markers. Bar = 200 μm.

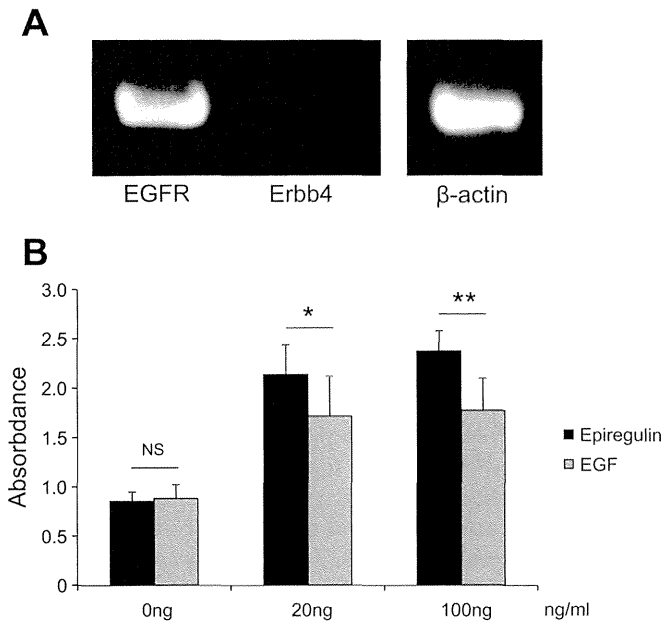

Fig. 5. Proliferation assay of LPCs cultured with recombinant epiregulin or epidermal growth factor (EGF). *A*: EGFR, but not ErrB4, was expressed in EpCAM+ LPCs. *B*: proliferation assay of LPCs cultured with recombinant epiregulin or EGF at 3 different concentrations (0, 20, 100 ng/ml). Recombinant epiregulin promoted the proliferation of EpCAM+ LPCs in a concentration-dependent manner. Furthermore, the proliferation efficacy was significantly higher in epiregulin than EGF. The water-soluble tetrazolium salt assay was repeated 8 times. Means ± SD, $^{**}P < 0.01$, $^{*}P < 0.05$.

active induction of epiregulin is required during liver regeneration to maintain homeostasis in vivo.

Komurasaki et al. (12) have reported that epiregulin promoted the proliferation of primary cultured hepatocytes in vitro and that its expression was upregulated after PH in rats. However, the precise relationship between epiregulin and LPCs has remained unclear. Furthermore, although epiregulin acts via the EGF receptor (11), neither its cells of origin nor the trigger for its expression have yet been proven. It is now known that LPCs originate from the canal of Hering and have bipotentiality for differentiation into either hepatocytes or cholangiocytes in injured adult liver (7). However, the mechanism of action and the origin of LPCs are still controversial.

Our study of the epiregulin level in human serum showed that it was significantly increased in patients with acute liver failure and did not differ significantly among several liver diseases, including acute hepatitis and cirrhosis. Epiregulin protein expression was also increased in both the liver and serum of DDC-fed mice. These data collectively imply that the expression of epiregulin is limited to conditions associated with critical liver injury in which there is insufficient regeneration of residual hepatocytes. In other words, increased epiregulin expression is related to the emergence of LPCs. HGF and TGF-α are known to be increased in severe liver injury (25, 27), and here we confirmed for the first time that the serum epiregulin level was elevated in patients with acute liver failure. Epiregulin may therefore be a useful biomarker for monitoring or assessing patients with severe liver injury, as is the case for HGF, which has been used as a prognostic indicator in patients with severe liver injury (28).

Epiregulin was expressed from 1 wk on the DDC diet and then gradually increased, reaching a peak at 4–5 wk. This course of expression was identical to that of CK19+ in LPCs. We have also shown that epiregulin was expressed in the area of LPCs forming ductular structures, i.e., the so-called stem cell niche, and was partially coexpressed by Thy1-positive cells. It has been reported that immune cells or mesenchymal cells emerge around the LPCs (10, 21). Recently, FGF7, another growth factor, was shown to be expressed by Thy1-positive cells forming the stem cell niche and to contribute to the maintenance of liver regeneration (15). Taken together, the data suggest that epiregulin may also contribute to liver regeneration through expression by Thy1-positive cells, which belong to the LPC niche. However, because epiregulin was partially coexpressed with Thy1, other candidate epiregulin-producing cells may exist. In fact heparin-binding-EGF, a hepatocyte growth-promoting factor belonging to the EGF family, is expressed by both endothelial cells and Kupffer cells (9).

In our in vivo study of mice with epiregulin overexpression, we have confirmed that epiregulin promoted the synthesis of DNA by mature hepatocytes and did not affect LPCs alone. The present study is the first study describing the ability of epiregulin to promote DNA synthesis in vivo. EGF was originally characterized as a growth factor that binds to specific receptors on the cell surface, stimulating protein tyrosine kinase activity leading to cellular proliferation and DNA synthesis (3, 4, 29). However, the signaling potencies of epiregulin and EGF were considered to be different (12). In particular, epiregulin is reported to exhibit modest activating effects for EGFR and MAPK phosphorylation although activating effects of epiregulin were supposed to be prolonged compared with those of EGF (12). In our in vitro study, epiregulin promoted the proliferation of LPCs, and its efficacies were higher than EGF. This biological difference may be derived partially due to the weak activating capabilities of epiregulin, which in turn affected to prevent the negative downstream regulations.

In this study, although epiregulin was shown to promote the induction and proliferation of LPCs, further analysis will be needed to confirm whether epiregulin is really essential for LPC induction, perhaps employing epiregulin gene-knockout mice. Although epiregulin gene-knockout mice have already been established, there have been no reports indicating that they have developmental anomalies in the liver (20). Using these epiregulin-knockout mice fed a DDC diet, we were able to evaluate whether LPCs were induced. Further analyses will be needed to determine whether there are unspecified epiregulin-expressing cells that are Thy1 negative.

In conclusion, epiregulin *1*) is expressed in the stem cell niche, which includes mesenchymal cells around the LPCs during severe liver injury, *2*) contributes to liver regeneration by inducing LPCs, and *3*) promotes DNA synthesis by mature hepatocytes. In addition, the serum epiregulin level is significantly elevated in both patients and mice with liver injury. Taken together, these data indicate that epiregulin would be a useful biomarker of liver regeneration.

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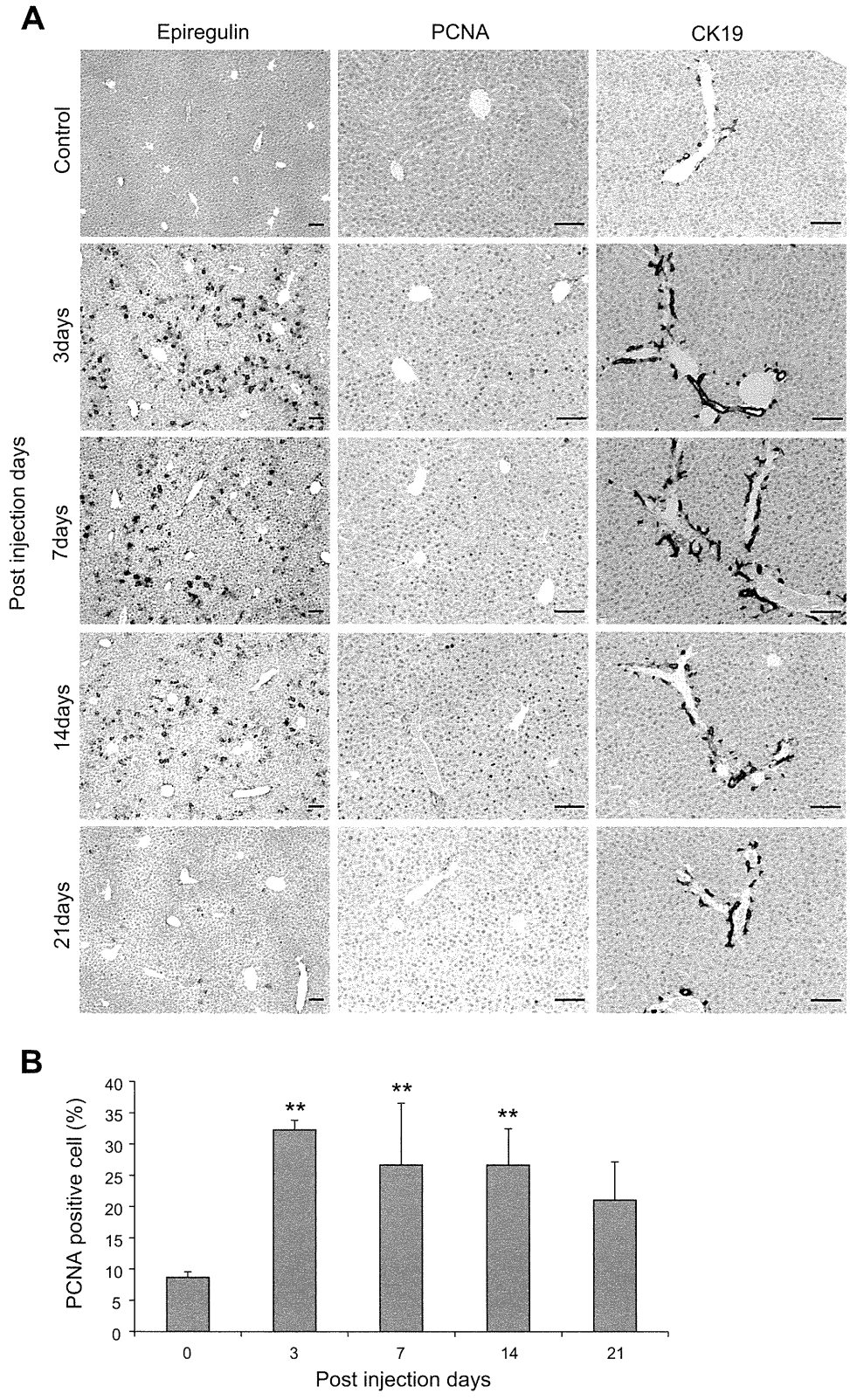

Fig. 6. The epiregulin gene was overexpressed using the hydrodynamic tail vein injection method in C57BL/6J mice. A: immunohistochemistry for epiregulin confirmed expression of the protein from 3 to 21 days after gene delivery. Control mice were studied 3 days after injection with empty expression plasmid vector. Along with epiregulin expression, the ratio of PCNA-positive cells was significantly increased at 3 to 14 days. In addition, ductular proliferation consisting of CK19-positive LPCs was confirmed at 3 to 21 days. Bar = 200 μm. *B*: ratio of PCNA-positive cells relative to the value in normal mice. The PCNA positivity ratio was significantly increased up to 14 days and peaked at 3 days after gene delivery. Means ± SD, $^{**}P < 0.01$.

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GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: K.T., H.H., and Y.U. conception and design of research; K.T., H.H., K.M., and T.K. performed experiments; K.T. and H.H. analyzed data; K.T., H.H., K.M., T.K., C.S., K.O., Y.N., H.W., T.S., and Y.U. interpreted results of experiments; K.T. and H.H. prepared figures; K.T., H.H., and Y.U. drafted manuscript; K.T., H.H., and Y.U. edited and revised manuscript; K.T., H.H., and Y.U. approved final version of manuscript.

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Original Article

Clinical manifestations of liver injury in patients with anorexia nervosa

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Aim: The number of Japanese patients with anorexia nervosa (AN) is increasing as society changes. Mild liver injury is a complication of AN in around 30% of cases. In some rare instances, patients present with severe liver injury similar to acute liver failure. However, there are numerous uncertainties over the clinical characteristics of this condition. The objective of the present study was to clarify the clinical characteristics of AN complicated by liver injury and to investigate the factors related to hepatic complications.

Methods: Thirty-seven patients hospitalized at our institution with a diagnosis of AN were enrolled as the study subjects. The study used clinical data obtained at the time of hospitalization. The enrolled patients underwent subgroup analysis and were categorized into three groups: (i) normal alanine aminotransferase (ALT), (ii) moderately elevated ALT, and (iii) highly elevated ALT.

Results: All of the study subjects were female with a median age of 24 years and presenting with marked weight loss (mean body mass index, 13 kg/m^2). Thirteen of the subjects had liver injury. We found that patients in the highly elevated ALT group had a significantly high blood urea nitrogen (BUN)/creatinine ratio, and a low blood sugar level.

Conclusions: Our present findings indicate that AN patients with highly elevated ALT have a severe dehydration. This suggests that dysfunction of hepatic circulation accompanying severe dehydration due to malnutrition may be an important factor in the development of liver injury in AN patients.

Key words: dehydration, eating disorder, emaciation, hypoxic hepatitis, liver enzyme

INTRODUCTION

ANOREXIA NERVOSA (AN) affects mainly adolescent females in developed countries including the USA, Europe and Japan. The majority of patients develop AN due to abnormal eating habits resulting from a desire to be lean or fear of becoming obese after exposure to psychiatric stress. In Japan, the prevalence of AN has been increasing rapidly;[1] according to annual reports issued by the Ministry of Health, Labour and Welfare, the incidence of eating disorders increased 10-fold in the 20 years since 1980, and the number of AN cases in particular increased fourfold during the 5 years since the mid 1990s. The prevailing explanation for this increase is the change of lifestyle in Japan including the increased variety of social circumstances. AN is associated with a number of complications including liver injury, especially elevation of the serum alanine aminotransferase (ALT) level in more than 30% of cases.[2] Furthermore, rare cases of severe liver injury resulting in acute liver failure have been reported.[3–5] However, the precise mechanism involved in the pathogenesis of liver injury associated with AN remains unclear. Moreover, few reports have documented the clinical features of AN complicated by liver injury. Some have indicated an association with low body mass index (BMI),[6,7] although the roles of other clinical surrogate markers are unclear.

The aim of the present study was to clarify the clinical features of AN complicated by liver injury and the

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