In daily practice, the concept of fiberoptic intubation in the awake patient is not clearly defined. In most cases, the choice of technique is dependent on institutional and personal preferences. Ultimately, such a choice is a compromise between safety, practicability, and acceptance. The technique as shown in the video is a thoroughly documented, well-tested method that has not been changed for many years.[5]

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Since publication of his article, the author reports no further potential conflict of interest.

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Deferoxamine for Advanced Hepatocellular Carcinoma

TO THE EDITOR: We have previously reported that the iron chelator deferoxamine can prevent liver injury as well as the development of preneoplastic lesions in rats,[1,2] and we have proposed the use of deferoxamine as an anticancer drug. The antiproliferative effect of deferoxamine arrests the cell cycle and induces apoptosis.[3] To our knowledge, no clinical study has been performed to evaluate deferoxamine therapy in patients with hepatocellular carcinoma.[4]

Our study involved 10 patients (6 men and 4 women) who had advanced hepatocellular carcinoma and did not have a response to hepatic arterial infusion chemotherapy with anticancer drugs. The average age of the patients was 64 years (range, 43 to 77). Written informed consent was obtained before the study, which was approved by the institutional review board of Yamaguchi University Hospital. Seven patients had hepatitis C virus infection, 2 patients had hepatitis B virus infection, and 1 patient did not have either type of infection. The tumor stages were classified as II, IVA, and IVB (according to the Liver Cancer Study Group of Japan criteria) for 1, 2, and 7 patients, respectively. The Child–Pugh class was A, B, and C for 3, 5, and 2 patients, respectively. (In the Child–Pugh classification of liver disease, class A indicates the least severe disease, class B moderately severe disease, and class C the most severe disease.) The patients received an arterial infusion of deferoxamine (at a dose of 10 to 80 mg per kilogram of body weight) over 24 hours on alternate days, through the injection port.

Deferoxamine was administered an average of 27 times (range, 9 to 78). Two, three, and five patients had a partial response, stable disease, and progressive disease, respectively (according to the Eastern Cooperative Oncology Group criteria). The overall response rate was 20%.

Tumor-marker levels (alpha-fetoprotein, des-γ-carboxyprothrombin, alpha-fetoprotein L3, or all of these levels) decreased in patients with a partial response. In one patient, a massive hepatocellular tumor with lung metastases disappeared with deferoxamine treatment (Fig. 1). The 1-year cumulative survival rate was 20%. Four patients had grade 2 or 3 interstitial pneumonia (according to the Common Terminology Criteria for Adverse Events, version 4.0), and one patient had grade 2 renal dysfunction. However, no grade 4 adverse events were observed.

Sorafenib, a multikinase inhibitor, has recently been established as the standard of care for patients with advanced hepatocellular carcinoma and preserved liver function (Child–Pugh class A) because it increases survival.[5] However, its safety and efficacy for patients with Child–Pugh class B or C disease is still unknown. Deferoxamine may warrant testing in patients with Child–Pugh class B or C hepatocellular carcinoma.

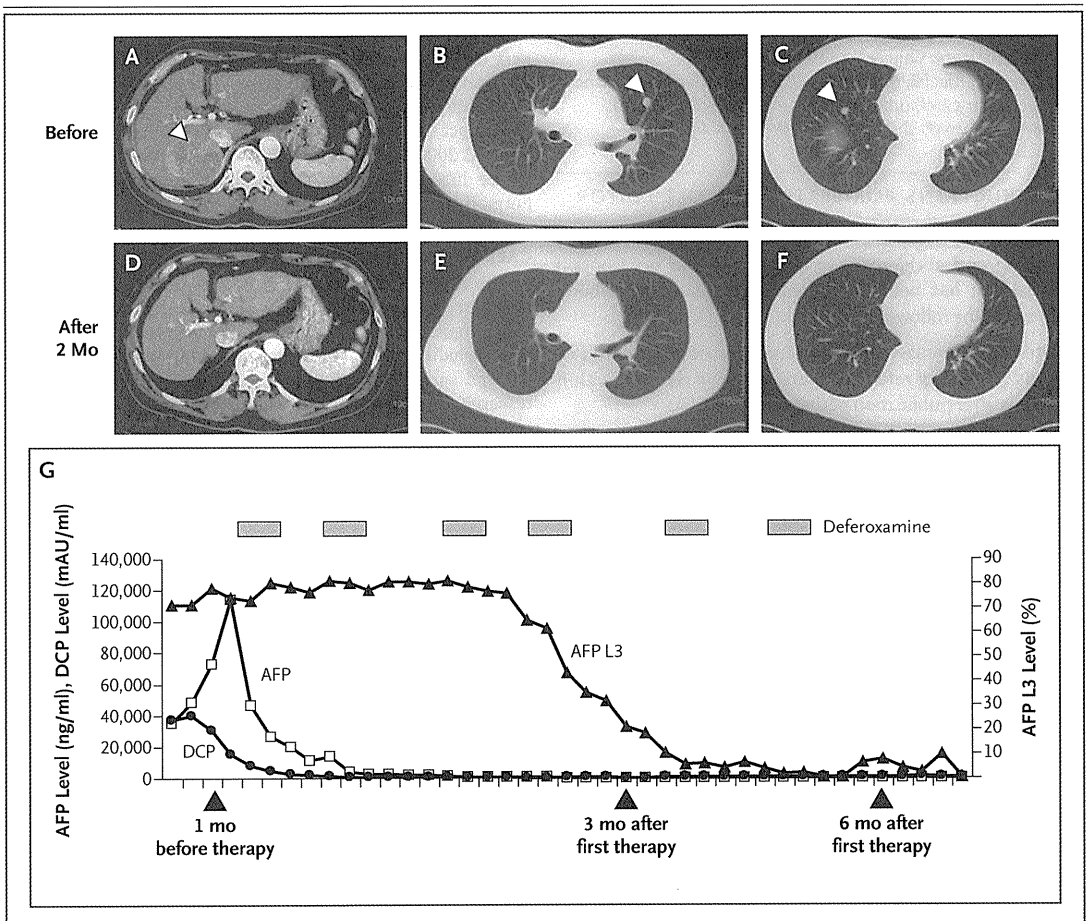

Figure 1. **Deferoxamine Treatment in One Patient.**

A 62-year-old man presented with a massive hepatocellular tumor with multiple small lung metastases (stage IVB). The hepatic reserve function was Child–Pugh class B (a score of 8) before treatment (in the Child–Pugh classification of liver disease, class A [a score of 5 or 6] indicates the least severe disease, class B [7 to 9] moderately severe disease, and class C [10 to 15] the most severe disease). In Panel A, abdominal dynamic computed tomography (CT) before treatment shows a tumor (arrowhead). In Panels B and C, plain chest CT scans obtained before treatment show multiple lung metastases (arrowheads). Two months after arterial infusion of deferoxamine (at a dose of 50 mg per kilogram of body weight per day), the liver tumor and multiple lung metastases were not observed on abdominal dynamic CT (Panel D) and plain chest CT (Panels E and F). As shown in Panel G, after arterial infusion of deferoxamine, the levels of three tumor markers (alpha-fetoprotein [AFP], des-γ-carboxyprothrombin [DCP], and AFP L3) decreased. The patient was considered to have a partial response according to the Eastern Cooperative Oncology Group criteria. The patient survived 20.9 months with deferoxamine treatment.

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Disclosure forms provided by the authors are available with the full text of this letter at NEJM.org.

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Autologous bone marrow cell infusion therapy for liver cirrhosis patients

Shuji Terai · Isao Sakaida

Published online: 15 July 2010

Abstract We developed a novel cell therapy, autologous bone marrow cell infusion (ABMi) therapy, using autologous bone marrow, for liver cirrhosis patients. Our study depends on the findings from basic studies that bone marrow cell infusion repairs liver fibrosis in the cirrhotic liver, and improves liver function and the survival rate. Beginning in November 2003, we started a clinical study and found that ABMi therapy was safe and effective for liver cirrhosis patients. Multicenter trials in Japan and Korea have also shown the effectiveness of ABMi therapy. In this review, we report the current status of ABMi therapy for liver cirrhosis patients.

Keywords ABMi therapy · Bone marrow cell · Liver cirrhosis · GFP/CCl4 model · Liver repair cell

Abbreviations

CCl4	Carbon tetrachloride
GFP	Green fluorescent protein
BMI	Bone marrow cell infusion
ABMi	Autologous bone marrow cell infusion
MMP	Matrix metalloproteinase
LC	Liver cirrhosis
MNC	Mononuclear cell
FACS	Fluorescent-activated cell sorter
LR cell	Liver repair cell

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Introduction

We began a clinical trial of autologous bone marrow cell infusion (ABMi) therapy for liver cirrhosis (LC) patients in November 2003 [1]. We then conducted a multicenter trial in Japan, in collaboration with a Korean group [2]. We have now performed ABMi therapy in 26 LC patients in Japan. The safety and effectiveness of ABMi therapy has been confirmed. In this review, we report the basic findings for the development of ABMi therapy and the current status of the clinical study.

Basic study for development of ABMi therapy

Possible stem cells have been identified in human bone marrow (BM) [3, 4]. Thus, BM was considered to be a novel source of cells for liver regenerative therapy [5–7]. We subsequently developed a green fluorescent protein (GFP)/carbon tetrachloride (CCl4) model that monitors the dynamic state of injected GFP-positive bone marrow cells (BMCs) in CCl4-induced persistent liver damage [6]. In the GFP/CCl4 model, we found that infused BMC repopulated in the cirrhotic liver and improved the function of the cirrhotic liver (Fig. 1). The infused BMC expressed matrix metalloproteinase (MMP9) and migrated into damaged areas [7]. Finally, BMC infusion alleviated liver fibrosis and improved the liver microenvironment in cirrhotic mice [8]. In this step, fibroblast growth factor 2 (FGF2) is important for the repopulation of BMC in the cirrhotic liver and for the repair of the liver microenvironment [9, 10]. In BM, liver repair (LR) cells may exist that repair the cirrhotic liver. From our previous data, we speculated that the Liv-8-negative fraction might be candidate LR cells [11–13]. Now we are analyzing the characteristics of candidate LR cells in

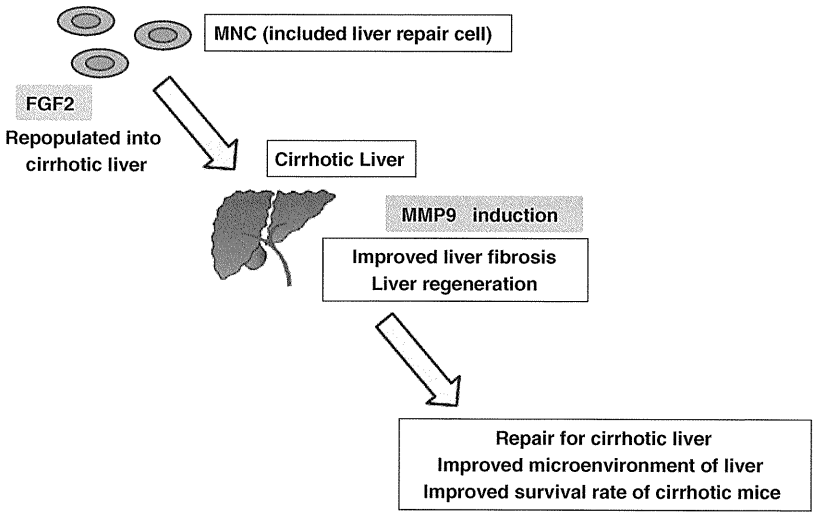

Fig. 1 Summary of the basic research. *FGF2*, Fibroblast growth factor 2; *MNC*, mononuclear cell; *MMP9*, matrix metalloproteinase 9

BM. The effects of stem cells on liver fibrosis were also analyzed by another group [14, 15]. Bone marrow cell (BMC) infusion and mesenchymal stem cell infusion were found to alleviate liver fibrosis in another mouse model. Based on these studies, BM cell infusion appears to improve the microenvironment in the cirrhotic liver [7, 8]. MMP9 expression in the cirrhotic liver was induced by BMC infusion. The MMP9 induction might be a key mechanism to understand the mechanism of repair of the cirrhotic liver. This repair mechanism is important for the development of ABMi therapy for LC patients, so we are analyzing the mechanism more precisely.

Clinical study: ABMi therapy for LC patients

We started a clinical trial of ABMi therapy for LC patients in November 2003. The subjects were LC patients with total bilirubin (TB) <3.0 mg/dL, platelets (Plt) >5 (10^{10}/L), and no viable hepatocellular carcinoma on diagnostic imaging. Autologous BM (400 mL) was isolated from the ilium under general anesthesia. Mononuclear cells (MNCs) were separated by cell washing and were infused via a peripheral vein. The protocol of ABMi therapy is shown in Fig. 2. It takes around 1 h to get BM from the ilium and around 3 h for cell processing. MNC characteristics were confirmed by fluorescence-activated cell sorter (FACS) analysis (CD34, CD45, c-kit). In the first report of our clinical study, we administered ABMi therapy in 9 LC patients [1]. From 400 mL of BM, we obtained $5.2 \pm 0.63 \times 10^9$ MNCs, and these were infused into LC patients. Liver function was monitored by blood examination for 24 weeks after ABMi therapy. We then monitored liver function using ultrasonography, computed tomography (CT), and laboratory tests. Significant improvements in serum albumin levels and total

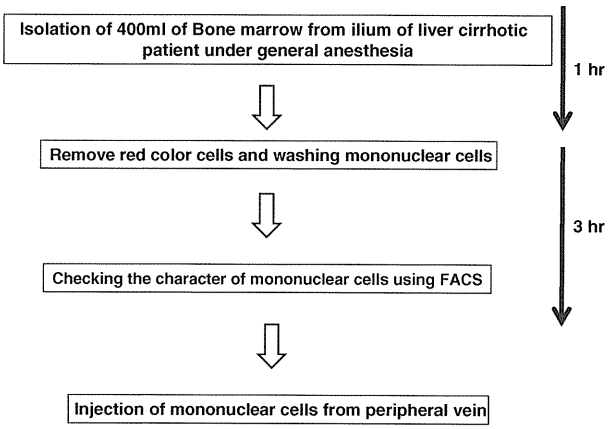

Fig. 2 The protocol for autologous bone marrow cell infusion (ABMi) therapy. *FACS*, Fluorescent-activated cell sorter

protein were seen at 24 weeks after ABMi therapy ($P < 0.05$). The Child-Pugh score had improved significantly at 4 and 24 weeks after ABMi therapy ($P < 0.05$). In addition, alpha fetoprotein (AFP) and proliferating cell nuclear antigen (PCNA) expression in liver biopsy tissue was significantly elevated after ABMi therapy ($P < 0.05$). These results suggested that ABMi therapy might induce repair of the cirrhotic liver [1]. No severe adverse effects were observed.

A multicenter trial of ABMi therapy in Japan was also carried out at Yamagata University, collaborating with Yamaguchi University, beginning in February 2006. At Yonsei University in Korea, the Yamaguchi-Yonsei collaboration study for ABMi therapy started in November 2006. In these clinical studies, the safety and effectiveness of ABMi therapy were also confirmed. In India and Brazil, cell therapy using BMCs for LC patient has also been studied, and its effectiveness has been confirmed [16].

These results suggested that LR cells in BM might be useful for the repair of the cirrhotic liver.

Future prospects

Based on previous clinical studies, we found that cell therapy using autologous BMCs was safe and effective for LC patients [1, 2]. Recently, in Iran, a similar study was performed and the effectiveness of cell therapy using BMCs was also confirmed [17, 18]. To develop more effective therapy and to cure patients with more severe LC, we are trying to identify the candidate LR cells in BM. As the next project, we intend to develop a method for the expansion of BM-derived LR cells and carry out new reparative cell therapy for LC patients.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Splenectomy enhances the anti-fibrotic effect of bone marrow cell infusion and improves liver function in cirrhotic mice and patients

Takuya Iwamoto · Shuji Terai · Yuko Mizunaga · Naoki Yamamoto · Kaoru Omori · Koichi Uchida · Takahiro Yamasaki · Yasuhiko Fujii · Hiroshi Nishina · Isao Sakaida

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Abstract
Background In 2003, we initiated a clinical trial to examine autologous bone marrow cell infusion (ABM*i*) therapy for cirrhotic patients and reported the clinical effect of the therapy. To analyze how splenectomy may potentiate the effects of bone marrow cell infusion on cirrhosis, we performed a mouse study and a clinical trial on patients with cirrhosis.
Methods In mice, we analyzed the effect of splenectomy on bone marrow cell infusion in four experimental groups (group A, splenectomy + bone marrow cell infusion + CCl_4; group B, sham operation + bone marrow cell infusion + CCl_4; group C, splenectomy + CCl_4; group D, sham operation + CCl_4). In clinical, we compared the effect of splenectomy on ABM*i* therapy.
Results We observed significantly increased average serum albumin levels and higher expression of green fluorescent protein (GFP), matrix metalloproteinase 9 (MMP9), and proliferating cell nuclear antigen in the livers of group A. We observed MMP9/GFP double-positive cells in the cirrhotic livers. A significant decrease in the liver fibrosis areas was observed in group A. Splenectomy enhanced the repopulation of bone marrow cells into the cirrhotic liver and improved the liver microenvironment via expression of MMP9 secreted from repopulating GFP-positive cells. Next, we performed a clinical trial to compare the effect of splenectomy on the efficacy of ABM*i* therapy. Cirrhotic patients who underwent splenectomy before ABM*i* therapy tended to have a greater improvement in liver function.
Conclusion ABM*i* therapy with splenectomy may be an effective therapeutic modality for cirrhosis.

Keywords Splenectomy · CCl_4 · Liver cirrhosis · Liver regeneration · Stem cell therapy · Autologous bone marrow cell infusion therapy

Abbreviations

ABM*i*	Autologous bone marrow cell infusion
PBS	Phosphate-buffered saline
Alb	Albumin
PT-INR	Prothrombin time international normalized ratio
P3P	Pro-collagen-III peptide
HGF	Hepatocyte growth factor
Plt	Platelet count
CCl_4	Carbon tetrachloride
GFP	Green fluorescent protein

Introduction

Cirrhosis is the end stage of chronic liver disease and is very difficult to treat. Regenerative therapies have the potential of allowing minimally invasive procedures with few complications. Bone marrow cell transplantation has been performed to treat hematological diseases, and several

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clinical studies have used bone marrow cell infusion to induce regeneration of the myocardium and blood vessels [1–6].

To assess the effect of cell therapy using bone marrow cells, we developed an in vivo mouse model (GFP/CCl_4 model) to monitor the dynamic state of the bone marrow cells, and our results suggest potential efficacy of bone marrow cell infusion for the treatment of liver failure [7–9]. In addition, in 2003, we initiated a clinical trial to examine autologous bone marrow cell infusion therapy (ABM*i* therapy) for cirrhotic patients and reported the safety and clinical effect of the therapy [10]. As a multi-center trial, ABM*i* therapy was performed for alcoholic liver cirrhosis patients and the effects were recorded [11]. ABM*i* therapy has also been performed in Korea and showed clinical improvement [12]. Cirrhotic patients often have hypersplenism as a result of the associated splenomegaly, so thrombocytopenia associated with hypersplenism is one of the major concerns in cirrhotic patients. To reduce the risk of hemorrhage, one of the eligibility criteria for the application of our autologous bone marrow cell infusion therapy protocol is a blood platelet count of $>5 \times 10^{10}$/L; some cirrhotic patients with splenomegaly do not satisfy this criterion. Splenectomy is often performed for hypersplenism [13, 14]. We examined the influence of splenectomy on the efficacy of bone marrow cell infusion for the treatment of cirrhosis. In addition, in three patients, we showed that splenectomy performed prior to ABM*i* therapy produced a greater improvement in liver function than therapy alone without prior splenectomy. In this paper, we describe the influence of splenectomy on the efficacy of bone marrow cell infusion in cirrhotic mice and in clinical trial patients.

Materials and methods

Investigation 1: basic study (mouse experiment)

Animals

C57 BL/6/Tg14 (act-EGFP) Osb Y01 mice (GFP transgenic mice) were kindly donated by Dr. Masaru Okabe (Osaka University Genome Research Center, Osaka, Japan). C57 BL/6 female mice were purchased from Japan SLC (Shizuoka, Japan). These mice were quarantined for 1 week and housed in a room under controlled temperature (25°C), humidity, and lighting (12 h light, 12 h dark). Access to food and tap water was ad libitum throughout the study period.

Bone marrow cell preparation and transplantation

For the isolation of bone marrow cells, C57 BL6/Tg 14(act-EGFP) Osb Y01 mice (6 weeks-old) were killed by cervical dislocation, and their limbs were removed. GFP-positive bone marrow cells were flushed from the medullary cavities of tibias and femurs with phosphate-buffered saline (PBS) culture solution using a 25G needle. The cell solution was filtered through a cell strainer (16 μm) to remove particulate matter and centrifuged at 500*g* for 5 min. After centrifugation, the supernatant was removed and cells were resuspended to prepare 1.0×10^5 cells/mL GFP positive bone marrow cell solution. Preparation of bone marrow cell took approximately 1.5 h.

Experimental protocol

To analyze the influence of splenectomy on the efficacy of autologous bone marrow cell infusion therapy, we used the GFP/CCl_4 mouse model of liver damage, in which the dynamic state of GFP-positive bone marrow cells can be monitored [7, 8, 15]. GFP transgenic mice and female C57BL/6 mice were used. CCl_4 was administered by intraperitoneal injection of 6-week-old female mice, at a dose of 0.5 mg/kg twice a week for 4 weeks, to induce chronic hepatopathy [16]. We also measured the weight of the spleen after CCl_4 injection. After 4 weeks, either a sham operation (CCl_4 + sham) or splenectomy (CCl_4 + splenectomy) was performed in the mice. CCl_4 was not administered for 2 weeks after the operation to allow healing of the wound. Then, the same dose of CCl_4 (0.5 mg/kg) was administered twice weekly to obtain sustained chronic hepatopathy. Four weeks after the operation, 1×10^5 GFP-positive bone marrow cells were infused via the caudal vein into the animals. For comparison, a group injected with only CCl_4 was also analyzed. There were four groups in this study: group A (CCl_4 + splenectomy + bone marrow cell infusion; $n = 21$), group B (CCl_4 + sham + bone marrow cell infusion; $n = 21$), group C (CCl_4 + splenectomy; $n = 12$), and group D (sham + CCl_4; $n = 12$). Changes in the severity of liver fibrosis and the degree of liver regeneration were investigated 7, 14, 21 and 28 days after bone marrow cell infusion. The therapeutic protocol is shown in Fig. 1a.

Measurement of laboratory data

Serum samples were obtained from the abdominal aorta as the mice were sacrificed. The blood levels of albumin (ALB) and platelet counts were measured.

Fluorescence-activated cell sorting analysis

Cells were analyzed in BD FACS Calibur™ (BD Bioscience, NJ, USA); 1×10^5 cells were routinely analyzed. Antibodies were precalibrated to determine optimal concentration. Anti-CD11b (Beckman Coulter, Inc, Fullerton, CA, USA), anti-CD44 (Beckman Coulter, Inc), and

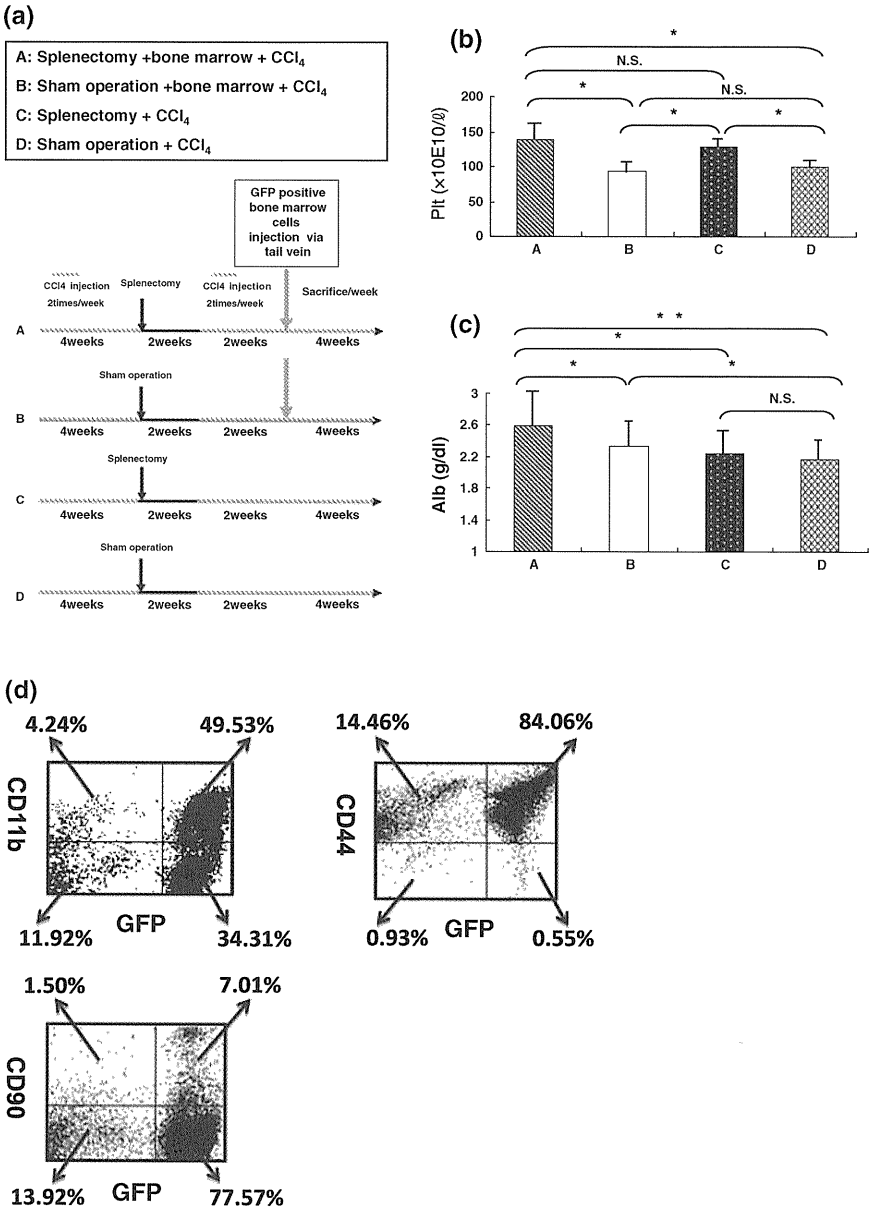

Fig. 1 Mouse model, blood parameters, fluorescence-activated cell sorting analysis, and experimental protocol. **a** Mouse models (*A* splenectomy + bone marrow cell infusion + CCl_4, *B* sham operation + bone marrow cell infusion + CCl_4, *C* splenectomy + CCl_4, *D* sham operation + CCl_4). Experimental protocol; GFP-transgenic mice and female C57BL/6 mice were used. CCl_4 was administered by intraperitoneal injection into 6-week-old female mice at a dose of 0.5 mg/kg twice a week for 4 weeks to induce chronic hepatopathy. After 4 weeks, either a sham operation (CCl_4 + sham operation) or splenectomy (CCl_4 + splenectomy) was performed in the mice. Four weeks after the splenectomy, 1×10^5 GFP-positive BMCs were infused via the caudal vein into the animals. The changes in the severity of liver fibrosis and degree of liver regeneration were investigated 7, 14, 21 and 28 days after the bone marrow cell infusion. **b** Average platelet counts (*A* splenectomy + bone marrow cell infusion + CCl_4, *B* sham operation + bone marrow cell infusion + CCl_4, *C* splenectomy + CCl_4, *D* sham operation + CCl_4). **c** Average serum albumin levels. Groups are as in **b**. Data are the means ± SD. $*p < 0.05$, $**p < 0.01$. **d** Fluorescence-activated cell sorting analysis. Bone marrow cells were obtained from C57 BL/6/Tg14 (act-EGFP) Osb Y01 mice (GFP transgenic mice). 1×10^5 cells were routinely analyzed. Antibodies were precalibrated to determine optimal concentration. The table shows the results of various surface markers

anti-CD90 (Beckman Coulter, Inc) were examined. The surface markers of bone marrow cells were showed in Fig. 1. CD11b is one of the markers of macrophages, CD90 is one of the markers of stromal cells. CD44 expressed in a large number of mammalian cell types and identified recently as a antigen of Liv-8 [17].

Histological examination

Immunohistochemical analysis In all experiments, 5-μm-thick sections were prepared from the right lobe of the mouse livers, fixed in 10% formalin for 24 h, embedded in paraffin, stained with Azan and Sirius red, and processed for immunohistochemical analysis. For immunohistochemical analysis, primary antibodies against green fluorescent protein (GFP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), matrix metalloproteinase 9 (MMP9) (Santa Cruz Biotechnology), alpha-smooth muscle actin (α-SMA) (Abcam Inc, Cambridge, MA, USA), F4/80 (Abcam Inc, Cambridge, MA, USA) and proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology) were used. Staining was visualized with the avidin–biotin–peroxidase complex method using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA). To estimate the Azan- and Sirius red-positive areas, image analysis software MetaMorph (Universal Imaging Corporation, Downingtown, PA, USA) was used on samples viewed under 40× magnification. Seven different randomly selected areas per specimen were analyzed. The percentages of the positive areas were calculated by averaging the percentages of the total area. To estimate the positive areas, GFP and MMP9 were examined by the same method as described above for estimating the Sirius red-positive area.

Double fluorescent immunohistochemical analysis For fluorescence immunohistochemistry, tissues were incubated with Alexa Fluor R488 and 568 donkey anti-goat IgG (H + L) conjugate, Alexa Fluor R488 goat anti-rabbit IgG (H + L) conjugate, and Alexa Fluor R568 goat anti-rat IgG (H + L) conjugate (Molecular Probes, Eugene, OR, USA) as secondary antibodies as appropriate. Positive cells in the liver were quantified using a Provis microscope (Olympus, Tokyo, Japan) equipped with a charge coupled device camera. Computer-assisted image analysis with MetaMorph software was used (Universal Imaging).

Western blot analysis Liver samples were obtained at week 4 after the bone marrow cell infusion and after the same period in the CCl_4 groups as a control. The liver samples (approximately 40 mg) were homogenized in 1 mL of cell lysis buffer (Cell Signal Technology Inc., Danvers, MA) containing 1 mM phenylmethanesulfonyl fluoride (PMSF) and complete mini (Roche Diagnostic, Pleasanton, CA, USA) and centrifuged (Centrifuge conditions 15,000 rpm, 5 min, 4°C). The supernatant represented whole protein. Then, 40 μg of the protein sample was mixed with the same volume of loading buffer (5% 2-mercaptoethanol and 95% Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA), heated for 3 min at 100°C, and separated by 10% SDS-PAGE. The separated bands were transferred to Immobilon-P transfer membrane (Millipore, Billerica, MA, USA), followed by blocking the membranes for 1 h with blocking buffer [0.1% Tween-20 (Wako Pure Chemical Industries, Ltd., Osaka, Japan)], 0.2% I-Block™ reagent (Tropix, Inc., Bedford, MA, USA), and 1 mM Tris–HCl buffer (pH 7.5) (Invitrogen Corp., Carlsbad, CA, USA). The membranes were then washed with washing buffer (0.1% Tween-20, 1 mM Tris–HCl buffer, pH 7.5) and incubated for 1 h at room temperature with primary antibodies against MMP9 (Santa Cruz Biotechnology, Inc.), α-SMA (Abcam Inc.), or β-actin (Abcam Inc.) in blocking buffer. After washing, the membranes were incubated for 1 h at room temperature with the appropriate secondary antibodies. Reactive bands were identified using ECL (Amersham Biosciences, Piscataway, NJ, USA) and autoradiography, according to the manufacturer's instructions.

Real-time quantitative PCR analysis Total RNA was isolated from the livers of mice 4 weeks after the bone marrow cell infusion or control CCl_4 treatment. The mRNA expression of TGF-β1 was evaluated using real-time quantitative PCR. The primer was designed according to a previous report [18]. Total RNA was extracted using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany). For cDNA synthesis, AMV reverse transcription reagents were used according to the manufacturer's instructions (Roche Diagnostic). Real-time PCR was performed with SYBR Green Master Mix (Roche Diagnostic). The specific primers used for TGF-β1 were: 5′-GAA GCC ATC CGT GGC CAG AT-3′ (forward) and 5′-GAC GTC AAA AGA CAG CAC T-3′ (reverse). The PCR primers used for mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as a positive control, were: 5′-GTC TTC ACC ACC ATG GAG AAG GC-3′ and 5′-ATG CCA GTG AGC TTC CCG TTC AGC-3′. PCR amplification was performed in triplicate using the following cycle conditions: 40 cycles of 90°C for 30 s, 55–60°C for 45 s, 72°C for 1 min. The validity of GAPDH as a reference gene was confirmed experimentally by measuring the slope of the plot of log input RNA amount versus the difference in thermal cycle, as suggested by the user manual (Roche Diagnostic).

Investigation 2: clinical study (autologous bone marrow cell infusion therapy)

Participants

Participants were patients with liver cirrhosis with serum total bilirubin levels of <3.0 mg/dL, platelet counts of $>5 \times 10^{10}$/L, and no visible hepatocellular carcinoma on computed tomography and/or magnetic resonance imaging. In addition, patients' heart and lung functions were normal.

Patients receiving autologous bone marrow cell infusion therapy between November 2003 and December 2007 were included in our study. Three patients underwent splenectomy for the treatment of thrombocytopenia and esophagogastric varices prior to the therapy. We compared the results in nine patients who received autologous bone marrow cell infusion therapy alone to those in these three patients who underwent splenectomy prior to the autologous bone marrow cell infusion therapy. The follow-up protocol and details of autologous bone marrow cell infusion have been reported previously [10]. The protocols were approved by the Ethics Committee of Yamaguchi University, and written informed consent was obtained from each of the study participants (ID: [20020109 2-5]).

Statistical analysis

We analyzed the changes in the variables from baseline (before bone marrow cell infusion) to 4 or 24 weeks after the bone marrow cell infusion. Values are shown as the means ± SD. Data were analyzed by analysis of variance with Fisher's projected least significant difference test.

Effect of splenectomy on improvement in liver function following bone marrow cell infusion

Result 1 (mouse experiment)

CCl_4 injection induced liver cirrhosis, and spleen weights were significantly increased compared to the control group (Table 1). A greater increase in the average platelet count was observed in groups A and C as compared with those of groups B and D ($p < 0.01$) (Fig. 1b), suggesting that the splenectomy was effective in improving the blood platelet count in the mice. The average serum albumin level was the highest in group A, and the difference in the serum albumin level between groups A and B was significant ($p < 0.05$) (Fig. 1c). In group C, the simple splenectomy group, a slightly greater increase in the average serum albumin level was observed as compared with that in group D, the control group, but the difference was not significant. As shown in Fig. 1d, GFP positive cell mainly expressed CD44 (84.06%), CD11b (49.53%) and CD90 (7.01%). FACS analysis showed that heterogeneous bone marrow cells were infused in this study.

The expression levels of GFP, PCNA, MMP9, and α-SMA in liver cells were investigated by immunohistochemical analysis. Significantly higher expression levels of GFP were observed in group A compared to those of group B (Fig. 2a, b), indicating that splenectomy increased the migration rate of the bone marrow cells that were infused into the cirrhotic liver (Fig. 2a–e). Expression levels of PCNA observed in group A were also higher than those in group B (Fig. 2f, g), suggesting that bone marrow cell infusion combined with splenectomy induced liver regeneration more effectively than bone marrow cell infusion alone ($p < 0.01$) (Fig. 2f–j). The greatest decrease in the area of liver fibrosis, as measured by Sirius red staining, was observed in group A, with the area differing significantly between group A and group B ($p < 0.01$). There was no significant difference in the area of liver fibrosis between group C and group D (Fig. 3a–e). Higher expression levels of MMP9 were observed in group A as compared with those in group B (Fig. 3f–j), consistent with the elevated expression of the MMP9 protein in the cells (Fig. 3k). The decrease in expression of α-SMA was most pronounced in group A (Fig. 3l–p), and this result was confirmed with western blot analysis (Fig. 3q). We measured the mRNA expression levels of TGF-β1 mRNA with real-time PCR to evaluate the degree of fibrogenesis in the liver. The decrease in TGF-β1 mRNA expression was most pronounced in the splenectomy groups (Fig. 4j). In addition, many MMP9/GFP double-positive cells, a few F4/80/GFP-positive cells, and α-SMA/GFP double-positive cells were observed in the liver (Fig. 4a–i). The MMP9/GFP double-positive cells were round in shape. This finding suggested that MMP9 was mainly secreted from the migrating GFP-positive bone marrow cells that had been infused. We therefore speculate that splenectomy enhanced the migration rate of the GFP-positive bone marrow cells into the liver, and that the MMP9/GFP double-positive cells were involved in the repair of the liver fibrosis. In addition, splenectomy also decreased the TGF-β1 mRNA expression levels.

Table 1 Comparison of spleen weight (control group and CCl_4 group)

	Body weight (g)	Spleen weight (mg)	Spleen weight/body weight (mg/g)
Control	26.3 ± 1.4	76.2 ± 8.6*	2.9 ± 0.3*
CCl_4	24.8 ± 2.2	104.2 ± 14.7*	4.2 ± 0.4*

Spleen weights and spleen weights per body weights were analyzed. Control, 10-week-old female mice; CCl_4, 10-week-old female mice, administered CCl_4 by intraperitoneal injection at a dose of 0.5 mg/kg twice a week for 4 weeks

* $p < 0.01$

Result 2 (effect of splenectomy on the migration rate of the infused bone marrow cells into different organs)

In the GFP/CCl_4 model, a large number of GFP-positive bone marrow cells were observed in the lung and spleen

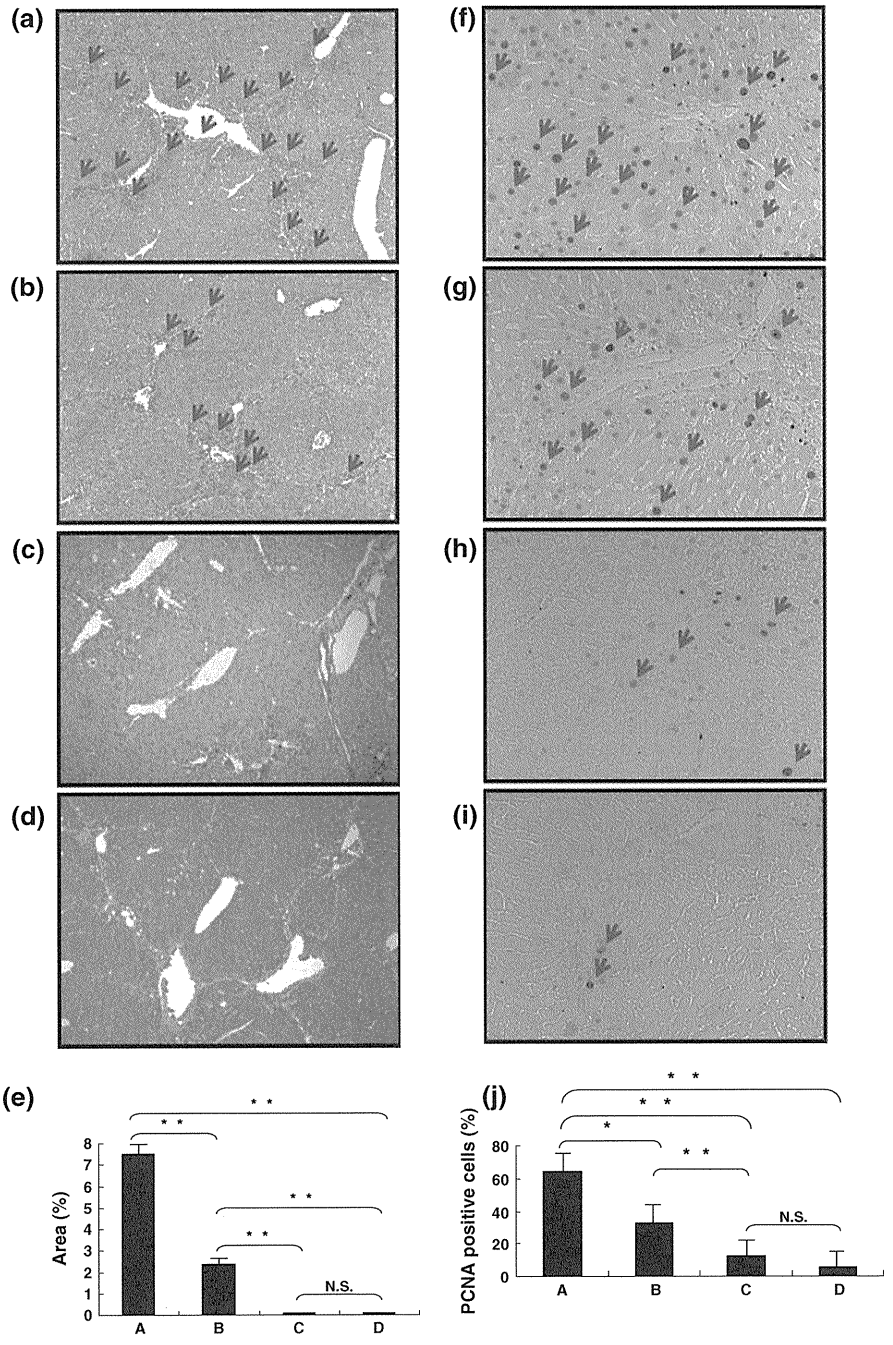

Fig. 2 Immunohistochemical analysis of GFP and PCNA expression in the liver. Immunohistochemistry for GFP [**a–d**, ×40] and PCNA [**f–i**, ×100]. The *red arrows* indicated GFP, PCNA positive cells. **a**, **f** Splenectomy + bone marrow cell infusion + CCl_4, **b**, **g** sham operation + bone marrow cell infusion + CCl_4, **c**, **h** splenectomy + CCl_4, **d**, **i** sham operation + CCl_4. The *red arrows* indicate GFP-positive cells (**a**, **b**) or PCNA-positive cells (**f–i**). Image analysis of GFP (**a–d**)-positive areas (**e**) and PCNA-(**f–i**) positive areas. Data are the means ± SD. $^{**}p < 0.01$; $^{*}p < 0.05$

within 1 week of the bone marrow cell infusion, and they decreased thereafter (Fig. 5a–d). There were no significant differences in the rates of captured bone marrow cells in the lungs between the splenectomy group and the bone marrow cell infusion-only group (Fig. 5b). Thus, many bone marrow cells may have homed to the liver without being trapped in the lung, and therefore splenectomy may have enhanced the migration of the infused bone marrow cells into the liver.

Result 3 (clinical study)

The splenectomy group was observed at least 4 months after splenectomy; there were no significant changes in the liver function within the period (Fig. 6a–c). The patient characteristics are shown in Table 2. Except for the blood platelet count, there were no significant differences in the clinical data between the splenectomy group and the non-splenectomy group. A significantly greater increase in the

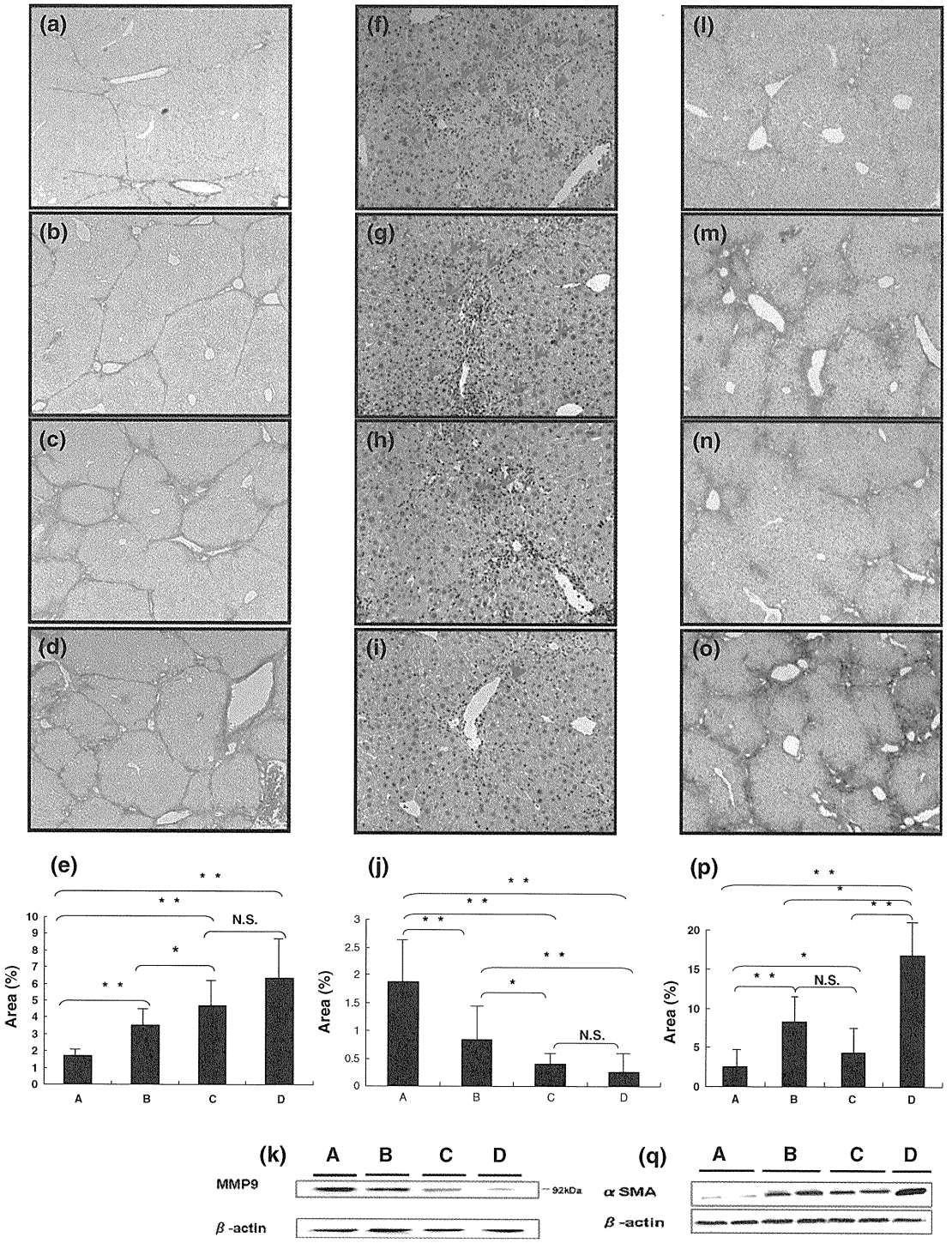

Fig. 3 Sirius red staining and immunohistochemical analysis of MMP9 and α-SMA. Sirius red staining [**a–d**, ×40]. Immunohistochemistry for MMP9 [**f–i**, the *red arrows* indicated MMP9 positive cells ×40] and α-SMA [**l–o**, ×40]. **a**, **f**, **l** Splenectomy + bone marrow cell infusion + CCl_4, **b**, **g**, **m** sham operation + bone marrow cell infusion + CCl_4, **c**, **h**, **n** Splenectomy + CCl_4, **d**, **i**, **o** sham operation + CCl_4, the *red arrows* indicate MMP9-positive cells (**f–i**). Image analysis of Sirius red (**a–d**)-positive areas (**e**), MMP9 (**f–i**)-positive areas (**j**), and α-SMA (**l–o**)-positive areas (**p**). Data are the means ± SD. $^{**}p < 0.01$; $^{*}p < 0.05$. Western blot analysis of MMP9 (**k**) and α-SMA (**q**). *A* Splenectomy + bone marrow cell infusion + CCl_4, *B* sham operation + bone marrow cell infusion + CCl_4, *C* splenectomy + CCl_4, *D* sham operation + CCl_4

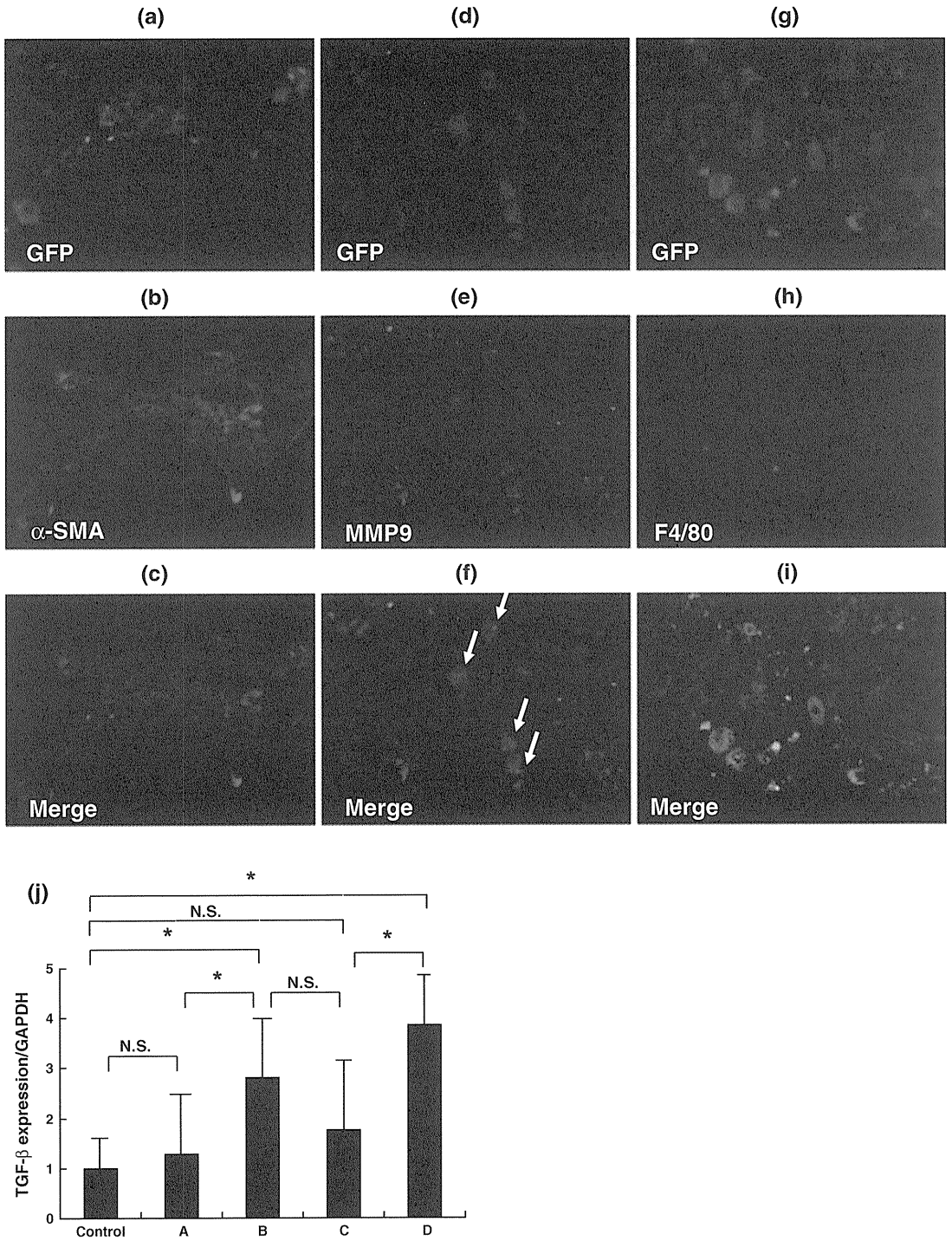

Fig. 4 Double fluorescent immunohistochemical analysis of GFP, α-SMA, F4/80, and MMP9 expression and TGF-β1 mRNA expression in the liver. Liver samples were obtained 4 weeks after bone marrow cell infusion (group A). **a–c** Double fluorescent immunohistochemical analysis of GFP and α-SMA (×100). **d–f** Double fluorescent immunohistochemical analysis of GFP and MMP9 (×100). The *white arrows* indicated GFP/MMP9 double positive cells. **g–i** Double fluorescent immunohistochemical analysis of GFP and F4/80 (×100). **j** TGF-β1 mRNA expression with real-time PCR. Total RNA was isolated from the livers of mice 4 weeks after the bone marrow cell infusion or control CCl_4 treatment. The *x* axis represents the following groups: *Control* normal mice not treated with CCl_4. *A* Splenectomy + bone marrow cell infusion + CCl_4, *B* sham operation + bone marrow cell infusion + CCl_4, *C* splenectomy + CCl_4, *D*: sham operation + CCl_4. The *y* axis represents the real-time PCR values normalized to the internal control GAPDH. Data are the means ± SD. $^*p < 0.01$

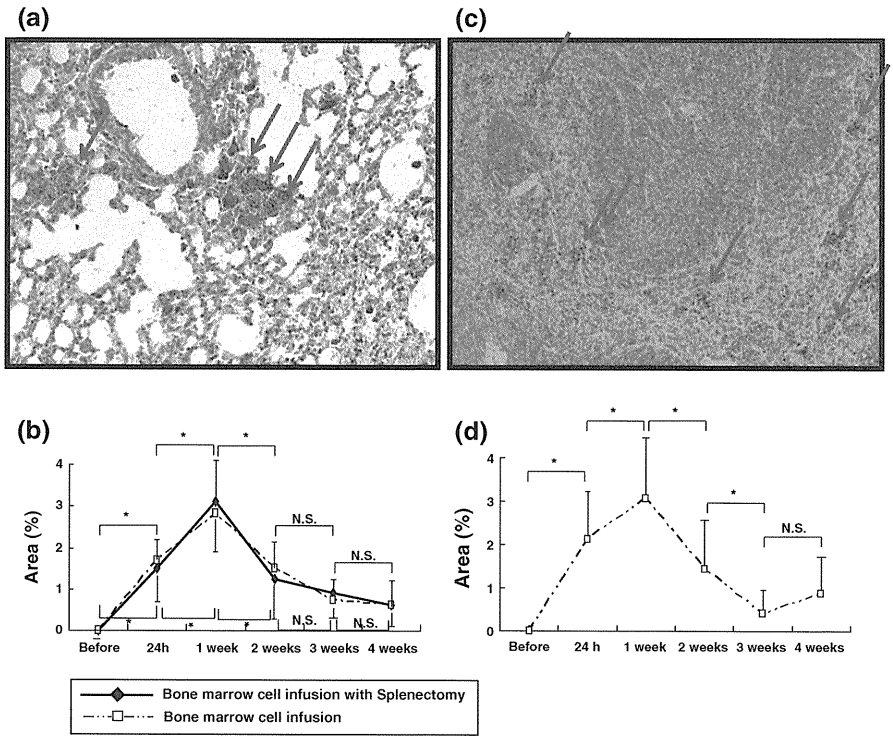

Fig. 5 Immunohistochemical analysis of GFP expression in the lung and spleen. **a** At 1 week, GFP was detected with an anti-GFP antibody in the lung (×100). The *red arrows* indicated GFP positive cells. **b** Image analysis of GFP-positive areas in the lung. **c** At 1 week, GFP was detected with an anti-GFP antibody in the spleen (×100). The *red arrows* indicated GFP positive cells. **d** Image analysis of GFP-positive areas in the spleen. $*p < 0.05$

serum albumin level was observed in the ABM*i* therapy with splenectomy group than in the ABM*i* therapy only group at 4 and 24 weeks (4 weeks, 3.2 ± 0.3 vs. 2.8 ± 0.3 g/dL; 24 weeks, 3.4 ± 0.4 vs. 2.9 ± 0.3 g/dL) (Fig. 6a). The Child-Pugh score decreased in the ABM*i* therapy with splenectomy group (4 weeks, 6.3 ± 0.6 vs. 7.7 ± 0.8; 24 weeks, 6.0 ± 1.0 vs. 7.4 ± 0.8) (Fig. 6b) [19]. A significantly greater decrease in the prothrombin time international normalized ratio (PT-INR) was observed in the ABM*i* therapy with splenectomy group as compared with that in the autologous bone marrow cell infusion-only group at 24 weeks (1.21 ± 0.10 vs. 1.34 ± 0.11) (Fig. 6c). A significant decrease in the serum level of type III procollagen-N-peptide (P3P) was observed in ABM*i* therapy with splenectomy group at 24 weeks (Fig. 6d) [20]. The serum level of serum hepatocyte growth factor (HGF) was higher in the ABM*i* therapy with splenectomy group than in the ABM*i* therapy only group (4 weeks, 0.51 ± 0.15 vs. 0.41 ± 0.10; 24 weeks, 0.55 ± 0.19 vs. 0.46 ± 0.08) (Fig. 6e) [21, 22]. Collectively, these data suggest that patients who underwent splenectomy prior to the autologous bone marrow cell infusion therapy showed a greater degree of improvement in liver function.

Discussion

In this paper, we report results using both a mouse model and a clinical study to show that splenectomy enhances the efficacy of autologous bone marrow cell infusion for alleviating cirrhosis. One of the eligibility criteria for application of our therapeutic protocol for autologous bone marrow cell infusion therapy is a platelet count of $>5 \times 10^{10}$/L. However, many cirrhotic patients with splenomegaly suffer from hypersplenism as a complication and do not satisfy this eligibility criterion. It has been reported that splenectomy enhances the degree of liver regeneration after partial hepatectomy [22]. In addition, splenectomy was shown to have a positive effect on liver transplantation from a living donor [23–27].

To understand the effects of splenectomy, we used a GFP/CCl_4 mouse model with CCl_4-induced cirrhosis in which one can monitor the dynamic state of the GFP-positive BMCs. The results of our analysis revealed the most pronounced improvement in liver function, as measured by the serum albumin levels, in group A (splenectomy + BMI + CCl_4) (Fig. 1c). The expression level of GFP was also the highest in group A (Fig. 2a–e). A larger number of GFP-positive cells migrated to the damaged liver in the splenectomy group compared to the non-splenectomy group, although there were no significant differences in the rate of captured bone marrow cells in the lungs between the splenectomy and bone marrow cell infusion-only groups. Thus, the increase in the migration rate of bone marrow cells into the liver in the splenectomy group appears to be due to the absence of captured bone marrow cells in the enlarged spleen following splenectomy. In the mouse model, the expression levels of PCNA were

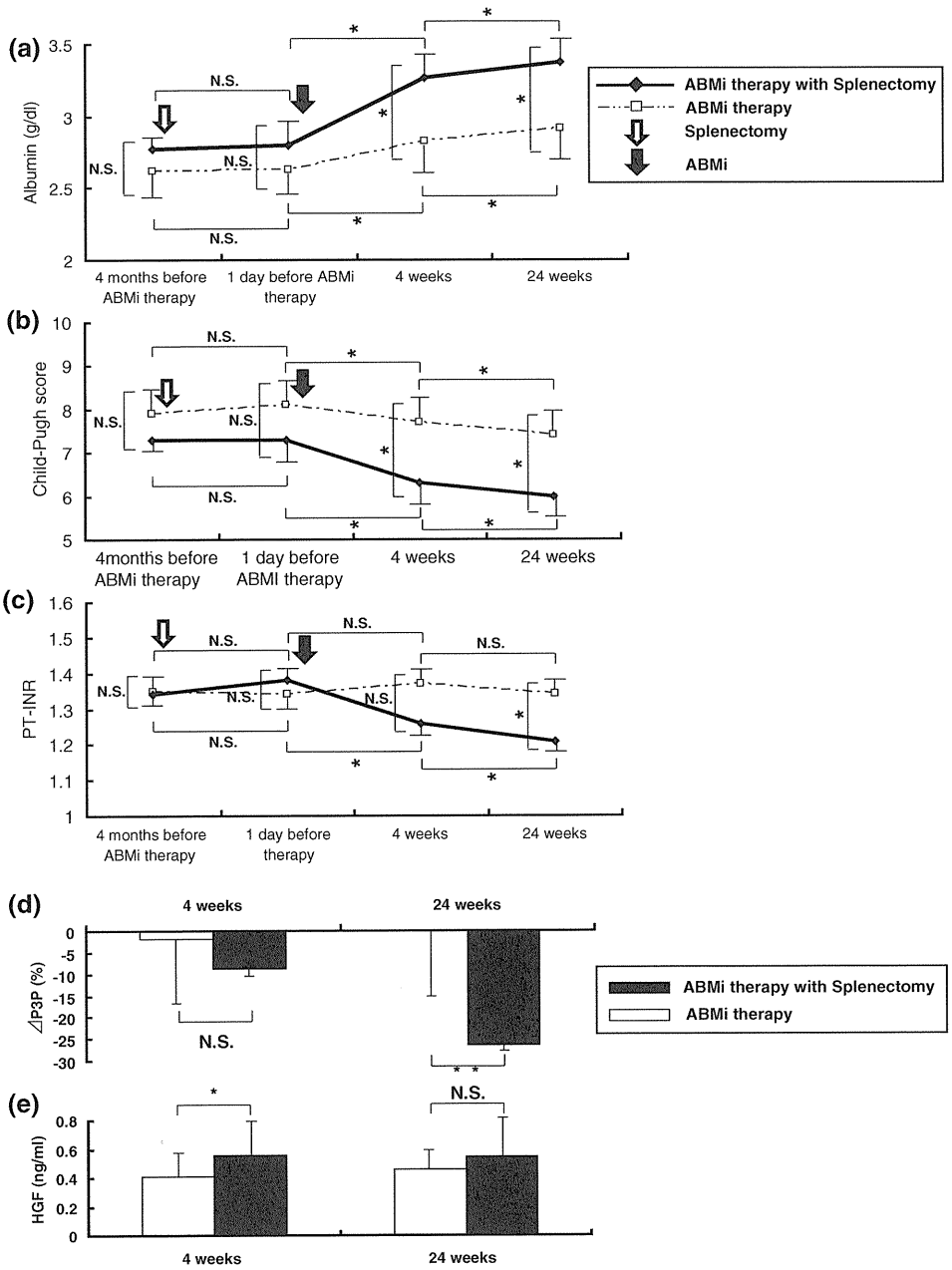

Fig. 6 Clinical blood parameters (ABM*i* therapy with splenectomy and ABM*i* therapy). **a–c** were indicated clinical blood parameters before and after autologous bone marrow cell infusion. The *white arrows* indicate the time of splenectomy. The *black arrows* indicate the time of ABM*i* therapy. **a** Average serum albumin levels. **b** The Child-Pugh score. **d** and **e** were measured at 4 and 24 weeks after ABM*i* therapy. **c** Prothrombin time international normalized ratio (PT-INR). **d** Reduction rate of serum pro-collagen-III peptide (P3P). **e** Hepatocyte growth factor (HGF). Data are the means ± SD. $*p < 0.05$, $**p < 0.01$

significantly higher in group A than in group B, suggesting that the cells were dividing more and bone marrow cell infusion combined with splenectomy resulted in more effective liver regeneration. Sirius red staining revealed a greater degree of alleviation of liver fibrosis in group A than in the other groups (Fig. 3a–e). To further examine the mechanism underlying this finding, we analyzed the expression levels of MMP9, α-SMA, and TGF-β1 in the liver. The expression levels of MMP9 were higher in the bone marrow cell infusion groups, as previously reported by us (Fig. 3f–k) [8], and significantly higher in group A than in group B (Fig. 3j, k). The decrease in expression of α-SMA was most pronounced in group A (Fig. 3l–q). We also measured the TGF-β1 mRNA expression levels with

Table 2 Clinical characteristics before ABM*i* therapy between splenectomy group and non-splenectomy group

	Age	Gender (male/female)	Etiology	Alb (g/dL)	PT (%)	ALT (IU/L)	P-3-P (U/mL)	HGF (ng/mL)	Plt ($\times 10^{10}$/L)	Child-Pugh score
ABMi	61.4 ± 5.0	(8/1)	HBV: 3 HCV: 5 Unknown: 1	2.6 ± 0.4	66.4 ± 8.1	42.3 ± 18.8	1.3 ± 0.3	0.40 ± 0.08	7.5 ± 2.5	8.3 ± 0.7
ABMi + splenectomy	62.0 ± 1.7	(2/1)	HBV: 1 HCV: 1 HBV + HCV: 1	2.9 ± 0.1	65.7 ± 6.8	33.6 ± 3.2	1.6 ± 0.3	0.53 ± 0.16	20.4 ± 2.5	7.0 ± 0.0
	NS	NS		NS	NS	NS	NS	NS	$p < 0.01$	NS

Alb albumin, *PT* prothrombin time, *ALT* alanine aminotransferase, *P-3-P* pro-collagen-III peptide, *HGF* hepatocyte growth factor, *Plt* platelet count

real-time PCR to evaluate the fibrogenetic process in the liver. TGF-β1 is a cytokine that causes activation of hepatic stellate cells and stimulates the production of extracellular matrix [28], and is a major factor for promoting liver fibrosis. In a previous study, TGF-β1, produced by splenic macrophages, was reported to possibly inhibit hepatocyte proliferation in the damaged liver. Therefore, in this model, removal of the enlarged spleen was thought to possibly stimulate regeneration of the damaged liver. One potential mechanism underlying the present results could be increased portal blood flow and changes in intrahepatic blood flow, associated with changes in shunt blood flow and changes in TGF-β1 kinetics due to splenectomy [28, 29]. Some previous studies have shown that splenectomy decreases the mRNA expression levels of TGF-β1 and α-SMA, and improves liver fibrosis, possibly resulting in more effective liver regeneration [28–30]. We obtained a similar result in our models. A more pronounced decrease in TGF-β1 mRNA and α-SMA expression were observed in our splenectomy groups (Fig. 4j). Splenectomy itself has the potential which improve liver fibrosis through the decrease of TGF-β1 and α-SMA expression levels. Furthermore, we also observed numerous MMP9/GFP double-positive cells, a few F4/80/GFP positive cells, and α-SMA/GFP double-positive cells in the liver (Fig. 4a–i). These results suggest that MMP9 was mainly secreted by the migrating GFP-positive BMCs in the liver. In addition, splenectomy appears to have enhanced the migration rate of the GFP-positive bone marrow cells into the liver, and MMP9/GFP double-positive cells appear to have been involved in the repair of the liver fibrosis. Thus, bone marrow cell infusion combined with splenectomy may alleviate liver fibrosis to a greater degree than bone marrow cell infusion alone because splenectomy enhanced the migration rate of GFP-positive BMCs into the liver, and the increased expression of MMP9 and decreased expression of TGF-β1 and α-SMA in these cells were involved in improving liver regeneration. Splenectomy itself would have the effect of reducing TGF-β1 and α-SMA, and recent reports from basic research revealed that increase of platelet level improved liver fibrosis [31], but the liver function did not significantly change in our basic and clinical study. We think that splenectomy alone is not sufficient for the improvement of liver function, and the main effect of splenectomy is to increase the number of bone marrow cells in the liver to prevent trapping in the enlarged spleen. On the other hand, this time we infused heterogeneous bone marrow cells (Fig. 1d). We previously reported that Liv-8 negative fraction has a greater improvement effect on liver fibrosis in GFP/CCl4 model [32]. Recently, Liv8 was identified as the CD44 protein [17]. From the results of FACS analysis (Fig. 1d), a small fraction of Liv8 negative was infused into cirrhosis mice.

In our clinical study, three patients underwent splenectomy for thrombocytopenia, with improvement of the platelet counts prior to the autologous bone marrow cell infusion. In this study, we observed patients at least 4 months after splenectomy, but liver function was not significant changed during that time. Compared with the finding in the non-splenectomy group, greater improvement in the Child-Pugh score was observed in the ABM*i* therapy with splenectomy group. After ABM*i* therapy, the average serum albumin level increased, and the PT-INR and serum level of P3P improved. More significant improvement in the Child-Pugh score was observed in patients who underwent splenectomy prior to ABM*i* therapy as compared with those in the ABM*i* therapy only group (Fig. 6a–d).

Therefore, based on the basic research, we suggest that splenectomy may be effective in alleviating liver fibrosis. In addition, splenectomy may allow more effective utilization of the infused bone marrow cells by preventing their capture by the spleen [33]. Clinical trials investigating liver diseases using bone marrow cells have attracted worldwide attention and have been reported from seven countries [34]. In patients with thrombocytopenia, splenectomy can enhance the efficacy of ABM*i* therapy and expand the indications for ABM*i* therapy. It will be necessary to increase the number of cases examined, but we believe that ABM*i* therapy combined with splenectomy may be a more effective therapy for cirrhosis than bone marrow infusion alone.

In conclusion, splenectomy enhanced the effect of bone marrow cell infusion for alleviating liver fibrosis in mice. It appears that ABM*i* therapy with splenectomy may prove to be an effective therapeutic modality for treating cirrhosis.

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Conflict of interest All authors declare that no competing interests exist.

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Forum Minireview

Novel Findings for the Development of Drug Therapy for Various Liver Diseases:

Current State and Future Prospects for Our Liver Regeneration Therapy Using Autologous Bone Marrow Cells for Decompensated Liver Cirrhosis Patients

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Abstract. We have developed an in vivo mouse model [the green fluorescent protein (GFP) / carbon tetrachloride (CCl_4) model] and reported that infused GFP-positive bone marrow cells administered via a tail vein efficiently repopulated cirrhotic liver tissue under conditions of persistent liver damage induced by CCl_4. Moreover, bone marrow cells infused into the liver improved liver function and ameliorated liver fibrosis with higher expression of matrix metalloproteinase 9 (MMP-9), consistent with improved survival rate. Based on these findings, we started a multicenter clinical trial of autologous bone marrow cell infusion (ABM*i*) therapy for decompensated liver cirrhosis patients and demonstrated the efficacy of this approach without unexpected complications. However, this therapy involves bone marrow aspiration under general anesthesia and is not indicated for patients for whom general anesthesia is difficult. We therefore aimed to develop a new liver regeneration therapy in which cells having a curative effect on liver cirrhosis are isolated and cultured from a small amount of autologous bone marrow aspirated under local anesthesia and infused back into the same subject. Herein, we present results for the GFP/CCl_4 model and ABM*i* therapy and future prospects for a new liver regeneration therapy.

Keywords: liver cirrhosis, bone marrow, stem cell, liver regeneration, autologous bone marrow cell infusion (ABM*i*) therapy, liver disease

1. Introduction

Today, anti-hepatitis virus therapy using interferon for hepatitis C patients is well developed, but interferon is not indicated for patients with decompensated liver cirrhosis. When decompensated liver cirrhosis or other severe liver disease occurs, the only curative therapy is currently liver transplant (liver transplant from either a living or a brain dead donor). However, transplants are not widely performed in Japan, due to various problems including a chronic donor shortage, surgical invasiveness, risk of immunological rejection, and medical costs. In many cases, symptomatic treatment is the only option. To compensate for this, development of new regenerative therapies for liver cirrhosis is an urgent task.

In 2000, Theise et al. reported the existence of Y chromosome–positive cells in livers with chronic inflammation in autopsy female cases of therapeutic bone marrow transplantations with male donors [transplantation from male (XY) donors to female (XX) patients], and those findings suggested the existence of pluripotent stem cells among bone marrow cells (1, 2). Since then, attention has been focused on bone marrow (stem) cells as a cell source for a liver regenerative therapy worldwide (3).

We have established a mouse model to evaluate differentiation from bone marrow cells to liver cells [the green fluorescent protein (GFP) / carbon tetrachloride

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