albumin, Child-Pugh score, and accumulation of ascites with *in vitro* expansion of CD34-positive cells induced by G-CSF and administration via a hepatic artery in subjects with alcoholic liver cirrhosis. A report by Han *et al.* (2008) described G-CSF administration for hepatitis B virus-related decompensated liver cirrhosis. In this study, administration of peripheral blood mononuclear cells induced with G-CSF showed significantly improvement effects on serum albumin and Child-Pugh score at 6 months after treatment, in comparison with those in patients with only G-CSF mobilization for 4 days. However, rupture of the spleen during peripheral blood stem-cell mobilization by administration of G-CSF has been reported even in healthy individuals (Falzetti *et al.*, 1999), and caution is needed in the administration of G-CSF to liver cirrhotic patients with splenomegaly.

Conclusion

Previous clinical studies using autologous bone marrow cells for chronic liver disease including liver cirrhosis suggested the efficacy and the safety of the therapy. However, to clearly show the efficacy and the safety of liver regeneration, randomized controlled study will be necessary. And issues that need to be investigated will include identification of cells showing treatment effects from bone marrow cell fractions, and clarification of the underlying mechanisms. When cells with liver regeneration and restorative activity can be isolated from small amounts of bone marrow fluid under local anesthesia, cultured, and then re-administered, the indications would be able to be expanded, and that genuine treatment methods using less-invasive bone marrow-derived cultured cells could be developed.

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Disclosure

The authors report no conflicts of interest.

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REVIEW

Timeline for development of autologous bone marrow infusion (ABM*i*) therapy and perspective for future stem cell therapy

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Abstract Liver cirrhosis patients generally progress to liver failure. To cure this progressive disease, we developed a novel cell therapy using bone marrow cells; autologous bone marrow cell infusion (ABM*i*) therapy. We previously described the possible action mechanism of ABM*i* therapy in the cirrhotic liver, and showed the timeline and results of clinical studies of ABM*i* therapy. We have also carried out other clinical studies using bone marrow cells and granulocyte colony-stimulating factor. Here, we report a new randomized clinical trial to evaluate the effects of ABM*i* therapy. However, ABM*i* therapy may not be possible in patients who are unable to undergo general anesthesia; therefore, we have started to develop a next-generation stem cell therapy using cultured mesenchymal stem cells.

Keywords ABM*i* therapy · Liver cirrhosis · Liver regeneration · Cell therapy

Introduction

The development of novel therapies is necessary for patients with liver failure. We started clinical studies of autologous bone marrow cell infusion (ABM*i*) therapy for patients with liver cirrhosis (LC) in November 2003, and to date, we have performed ABM*i* in 19 LC patients. The safety and efficacy of ABM*i* therapy have been confirmed, and no severe side effects have been detected. Multicenter trials have been performed in Japan and Korea. In this paper, we present the current status of ABM*i* therapy and the future prospects for next-generation stem cell therapies using bone marrow cells (BMCs).

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How does bone marrow cell infusion affect LC in murine models?

In 2000, Theise et al. [1] reported the presence of Y-chromosome-positive hepatocytes in females who died after BMC transplantation. The mechanism of differentiation of hepatocytes from BMCs was subsequently debated, with trans-differentiation and cell fusion found to be key mechanisms [2–4]. We therefore started basic studies to analyze the possibility of cell therapy in LC patients.

We first developed a new model to monitor the differentiation of BMCs into hepatocytes, using GFP transgenic (Tg) mice [2]. This model allowed us to continue carbon tetrachloride (CCl_4)-induced liver damage after GFP-positive BMC infusion. The injected BMCs were heterogeneous cells, and we found that GFP-positive BMCs repopulated the CCl_4-induced cirrhotic liver and expressed matrix metalloproteinase (MMP)-2 and MMP-9. Using this model, we confirmed that BMC-derived MMP attenuated liver fibrosis, subsequently improving liver function [5]. We also found that A6-positive cells (oval cells, liver progenitor cells) and Liv2-positive hepatoblasts were activated after GFP-positive BMC infusion. We believe that BMCs activated the generation of hepatoblasts and liver progenitor cells.

On the other hand, another group reported that the introduction of MMP-9 using an adenoviral system attenuated liver fibrosis [6–8], which may be an important mechanism for reversing LC. With regard to other cytokines, we found that fibroblast growth factor 2 (FGF2) accelerated the repopulation rate of BMCs and attenuated liver fibrosis [9, 10]. The inflammation system is also a key factor in considering the effects of BMC infusion.

Subsequently, we analyzed how BMCs affected liver fibrosis, using TNF-R1KO mice and a tumor necrosis factor (TNF)-alpha antagonist. We found that liver fibrosis was not attenuated after BMC infusion without TNF-alpha signaling [11]. Similarly, SEK/JNK signaling is essential for the generation of hepatoblasts [12]. We also found that granulocyte colony-stimulating factor (G-CSF) and interleukin (IL)-1beta were markedly affected by BMC infusion [26]. These results suggest that BMC infusion itself markedly affects cytokine levels (Fig. 1).

Protocol of ABM*i* therapy

ABM*i* therapy was started in November 2003. Recruitment commenced in October 2003, and the first patient began treatment on November 14, 2003. Eligible patients were between 18 and 75 years of age and had a clinical diagnosis of LC. To be eligible, the patients with LC had to have total bilirubin of less than 3.0 mg/dL, a platelet count of more than 5.0×10^{10}/L, and no visible hepatocellular carcinoma (HCC) on computed tomography (CT) or magnetic resonance imaging. Patients were excluded from the study if they had problems in organs other than the liver (e.g., the heart or lungs). Approximately 400 mL of bone marrow fluid was harvested from the ilium according to standard procedures, under general anesthesia, and collected into plastic bags containing heparin. After fat was removed from the tops of the bags, hydroxyethyl starch was added at a final concentration of 1.0 %. After 40-min incubation at room temperature, red blood cells (RBCs) were precipitated, and we then washed the cells with normal saline and centrifuged the bag in order to concentrate mononuclear cells (MNCs). The final concentrated and washed cell product was made up to a final volume of approximately 100 mL. Before the injection of MNCs into patients, 5 mL of the final cell product was submitted to the trypan blue dye exclusion test, endotoxin test, and fluorescence-activated cell sorting analysis. CD34-, CD45-, and c-kit-positive cells were determined by flow cytometry.

After cell preparation, MNCs were administered via the peripheral vein. Patients were followed up every week for 4 weeks, and laboratory data were analyzed monthly for 24 weeks. During the study period, the medication before and after ABM*i* therapy was not changed. Patients who changed medication or those who consumed alcohol were treated as dropouts. During the study, patients did not receive antiviral therapies such as interferon, lamivudine, ribavirin, or G-CSF. The primary outcomes of the study were the safety and feasibility of ABM*i* therapy. The Child–Pugh score (albumin, total bilirubin, prothrombin time, ascites, and encephalopathy) was used to evaluate the overall condition of the LC patients. All protocols were approved by the Ethics Committee of Yamaguchi University, and written informed consent was obtained from all patients (Fig. 2).

Results of first clinical study after ABM*i* therapy

Ten patients underwent ABM*i* therapy, and one of them was excluded after alcohol consumption. First, we obtained a sufficient volume of BMCs from the LC patients. This is the first trial to have obtained BMCs from LC patients and we believe that our method for obtaining BMCs is safe. From 400 mL of bone marrow fluid, we obtained, on average, $7.81 \pm 0.98 \times 10^9$ MNCs. After the cells had been washed, $5.20 \pm 0.63 \times 10^9$ MNCs were infused into LC patients. We analyzed the clinical course in 9 LC patients, who were followed for up to 6 months (etiology was hepatitis B virus [HBV] infection in 3 patients, HCV

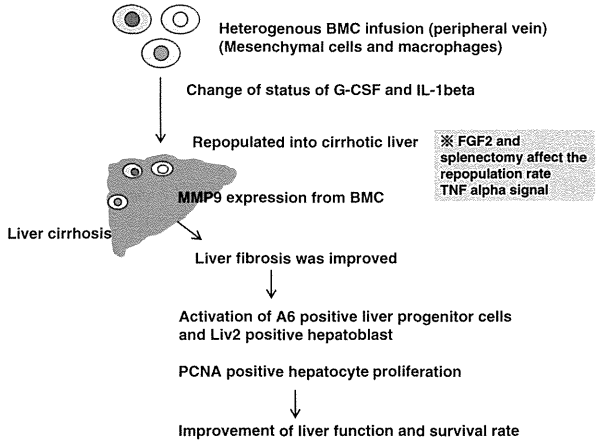

Fig. 1 Basic study in murine GFP/CCl_4 model

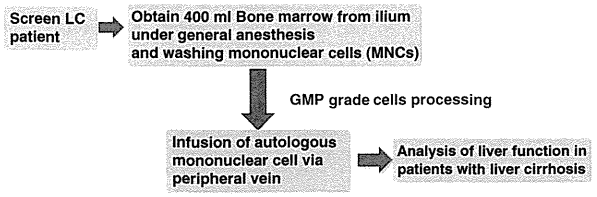

Fig. 2 Protocol for autologous bone marrow cell infusion (ABM*i*) therapy

infection in 5, and unknown in 1). Significant improvements in serum albumin levels and total protein were observed at 24 weeks after ABM*i* therapy ($p < 0.05$), and significantly improved Child–Pugh scores were seen at 4 and 24 weeks ($p < 0.05$). Alpha-fetoprotein and proliferating-cell nuclear antigen (PCNA) expression in liver biopsy tissue were also significantly elevated after ABM*i* therapy ($p < 0.05$) [13]. These results suggest that ABM*i* therapy activated the proliferation of resident hepatocytes in cirrhotic liver and improved liver function (Figs. 2, 3).

Second clinical trial of ABM*i* therapy for patients with HBV- and alcohol-related LC (collaboration study)

We also performed a collaboration study with Yonsei University in Korea, in which 10 patients with HBV-related LC received ABM*i* therapy. All 10 patients subsequently showed improved Child–Pugh scores and liver function. In this study, liver biopsies were performed before and at 1, 3, and 6 months after ABM*i* therapy, and areas showing hepatic progenitor cell (HPC) activation and positive reactions for cytokeratin (CK)-7 were evaluated. At 3 months after the ABM*i* therapy, CK-7-positive HPCs were elevated in the cirrhotic liver [14].

At Yamagata University, ABM*i* therapy for patients with alcohol-induced LC was performed in a collaboration study with other researchers at this University (Table 1). Five subjects and five control patients with alcoholic LC who had abstained from alcohol intake for 24 weeks prior to the study were enrolled. Autologous BMCs were washed and injected intravenously, and changes in serum liver function parameters and levels of type IV collagen 7S domain (a marker of fibrosis) were monitored for 24 weeks. The distribution of activated bone marrow was assessed by indium-111-chloride bone marrow scintigraphy [15]. The results suggested that BMC infusion activated bone marrow itself. Also, the serum albumin, prothrombin time, and Child–Pugh score were improved in the alcoholic LC patients.

In 2011, a clinical study of ABM*i* therapy for patients with HIV-plus HCV-related LC was started after approval was obtained from the Committee on the Guidelines for Clinical Research using Human Stem Cells in Japan. The results appear to be promising (Fig. 3).

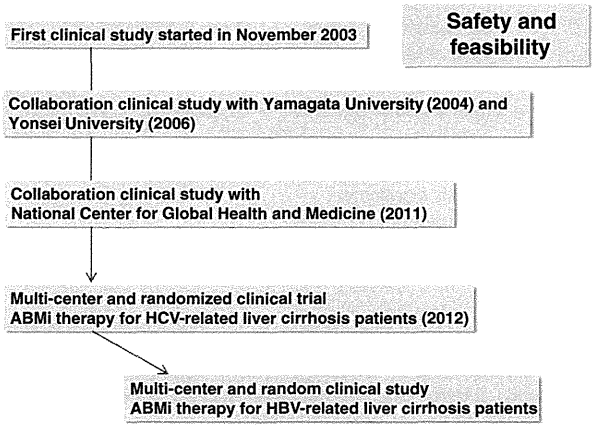

Fig. 3 Timeline of ABM*i* therapy studies

Other studies using autologous BMCs (Table 2)

Lyra et al. have also demonstrated the effectiveness of bone marrow stem cell infusion. Clinical studies by Lyra et al. [16, 17] in Brazil suggested the feasibility and safety of autologous BMC infusion through the hepatic artery rather than through a peripheral vein for patients with chronic liver disease awaiting liver transplantation. In other clinical studies, increased volumes in the left lateral hepatic segment were reported by am Esch et al. [18] with the intraportal administration of CD133-positive BMCs after portal venous embolization of right liver segments. Conversely, death due to radiocontrast nephropathy was reported as a result of the infusion of concentrated CD34-positive cells from 200 mL of bone marrow fluid through the hepatic artery into patients with decompensated LC, leading to discontinuation of that clinical study [19]. This occurrence indicates the need for clarification of treatment indications, as well as a full investigation into administration routes, cell concentrations, and speeds of drip infusion. Less invasive liver regeneration therapies using cultured autologous bone marrow-derived cells are also necessary.

Granulocyte-colony stimulation factor induction therapy

Various methods have been developed for cell therapy in liver disease, including the use of CD34-positive cells induced by G-CSF. Gordon et al. [20] collected CD34-positive cells from peripheral blood after induction with G-CSF, administered these cells via the hepatic artery, and reported improved serum levels of bilirubin and albumin in some patients, despite an observation period of only 60 days. In addition, Spahr et al. [21] administered G-CSF to patients with alcoholic LC and reported that G-CSF mobilized CD34 cells, increased hepatocyte growth factor (HGF) and induced HPCs to proliferate within 7 days of administration, increasing the proliferation of HPCs. Pai et al. [22] reported improvements in serum albumin and the

Table 1 Clinical study of ABM*i* therapy

Authors	Type of infused cells	Number of infused cells	Improvement after the infusion	No. of patients and disease etiology
Terai et al. [13]	BMNCs from iliac crest (400 mL)	$5.20 \pm 0.63 \times 10^9$	Improved serum albumin and CP score	5 HCV, 3 HBV, 1 unknown
Kim et al. [14]	BMNCs from iliac crest (500–750 mL)	$0.48–1.48 \times 10^8$/kg	Activation of HPC (cytokeratin7$^+$ cells). Increased liver volume. Improved CP score	10 HBV
Saito et al. [15]	BMNCs from iliac crest (400 mL)	$8.0 \pm 7.3 \times 10^9$	Improved serum albumin, PT, and CP score	5 Alcohol + 5 controls

ABMi autologous bone marrow cell infusion, *BMNC* bone marrow mononuclear cell, *CP* Child–Pugh (score), *HCV* hepatitis C virus, *HBV* hepatitis B virus, *HPC* hepatic progenitor cell, *PT* prothrombin time

Table 2 Other clinical studies using BMCs and G-CSF

Authors	Type of infused cells	Number of infused cells	Improvement after the infusion	No. of patients and disease etiology
Gordon et al. [20]	CD34$^+$ cells from G-CSF-mobilized peripheral blood	$1 \times 10^6–2 \times 10^8$	Improved serum albumin and serum bilirubin	5 Alcohol
Spahr et al. [21]	Only G-CSF mobilization for 5 days	N/A	Elevated serum HGF, increased hepatic Ki67$^+$/cytokeratin7$^+$ cells	13 Alcohol + 11 ctrl.
Pai et al. [22]	Cultured CD34$^+$ cells from G-CSF-mobilized peripheral blood	2.3×10^8 (mean)	Improved serum bilirubin and CP score	9 Alcohol
Han et al. [23]	PBMCs from G-CSF-mobilized peripheral blood	$10^7–10^8$/kg	Improved serum albumin and CP score	20 HBV + 20 ctrl. (only G-CSF)
Peng et al. [27]	BMNCs (MSCs) from iliac crest (120 mL)	Not described	Improved serum albumin, bilirubin, PT, and MELD	53 HBV + 105 ctrl. in the early period. No difference of HCC development through 48 weeks
Lyra et al. [16]	BMNCs from iliac crest (maximum 50 mL)	At least 1×10^8	Improved serum albumin and bilirubin	10
Lyra et al. [17]	BMNCs from iliac crest (maximum 50 mL)	$3.78 \pm 2.69 \times 10^8$	Improved serum albumin, bilirubin, and CP for 60–90 days	15 + 15 ctrl.
am Esch et al. [18]	Bone marrow-derived CD133$^+$ cells (60–220 mL)	$2.4–12.3 \times 10^6$	Increased liver volume after liver resection	3 bearing liver tumors + 3 ctrl.
Mohamadnejad et al. [19]	Bone marrow-derived CD34$^+$ cells from iliac crest (200 mL)	5.25×10^6 (mean) (CD34$^+$, 90.5 %)	One patient developed radiocontrast nephropathy, and died	1 HBV, 1 PBC 1 AIH, 1 cryptogenic
Mohamadnejad et al. [30]	Cultured bone marrow-derived MSCs (80–100 mL)	31.7×10^6 (mean)	Improved MELD (2 of 4 patients)	3 Cryptogenic, 1 AIH
Kharaziha et al. [31]	Cultured bone-marrow derived MSCs (20 mL)	$3–5 \times 10^7$	Improved MELD, serum creatinine, prothrombin complex	4 HBV, 2 unknown 1 HCV
Amer et al. [32]	Cultured bone-marrow derived MSCs stimulated to hepatic lineage using HGF-containing medium (95 ± 25 mL)	2×10^7 hepatic lineage-committed cells in a total of 2×10^8 cells	Improved MELD and CP	20 HCV + 20 ctrl.

BMC bone marrow cell, *G-CSF* granulocyte colony-stimulating factor, *N/A* not applicable, *HGF* hepatocyte growth factor, *ctrl.* control, *CP* Child–Pugh (score), *PBMC* peripheral blood mononuclear cell, *HBV* hepatitis B virus, *BMNC* bone marrow mononuclear cell, *PT* prothrombin time, *MELD* Model for End-Stage Liver Disease, *AIH* autoimmune hepatitis, *MSC* mesenchymal stem cell, *PBC*

Child–Pugh score, as well as the attenuation of ascites accumulation, after the administration of CD34-positive cells (whose in vitro expansion had been induced by G-CSF) via a hepatic artery in subjects with alcoholic LC. A report by Han et al. [23] also described G-CSF administration for HBV-related decompensated LC. In that study, the administration of peripheral blood MNCs induced by G-CSF led to significant improvements in serum albumin and the Child–Pugh score at 6 months after treatment, as compared to findings in patients treated with G-CSF mobilization alone for 4 days. However, rupture of the spleen by the administration of G-CSF has been reported during peripheral blood stem-cell mobilization, even in healthy individuals [24]; thus, caution is necessary to avoid splenic rupture during the administration of G-CSF to LC patients with splenomegaly.

Splenectomy and serum markers

We subsequently examined the effects of splenectomy before ABM*i* therapy. From these studies, we found that splenectomy enhanced the effects of ABM*i* therapy, as infused MNCs repopulated the cirrhotic liver more readily owing to the decrease in trapping by the spleen [25]. We also analyzed changes in serum cytokine profiles after ABM*i* therapy. We compared human studies and murine GFP/CCl_4 models, and found marked decreases in G-CSF and IL-1beta after the ABM*i* therapy in the animal models as well as in the human studies. These changes in cytokines may be important for initiating the repair of the cirrhotic liver [26]. Further study is necessary in order to understand the mechanisms responsible (Figs. 2, 4).

Risk of HCC after BMC infusion

We have previously reported the efficacy and safety of ABM*i* therapy in LC patients without HCC in a multicenter clinical trial. However, as LC is highly oncogenic, evaluation of the effects of ABM*i* on the mechanisms of hepatocarcinogenesis is of great importance. In China, Peng et al. [27] recruited 527 patients with HBV-related decompensated LC, among whom 53 patients received 120 mL of autologous bone marrow fluid via the hepatic artery, and 105 patients did not. The results of analysis showed no adverse effects due to bone marrow administration. Patients were divided into a short-term observation group (1–48 weeks) and a long-term observation group (until 192 weeks), and the results showed improved hepatic function in the early period. Long-term observation showed no changes in the incidence of HCC after the administration of BMCs, thus suggesting the possibility of improved survival rate. This clinical study confirmed that BMC infusion does not enhance the risk of HCC in LC patients [27].

We also analyzed the effects of repeated BMC infusions in an *N*-nitrosodiethylamine (DEN)/GFP-CCl_4 model developed by administering DEN, followed by repeated administration of CCl_4 intraperitoneally. In the administration (ABM*i*) group, GFP-positive BMCs were infused through the tail vein. The kinetics of hepatocarcinogenesis were evaluated histologically at 4.5 months after DEN treatment. At 4.5 months, there was a significantly lower incidence of tumor foci and tumors in the ABM*i* group, and the foci were smaller in number than those in the non-ABM*i* group, while the size of the tumors was similar to that in the non-ABM*i* group. Livers in the ABM*i* recipient

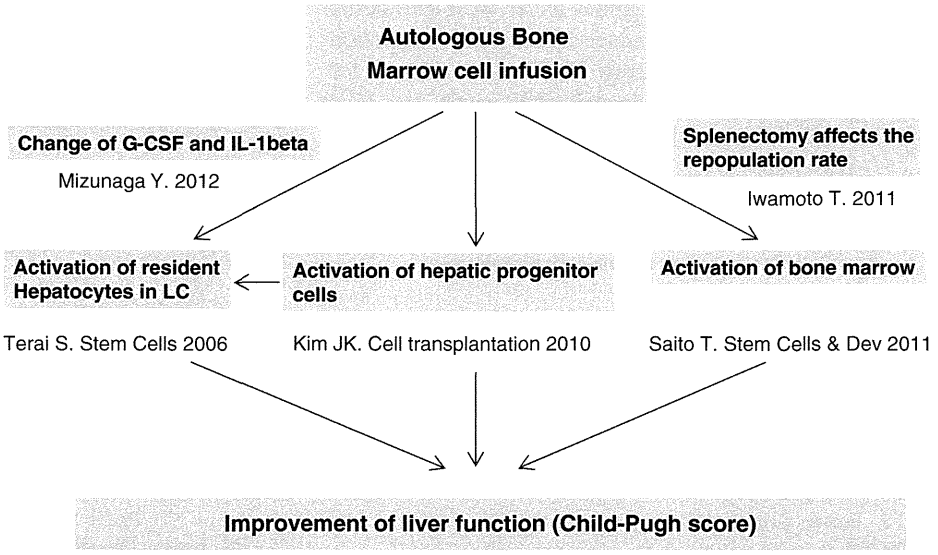

Fig. 4 Effects of autologous bone marrow cell infusion (ABM*i*) in clinical studies

mice showed significantly reduced liver fibrosis, consistent with significantly lower 8-hydroxy-2′-deoxyguanosine levels, and higher superoxide dismutase activity. These results confirmed that frequent BMC infusion may contribute to suppressing the generation of HCC during the early stages of hepatocarcinogenesis, consistent with improvements in liver fibrosis and the stabilization of redox homeostasis [28].

Future of stem cell therapy

ABM*i* therapy requires bone marrow obtained under general anesthesia; thus, there are limits with regard to which patients are candidates for ABM*i* therapy. To resolve this issue, we have begun to develop new methods for cell therapy. We are now trying to develop a new approach using cultured BMCs. To develop this therapy, we analyzed which cell fraction was most useful for improving liver function. In previous studies, we found that mesenchymal stem cells were candidates for such approaches. However, Forbes et al. [29] showed that macrophages were useful for repairing the cirrhotic liver, but in humans, macrophage proliferation is difficult.

Some clinical phase I trials using mesenchymal stem cells (MSCs) have been reported. Mohamadnejad et al. have shown improvements in the Model for End-Stage Liver Disease (MELD) score in some patients with peripheral vein administration of cultured MSCs in autologous BMCs [30]. Kharaziha et al. [31] also reported that the MELD score had decreased significantly, from 17.9 ± 5.6 to 10.7 ± 6.3, after the administration of cultured autologous MSCs in four hepatitis B LC patients, one hepatitis C LC patient, one alcoholic LC patient, and two cryptogenic LC patients. For that treatment, approximately 20 mL of autologous bone marrow fluid was aspirated from both posterior-superior iliac spines under local anesthesia. Mononuclear cells were separated by the Ficoll separation method. Separated bone marrow MNCs were cultured for 2 weeks, and were then collected, after which 3×10^7–5×10^7 cells expressing CD44, CD73, and CD105, consistent with MSC characteristics, were infused via the portal vein or peripheral vein.

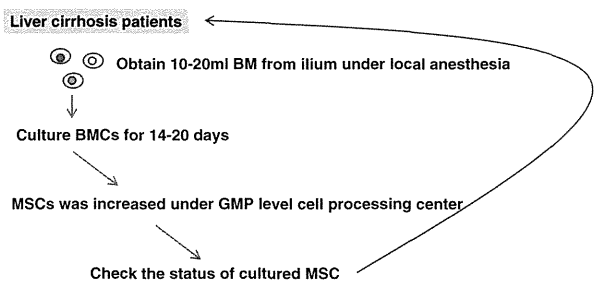

Fig. 5 Future of stem cell therapy using cultured mesenchymal stem cells (*MSCs*)

Of note, a recent clinical trial for patients with HCV-related liver failure was reported by Amer et al. [32]. They also aspirated autologous bone marrow fluid (approximately 120 mL) from the posterior-superior iliac crest under local anesthesia, and then directly injected autologous cultured bone marrow-derived MSCs, which had been stimulated to hepatic lineage using HGF-containing medium, into the spleen or liver, using an 18-gauge needle under abdominal ultrasound (US) guidance [32]. In these patients, MELD and Child–Pugh scores were significantly lower than those in the control group from 2 weeks to 6 months after the injection. No differences were observed between the intrasplenic route and intrahepatic route.

The Highway Program for the realization of regenerative medicine in Japan was started in 2011 to develop new stem cell therapies for humans. We are therefore receiving grants for basic proof of concept (POC) research into cell therapies for LC patients. After demonstrating POC, we plan to prepare protocols for clinical studies and to perform stem cell therapy for LC, but we must first conform to the Guidelines on Clinical Research using Human Stem Cells in Japan (Fig. 5).

Conclusions

Several clinical studies using autologous BMCs for advanced liver disease have suggested the efficacy and safety of this treatment. However, randomized clinical trials are necessary to clearly confirm the efficacy and safety of liver regeneration therapies using autologous bone marrow (stem) cells or cultured bone marrow-derived cells. We have planned multicenter and randomized clinical trials of ABM*i* therapy for LC patients, in accordance with the Guidelines on Clinical Research using Human Stem Cells in Japan. We are performing basic POC research into cell therapies for LC patients, after which we will prepare clinical study protocols. Stem cell therapy using BMCs is considered to be promising for LC patients.

Acknowledgments This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (JSPS); Ministry of Health, Labour and Welfare, Health and Labour Sciences Research Grants; and the Japan Science and Technology Agency (JST) Project for Realization of Regenerative Medicine and the Highway Program.

Conflict of interest The authors declare that they have no conflict of interest.

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REVIEW

Stem cell therapy in chronic liver disease

Taro Takami[a], *Shuji Terai*[b], *and Isao Sakaida*[b]

Purpose of review
To provide an overview of the current status of liver regeneration therapies for liver cirrhosis and future prospects.

Recent findings
Various clinical studies for liver disease have been reported, including hepatic administration of autologous CD34-positive cells induced by granulocyte colony-stimulating factor, portal vein administration of CD133-positive mononuclear cells, and administration of autologous bone marrow-derived mesenchymal stem cells. Effectiveness of these approaches has been shown in some patients. We have also reported improved liver fibrosis and function with infusion of autologous bone marrow cells in a basic study with mice, and on the basis of those results started autologous bone marrow cell infusion (ABM*i*) therapy for liver cirrhosis. The efficacy and safety of ABM*i* therapy has also been reported by other institutions.

Summary
Results of recent clinical studies strongly suggest that liver function-improving effects can be achieved using infusion of bone marrow (stem) cells for cirrhosis. New treatment methods using less-invasive bone marrow-derived cultured cells need to be developed.

Keywords
bone marrow, liver cirrhosis, liver regeneration, stem cell

INTRODUCTION

With the development of new antiviral medicines and advances in interferon therapy, viral elimination and cure of hepatitis can be expected even in some patients with chronic viral hepatitis, for which radical cure has been difficult. In many patients, however, liver cirrhosis has already occurred. In cases that have progressed to decompensated liver cirrhosis, the only radical treatment currently available is still liver transplantation. However, liver transplants are not widely performed worldwide due to problems such as chronic donor shortages, surgical invasiveness, risk of immunological rejection, and medical costs. To compensate for this, development of new regenerative therapies for liver cirrhosis is an urgent task. In 2000, Theise *et al.* [1] reported the existence of Y chromosome-positive cells in livers with chronic inflammation in autopsied women who had received therapeutic bone marrow transplantations from male donors, suggesting the existence of pluripotent stem cells among bone marrow cells. Since then, attention has been focused on bone marrow (stem) cells as a cell source for liver regenerative therapies [2–6,7▪▪]. Here, we present an overview of the current status of clinical trials and future prospects for liver regeneration therapies using stem cells, including autologous bone marrow-derived cells.

GRANULOCYTE COLONY-STIMULATING FACTOR

Various cell therapies for liver disease have been developed, including using CD34-positive cells induced with granulocyte colony-stimulating factor (G-CSF). Gordon *et al.* [8] collected CD34-positive cells from peripheral blood after induction with G-CSF, then administered these cells via a hepatic artery, and reported improved serum levels of bilirubin and albumin in some patients, despite a short

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KEY POINTS

- Previous clinical studies using autologous bone marrow cells suggest the efficacy and safety of this approach for advanced liver disease.
- Randomized controlled studies are needed to establish genuine efficacy because most of the published results have come from uncontrolled studies.
- New treatment methods using less-invasive bone marrow-derived cultured cell fractions showing treatment effects on liver fibrosis and functions need to be developed.

observation period of only 60 days. In addition, Spahr *et al.* [9] administered G-CSF to patients with alcoholic liver cirrhosis and reported increased proliferation of hepatic progenitor cells (HPCs), whereas Pai *et al.* [10] reported improvements in serum albumin, Child–Pugh score, and accumulation of ascites with in-vitro expansion of CD34-positive cells induced by G-CSF and administration via a hepatic artery in patients with alcoholic liver cirrhosis. A study by Han *et al.* [11] described G-CSF administration for hepatitis B virus (HBV)-related decompensated liver cirrhosis. In this study, administration of peripheral blood mononuclear cells induced with G-CSF showed significantly improved effects on serum albumin and Child–Pugh score at 6 months after treatment, compared with those in patients with only G-CSF mobilization for 4 days. However, rupture of the spleen during peripheral blood stem-cell mobilization by administration of G-CSF has been reported even in healthy individuals [12], and caution is needed in the administration of G-CSF to liver cirrhosis patients with splenomegaly.

NONCULTURED AUTOLOGOUS BONE MARROW CELLS

In our previous animal studies, we have developed an in-vivo mouse model [the green fluorescent protein (GFP)/carbon tetrachloride (CCl_4) model], and reported that GFP-positive bone marrow cells infused via a tail vein efficiently repopulated cirrhotic liver under conditions of persistent liver damage induced by CCl_4 [13]. In these processes, elevation in serum albumin levels [13], a significant increase in survival rate, and reduced liver fibrosis assessed by Sirius red staining were seen following infusion of GFP-positive bone marrow cells [14]. Infused bone marrow-derived GFP-positive cells were also confirmed to produce collagenases including matrix metalloproteinase (MMP) 2 and MMP-9 during these processes [14]. On the basis of the above basic study, infusion of autologous bone marrow cells via a peripheral vein in a chronic liver injury environment is thought to improve liver functions and reduce liver fibrosis, and to significantly improve vital prognosis in recipients. Our clinical study of autologous bone marrow cell infusion (ABM*i*) therapy for decompensated liver cirrhosis was therefore started in November 2003 [15]. In addition, a multicenter clinical trial of liver regeneration with cell transplantation was started in 2005. With that ABM*i* therapy, around 400 ml of autologous bone marrow cells was collected under general anesthesia, and the collected bone marrow fluid was concentrated and washed. Bone marrow mononuclear cells in that fluid were then purified and condensed according to standard operating procedures (SOP) at the regenerative and cell therapy center fully equipped with good manufacturing practice (GMP) grade facilities, and administered by drip infusion via a peripheral vein to the same patient. The course was observed for 6 months after ABM*i*, and efficacy and safety were evaluated using blood biochemistry tests, liver biopsy, abdominal ultrasonography, and abdominal computed tomography and so on. During the observation period, no changes in oral medications, antiviral drugs, or other agents were seen [15]. As a result, serum albumin levels, total protein levels, and Child–Pugh score at 6 months after ABM*i* were significantly improved in patients for whom the course could be observed for 6 months after ABM*i* [15]. Similar improvements were also seen in nine patients for whom the course could be observed for 15 months [16]. In 2011, Kim *et al.* [17▪] reported that ABM*i* improved serum albumin levels, Child–Pugh score, liver volume measured by MRI and accumulation of ascites in patients with HBV-related decompensated liver cirrhosis, and results from liver biopsies taken over time suggested the possibility of HPC activation as the underlying mechanism. All 10 patients who received ABM*i* also showed an improvement in quality of life, with no serious adverse events. Moreover, Saito *et al.* [18▪] recently reported the efficacy and safety of ABM*i* therapy for patients with alcoholic liver cirrhosis. Such results are gradually confirming the safety and efficacy of ABM*i* therapy. Therefore, we believe that ABM*i* therapy represents a promising treatment for advanced liver cirrhosis (Fig. 1).

Peng *et al.* [19▪▪] recruited 527 patients with HBV-related decompensated liver cirrhosis, divided into a group of 53 patients who received 120 ml of autologous bone marrow fluid via a hepatic artery and 105 patients who did not. Results of analysis showed no adverse effects from bone marrow administration.

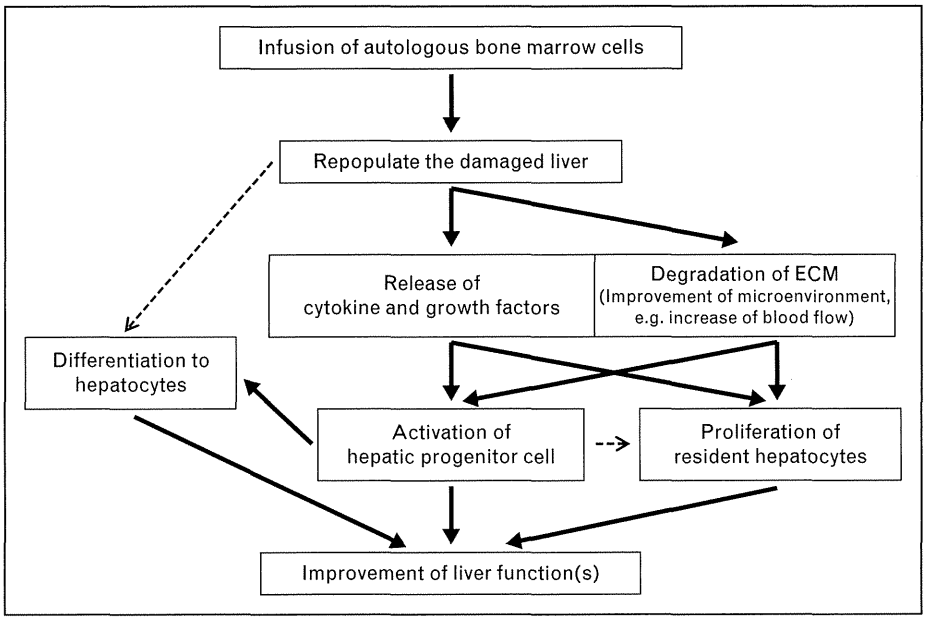

FIGURE 1. Possible mechanism(s) of autologous bone marrow cell infusion therapy. ECM, extracellular matrix.

Patients were divided into a short-term observation group (1–48 weeks) and a long-term observation group (until 192 weeks), and the results of analysis showed improved hepatic function in the early period. Long-term observation showed no change in the incidence of hepatocellular carcinoma (HCC) after the administration of bone marrow cells, suggesting the possibility of an improved survival rate [19▪▪]. Other studies to date on cell therapies using bone marrow (stem) cells for liver cirrhosis include not only those on our ABM*i* therapy [15,16] but also studies by Lyra *et al.* [20,21▪] on the effectiveness of infusion of bone marrow stem cells. Clinical studies by Lyra *et al.* in Brazil suggested the feasibility and safety of ABM*i* through a hepatic artery rather than a peripheral vein for chronic liver disease patients awaiting liver transplantation [20,21▪]. In other clinical studies, increased volumes of left lateral hepatic segments were reported by am Esch *et al.* [22,23] with intraportal administration of CD133-positive bone marrow cells after portal venous embolization of right liver segments. Conversely, death due to radiocontrast nephropathy has been reported as a result of infusion of concentrated CD34-positive cells from 200 ml of bone marrow fluid through a hepatic artery into patients with decompensated liver cirrhosis, and that clinical study was discontinued [24]. This indicates the need for clarification of the treatment indication criteria and full investigation of administration routes, cell concentrations and speed of drip infusion. The need for development of less-invasive liver regeneration therapies using cultured autologous bone marrow-derived cells is also suggested.

CULTURED AUTOLOGOUS BONE MARROW-DERIVED CELLS

Our ABM*i* 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 patient. The bone marrow contains two major sources of stem cells, hematopoietic stem cells (HSCs) and mesenchymal stem (stromal) cells (MSCs). Previous basic studies suggested the therapeutic effect on advanced liver diseases using these cell types in animal models. Therefore, issues that need to be investigated in the future will include identification of cells showing treatment effects from bone marrow cell fractions, and clarification of the mechanisms underpinning such actions. When cells with liver regeneration and restorative activity can be isolated from small amounts of bone marrow fluid, cultured, and then readministered, the indications would be able to be expanded, as collection of bone marrow fluid would no longer need to be performed under general anesthesia. However, safety evaluation guidelines for cultured cells are needed when autologous cultured bone marrow-derived cells

Table 1. Previous clinical trials in liver disease using autologous bone marrow (stem) cells

Type of infused cells	Number of infused cells	Improvement after the infusion	Number and etiology of patients	Reference
$CD34^+$ cells from G-CSF mobilized peripheral blood	1×10^6–2×10^8	Improved serum albumin and serum bilirubin	Five alcohol	[8]
Only G-CSF mobilization for 5 days	N/A	Elevated serum HGF. Increased hepatic $Ki67^+$/$cytokeratin7^+$ cells	24 alcohol, (11/24 ctrl.)	[9]
Cultured $CD34^+$ cells from G-CSF mobilized peripheral blood	2.3×10^8 (mean)	Improved serum bilirubin and CP	Nine alcohol	[10]
PBMCs from G-CSF mobilized peripheral blood	10^7–10^8/kg	Improved serum albumin and CP	40 HBV (20/40 ctrl., only G-CSF)	[11]
BMNCs from iliac crest (400 ml); ABM*i* therapy	$5.20 \pm 0.63 \times 10^9$	Improved serum albumin and CP	Five HCV, three HBV, one unknown	[15]
BMNCs from iliac crest (500–750 ml) ; ABM*i* therapy	0.48–1.48×10^8/kg	Activation of HPC ($cytokeratin7^+$cells). Increased liver volume Improved CP	10 HBV	[17▪]
BMNCs from iliac crest (400 ml); ABM*i* therapy	$8.0 \pm 7.3 \times 10^9$	Improved serum albumin, PT, and CP	10 alcohol (5/10 ctrl.)	[18▪]
BMNCs (MSCs) from iliac crest (120 ml)	Not described	Improved serum albumin, bilirubin, PT, and MELD in the early period No difference of HCC development through 48 weeks	158 HBV (105/158 ctrl.)	[19▪▪]
BMNCs from iliac crest (maximum 50 ml)	At least 1×10^8	Improved serum albumin and bilirubin	10	[20]
BMNCs from iliac crest (maximum 50 ml)	$3.78 \pm 2.69 \times 10^8$	Improved serum albumin, bilirubin, and CP for 60–90 days	30 (15/30 ctrl.)	[21▪]
Bone marrow-derived $CD133^+$ cells (60–220 ml)	2.4–12.3×10^6	Increased liver volume after liver resection	Six bearing liver tumors (3/6 ctrl.)	[22]
Bone marrow-derived $CD34^+$ cells from iliac crest (200 ml)	5.25×10^6 (mean) ($CD34^+$, 90.5%)	One case developed radiocontrast nephropathy and then died	One HBV, one PBC One AIH, one cryptogenic	[24]
Cultured bone marrow-derived MSCs (80–100 ml)	31.7×10^6 (mean)	Improved MELD (two of four patients)	Three cryptogenic, one AIH	[28]
Cultured bone marrow-derived MSCs (20 ml)	3–5×10^7	Improved MELD, serum creatinine, prothrombin complex	Four HBV, two unknown One HCV	[29]
Cultured bone marrow-derived MSCs stimulated to hepatic lineage using HGF-containing medium (95 ± 25 ml)	2×10^7 hepatic lineage-committed cells in a total of 2×10^8 cells	Improved MELD and CP	40 HCV (20/40 ctrl.)	[30▪]

ABM*i*, autologous bone marrow cell infusion; AIH, autoimmune hepatitis; BMNC, bone marrow mononuclear cell; CP, Child–Pugh score; ctrl., control; G-CSF, granulocyte colony-stimulating factor; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HGF; hepatocytes growth factor; HPC, hepatic progenitor cell; MELD, model for end stage liver disease; MSC, mesenchymal stem cell; N/A, not applicable; PBMC, peripheral blood mononuclear cell; PT, prothrombin time.

are used in liver cirrhotic patients, and a system conforming to SOP at a GMP-grade cell-processing center will be also essential.

Some clinical phase I trials using MSCs have been reported in patients with myocardial infarction [25], amyotrophic lateral sclerosis [26] and Crohn's disease [27]. We can check the current status of clinical trials in this database (*ClinicalTrials.gov*; http://www.clinicaltrials.gov/). About advanced liver disease, Mohamadnejad *et al.* [28] have shown improvements of the model for end-stage liver disease (MELD) score in some patients with peripheral vein administration of cultured MSCs in autologous bone marrow cells. Kharaziha *et al.* [29] also reported that liver function assessed by the MELD score decreased significantly from 17.9 ± 5.6 to 10.7 ± 6.3 after administration of cultured autologous MSCs in four hepatitis B, one hepatitis C, one alcoholic, and two cryptogenic cirrhotic patients. They aspirated around 20 ml of autologous bone marrow fluid from both posterior superior iliac spines under local anesthesia. The mononuclear cells were separated by the Ficoll separation method. Separated bone marrow mononuclear cells were cultured for 2 weeks, and then were collected. They infused about 3×10^7–5×10^7 cells expressing CD44, CD73, and CD105, consistent with MSCs characteristics to the same patient via a portal vein or peripheral vein. Moreover, recently, the clinical trial for patients with hepatitis C virus (HCV)-related liver failure has been reported by Amer *et al.* [30▪]. They also aspirated around 120 ml of autologous bone marrow fluid from the posterior superior iliac crest under local anesthesia, and then injected autologous cultured bone marrow-derived MSCs, which had been stimulated to hepatic lineage using hepatocytes growth factor (HGF) containing medium, into spleen or liver directly using a needle gauge 18 under abdominal ultrasonography guidance. In these patients, the MELD score and Child score were significantly lower than those in the control group from 2 weeks to 6 months after the injection. No difference between intrasplenic route and intrahepatic route was observed.

CONCLUSION

Previous clinical studies using autologous bone marrow cells for advanced liver disease suggested the efficacy and safety (Table 1). However, randomized controlled studies are needed to clearly show the efficacy and safety of liver regeneration therapies using autologous bone marrow (stem) cells or cultured bone marrow-derived cells. And new treatment methods using less-invasive bone marrow-derived cultured cells need to be developed.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:

- ▪ of special interest
- ▪▪ of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 291).

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REGULAR ARTICLE

Bone-marrow-derived cells cultured in serum-free medium reduce liver fibrosis and improve liver function in carbon-tetrachloride-treated cirrhotic mice

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Abstract We have previously developed autologous bone marrow cell infusion (ABM*i*) therapy for liver cirrhosis patients. One problem associated with ABM*i* therapy is that general anesthesia is required to obtain 400 ml bone marrow fluid from liver cirrhosis patients. However, many patients with decompensated cirrhosis do not meet the criteria, because of decreased liver function or an increased bleeding tendency. To overcome these issues, our aim is to derive liver repair cells from small amounts of autologous bone marrow aspirates obtained under local anesthesia and to use these cells in liver cirrhosis patients. Here, we conducted, by using a mouse model, basic research aimed at achieving novel liver regeneration therapy. We cultured bone marrow cells aspirated from the femurs of C57 BL/6 Tg14 (act-EGFP) OsbY01 mice (green fluoresent protein [GFP]-transgenic mice). After 14 days of culture with serum-free medium (good manufacturing practice grade), the obtained spindle-shaped GFP-positive cells were injected (1×10^4 cells) via the caudal vein into mice with carbon tetrachloride (CCl_4)-induced cirrhosis. Numerous cultured macrophages and some mesenchymal stem cells repopulated the cirrhotic liver. The results showed that serum albumin, liver fibrosis and liver function were significantly improved in the group treated with cultured bone marrow cells ($P<0.01$). Moreover, matrix metalloproteinase-9 expression was increased in the liver ($P<0.01$). Thus, infusion of bone-marrow-derived cultured cells improved liver function and liver fibrosis in mice with CCl_4-induced cirrhosis.

Keywords Bone marrow cell · CCl_4 · Liver cirrhosis · Liver regeneration · Stem cell therapy · Mouse

Abbreviations

BMC	Bone marrow cell
GFP	Green fluorescent protein
CCl_4	Carbon tetrachloride
ABM*i*	Autologous bone marrow cell infusion
MSC	Mesenchymal stem cell
GMP	Good manufacturing practice
PBS	Phosphate-buffered saline
FITC	Fluorescein isothiocyanate
PE	Phycoreythrin
PI	Propidium iodide
DAB	Diaminobenzidine
FBS	Fetal bovine serum
α-SMA	α-Smooth muscle actin
MMP9	Matrix metalloproteinase-9

Introduction

Cirrhosis is an end-stage condition in liver disease and its treatment is extremely difficult. One of the few available options is liver transplantation but donor supply, surgical invasiveness and costs continue to be problems. On the other hand, cell therapy with autologous stem cells is a promising approach for novel organ regeneration, without the risk of immunorejection and is compatible with biological tissue in patients.

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As a basic model to advance clinical indications for liver regeneration therapy with autologous bone marrow cells (BMCs), we have developed an in vivo mouse model (the green fluorescent protein [GFP]/carbon tetrachloride [CCl_4] model) and have reported that GFP-positive BMCs infused via a tail vein efficiently repopulate cirrhotic liver under conditions of persistent liver damage induced by CCl_4 (Terai et al. 2003). In evaluations with this model, we have found that the infusion of BMCs can improve liver fibrosis, restore liver function and increase survival rates (Sakaida et al. 2004; Terai et al. 2005). Based on these basic studies, in November 2003, we began clinical studies of liver regeneration therapy with autologous BMCs from the iliac crest for autologous BMC infusion (ABM*i*). To date, the treatment has been safe and as in the basic research, ABM*i* therapy has improved liver function in patients with decompensated liver cirrhosis (Terai et al. 2006).

A multi-center trial for ABM*i* therapy was performed in Korea and Yamagata and its efficacy was demonstrated (Kim et al. 2010; Saito et al. 2011; Terai and Sakaida 2011). However, a problem associated with ABM*i* is that, because general anesthesia is required to obtain about 400 ml bone marrow fluid, restrictions occur with regard to liver function. In addition, to ensure safe collection, this procedure is limited to patients with platelet counts of at least 50,000/μl. Thus, many patients with decompensated cirrhosis do not meet the criteria. To overcome these issues, we have developed a method whereby bone-marrow-derived liver repair cells can be cultured and grown from small amounts of autologous bone marrow aspirates obtained under local anesthesia.

Using BMCs, we have conducted basic research aimed at achieving novel liver regeneration therapy. Human mesenchymal stem cells (MSCs) are present in adult bone marrow, can replicate as undifferentiated cells, have the ability to develop into several different types of tissue and are regarded as pluripotent cells. MSCs were first reported by Friedenstein et al. (1982). From human bone marrow, MSCs can be isolated by simple seeding on a plastic dish in the presence of fetal bovine serum (FBS; Pittenger et al. 1999). On the other hand, macrophage fractions from mouse bone marrow tend to be increased in the same way. Macrophages have a broad repertoire of context-dependent immune, inflammatory, trophic and regulatory actions. Treatment of cirrhosis with macrophages has been reported (Thomas et al. 2011). Because our experimental concept is to study the effects of cultured BMCs obtained by simple culture methods without sorting, we decided to study the effects of a culture cell population that mainly comprised macrophage fractions in the mouse. For potential clinical application in humans, the culture medium used in this study was serum-free and of a good manufacturing practice (GMP) grade.

Here, we report the development of a method for culturing bone-marrow-derived cells in serum-free medium. We have also examined the efficacy of these cultured cells in improving liver fibrosis and liver function.

Materials and methods

Animals

Female C57 BL/6 mice were purchased from Nippon (Shizuoka, Japan) and male C57 BL/6 Tg14 (act-EGFP) OsbY01 mice (GFP-transgenic mice) were kindly provided by Dr. Masaru Okabe. Mice were maintained in specific pathogen-free housing at the Animal Experiment Facility of Yamaguchi University School of Medicine and were cared for in accordance with the requirements of Yamaguchi University School of Medicine for the ethical treatment of animals and after obtaining approval for the experimental protocol.

BMC preparation

For the isolation of BMCs, GFP-transgenic mice (aged 6 weeks) were killed by cervical dislocation and their limbs were removed. GFP-positive BMCs were flushed from the medullary cavities of tibias and femurs with phosphate-buffered saline (PBS; Life Technologies, Carlsbad, Calif., USA) by using a 25 G needle. The cell suspension was filtered through a cell strainer (100 μm) to remove debris, followed by centrifugation at 500*g* for 5 min. After centrifugation, the supernatant was removed and the cells were resuspended to give 1.0×10^7 cells/ml. The preparation of BMCs took approximately 1.5 h. Cellular characterization of uncultured and cultured cells was performed by flow cytometric analysis (Fig. 1; see also below).

Culture of bone-marrow-derived cells

GFP-positive BMCs were isolated from male GFP-transgenic mice as descried previously (Agata et al. 2009). Under standard culture protocols, BMCs are seeded at a density of $1\times10^{5-6}$/ml but the proliferation rate is insufficient in GFP-transgenic mice. By increasing the cell density for primary culture to 1×10^7/ml, BMCs could be grown more efficiently (Fig. 2). Thus, isolated GFP-positive BMCs (1×10^7) were cultured on a CELL-start-coated dish (9.6 cm^2/well) in StemPro MSC Xeno Free medium (Life Technologies) with 2 mML-glutamine (Life Technologies), StemPro LipoMax supplement (Life Technologies) and 100 μg/ml penicillin-streptomycin (Life Technologies). StemPro MSC Xeno Free medium was prepared under GMP and qualified by using a human MSC performance

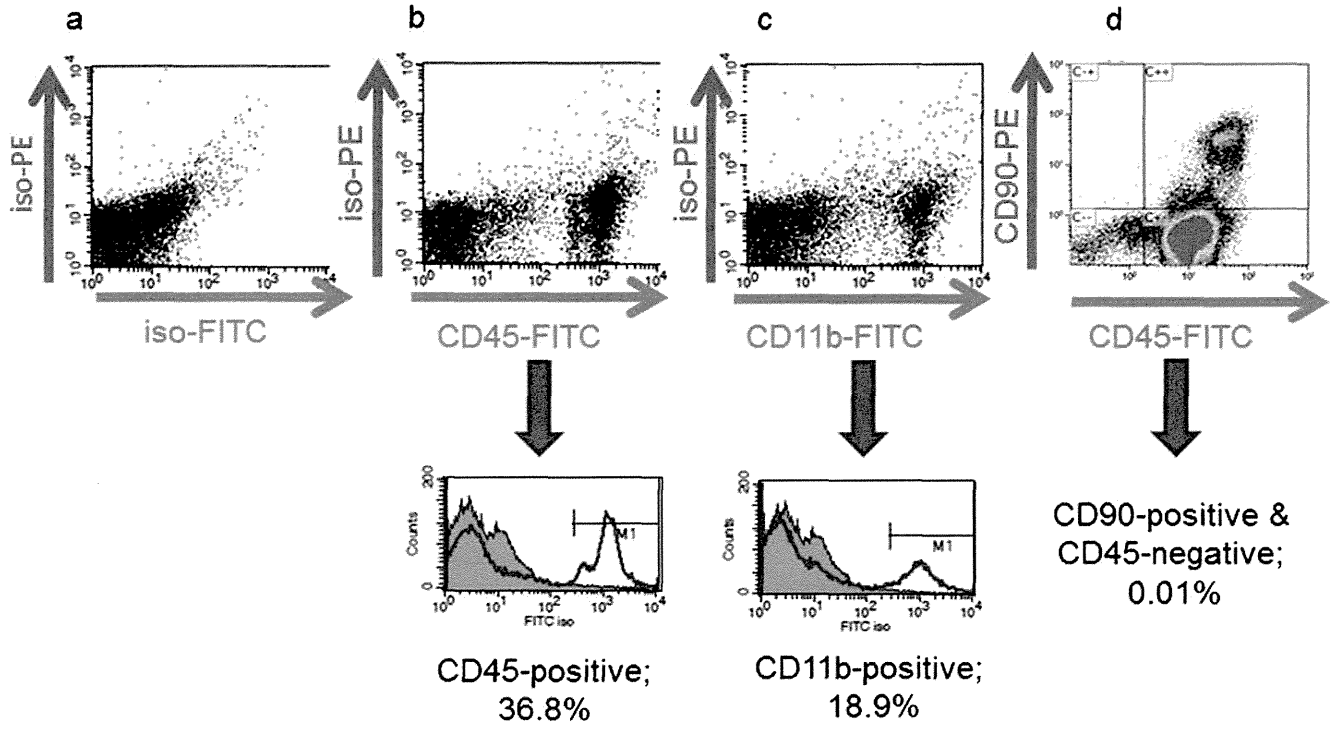

Fig. 1 Cellular characterization of uncultured bone marrow cells (BMCs) by flow cytometric analysis (*FITC* fluorescein isothiocyanate, *PE* phycoreythrin, *iso* isotype). Representative flow cytometric analyses for CD11b, CD45 and CD90 antigens in BMCs. **a** Control. **b** Expression of CD45. **c** Expression of CD11b. **d** Expression of CD45 and CD90

assay (Agata et al. 2009). At 24 h after seeding, floating cells were removed and the medium was replaced with a fresh medium. Thereafter, the medium was replaced every other day. After incubation for 14 days, cultured bone-marrow-derived cells that had attached to the dish were removed by using trypsin-EDTA (Life Technologies). Detached cells were collected in 15-ml centrifuge tubes and were then washed with cold 3% FBS (Life Technologies) in PBS. After centrifugation at 300*g* for 3 min, the cell pellet was recovered. Following further centrifugation, the supernatant was removed and the cells were resuspended to give a suspension of 1.0×10^5 GFP-

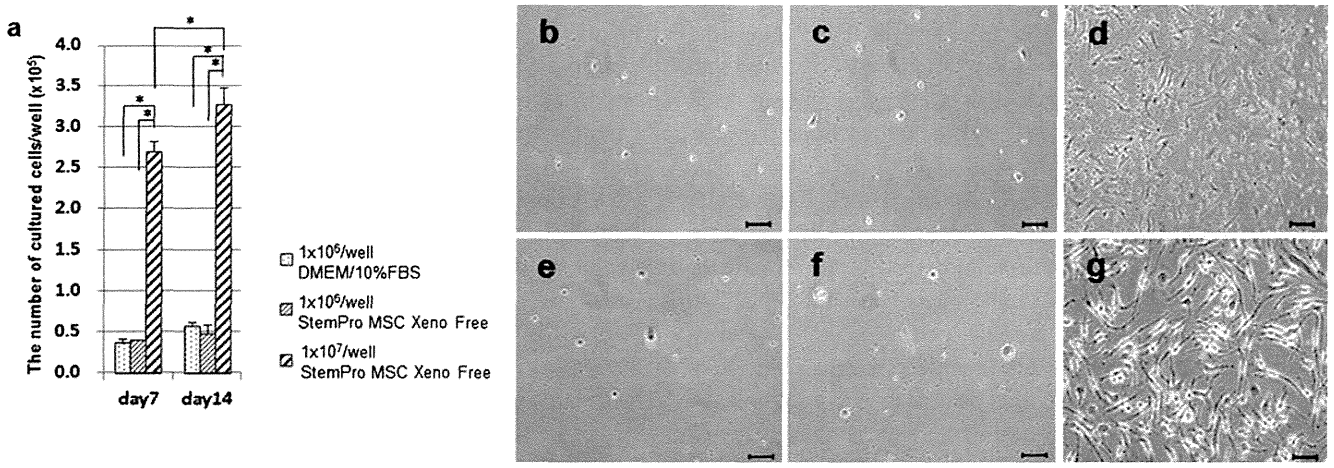

Fig. 2 Proliferation of green fluorescent protein (GFP)-transgenic murine bone-marrow-derived cells. **a** Number of cultured bone-marrow-derived cells (*error bars* SD). *$P<0.001$, $n=6$ each. **b–g** Morphology of cultured cells. Magnification ×100. **b–d** Cells at day 7. **e–g** Cells at day 14. **b**, **e** Cells seeded at 1.0×10^6/well (9.6 cm^2) on a non-coated dish with Dulbecco's modified Eagle's medium (*DMEM*) with 10% fetal bovine serum (*FBS*). **c**, **f** Cells seeded at 1.0×10^6/well on a CELL-start-coated dish with StemPro MSC Xeno Free medium with 2 mM L-glutamine and StemPro LipoMax supplement. **d**, **g** Cells seeded at 1.0×10^7/well on a CELL-start-coated dish in StemPro MSC Xeno Free medium with 2 mM L-glutamine and StemPro LipoMax supplement. *Bars* 100 μm (**b–g**)

positive cultured BMC per milliliter. Cellular characterization was performed by flow cytometric analysis.

Cellular characterization by flow cytometric analysis

For flow cytometric analysis, we isolated BMCs from male C57 BL/6 mice without expressing GFP (aged 6 weeks). We analyzed both uncultured and cultured BMCs. After incubation for 14 days, cultured bone-marrow-derived cells attached to the dish were trypsinized by using trypsin-EDTA (Life Technologies) and washed with cold PBS. After centrifugation at 300*g* for 3 min, the cell pellet was resuspended in cold PBS. Cells were incubated with mouse FcR Blocking Reagent (Miltenyi Biotec., Tokyo, Japan) for 20 min on ice to reduce nonspecific binding. Then, cells were labeled with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD45, phycoreythrin (PE)-rat anti-mouse CD11b, or allophycocyanin (APC)-rat anti-mouse CD90 (Beckman Coulter). For the isotype control, cells were labeled with FITC-rat IgG isotype control, PE-rat IgG isotype control, or APC-rat IgG isotype control (Beckman Coulter). Dead cells were stained with propidium iodide (PI). After 20 min of incubation, cells were washed with cold PBS and measured by a Gallios flowcytometer (Beckman Coulter). Data were analyzed with Flowjo software (Tree Star, Ashland, Ore., USA).

Experimental protocol

In 6-week-old female C57 BL/6 mice, CCl_4 at a concentration 1.0 ml/kg was repeatedly administered into the peritoneal cavity, twice weekly, for 4 weeks, in order to induce chronic liver injury. At 4 weeks after starting CCl_4 treatment, cultured cells (1×10^4 cells/animal) were administered, via the caudal vein, into the treated animals (cultured BMCs + CCl_4: group C; n=7). CCl_4 was continuously administrated for 4 more weeks, after which the mice were killed and blood and tissue samples were collected. As control groups, CCl_4-treated mice were administered, via the caudal vein, uncultured BMCs (1×10^5/animal) from C57 BL/6 GFP-transgenic mice (bone marrow infusion + CCl_4: group B; n=12) or PBS (CCl_4: group A; n=11).

Immunohistochemistry and double-immunofluorescence examination

In all experiments, 5-μm-thick sections were prepared from the right lobe of 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, the Vectastain ABC kit (Vector Laboratories, Burlingame, Calif., USA) with anti-α-smooth muscle actin (α-SMA) antibody (an indicator of stellate cell activation; Dako, Glostrup, Denmark), anti-matrix metalloproteinase-9 (MMP-9) antibody (a collagenase to improve liver fibrosis; Santa Cruz Biotechnology, Santa Cruz, Calif., USA), anti-GFP antibody (Santa Cruz Biotechnology) and anti F4/80 antibody (a marker of macrophages; Abcam, Cambridge, Mass., USA) were used according to the avidin-biotin-peroxidase complex method, as described previously (Jin et al. 2007). To estimate the Sirius-red- or MMP-9-positive areas, a computerized image analysis system utilizing MetaMorph software (Universal Imaging, Downingtown, Pa., USA) was used at ×40 magnification. Seven randomly selected areas per specimen were analyzed. Positive areas were expressed as a percentage of the total area of the specimen.

For fluorescence immunohistochemistry, tissues were incubated with Alexa Fluor R 488 and 568 donkey anti-goat IgG (H+L) conjugate, Alexa Fluor R488 goat anti-rabbit IgG (H+L) conjugate and Alexa Fluor R 568 goat anti-rat IgG (H+L) conjugate (Molecular Probes, Eugene, Ore., USA) as secondary antibodies. Positive cells in the liver were quantified by using a Provis microscope (Olympus, Tokyo, Japan) equipped with a charge-coupled device camera, followed by computer-assisted image analysis with MetaMorph software (Universal Imaging).

Serum albumin level analysis

Serum samples were obtained from the abdominal aorta. Serum albumin levels after 4 weeks of cell infusion were analyzed by using the SPOTCHEM EZ SP-4430 dry chemical system (Arkray, Kyoto, Japan).

Statistical analysis

Values are shown as means ± SD. Data were analyzed by analysis of variance with Fisher's projected least significant difference test. A P-value of less than 0.05 was considered statistically significant.

Results

Flow cytometric analysis of BMC and cultured BMC

As shown in Fig. 1, CD45- and CD11b-positive cells were included in the uncultured whole BMCs, showing that a macrophage fraction existed in uncultured BMCs. However, CD45-negative and CD90-positive cells indicating a mesenchymal fraction in whole BMCs were rare. The proliferation rate and character of BMCs cultured by StemPro MSC Xeno Free medium (Life Technologies) are shown in Fig. 2. Flow cytometric analysis demonstrated that the cultured BMCs from male C57 BL/6 mice were strongly

positive for CD11b and negative for CD90 (Fig. 3b, d). Only 0.047% of cultured cells were CD-90-positive and CD-45-negative, consistent with MSC characteristics (Fig. 3d). Flow cytometric analysis showed that mainly macrophage fractions were increased in the cultured system; these cells were subsequently used in our study.

Improvement in serum albumin after BMC and cultured BMC infusion

Serum albumin was lower in the CCl_4 injury group (group A) but was significantly higher in the BMC-treatment group (group B) and the cultured cell-treatment group (group C). No significant differences were apparent between groups B and C (Fig. 4). These results showed that the improvement of liver function was similar between primary BMC and 1/10 the amount of cultured BMC. Except for the serum albumin levels, no significant differences were observed in other blood data (e.g., alanine aminotransferase, total bilirubin; data not shown) between the three groups.

Improvement in liver fibrosis after BMC and cultured BMC infusion

Liver fibrosis was evaluated by Sirius-red staining. As compared with the CCl_4 injury group (group A), the fibrosis area was smaller in the BMC-treatment group and cultured-cell-treatment group (Fig. 5a-d). In groups B and C, GFP-positive cells were mainly observed in the portal region (Fig. 5e-h). In addition, MMP-9 expression was significantly elevated in groups B and C (Fig. 5i-l). Immunohistochemical analysis showed a few F4/80-positive cells around the liver in groups A and B (Fig. 6a, b). On the other hand, in group C, many F4/80-positive cells were mainly observed in the portal region (Fig. 6c). Double-immunostaining revealed that a few F4/80/

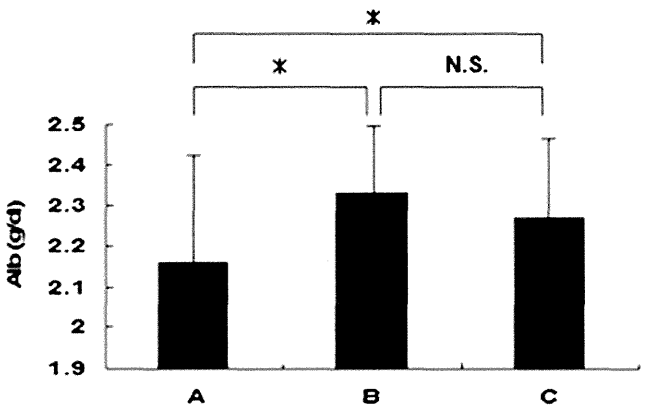

Fig. 4 Serum albumin (*Alb*). After 8 weeks of treatment with CCl_4 (4 weeks after BMC/cultured cell administration), mouse blood was collected and analyzed. CCl_4 group (*A*), CCl_4 + BMC group (*B*), and CCl_4 + cultured cell group (*C*). *$P<0.05$ (*N.S.* not significant)

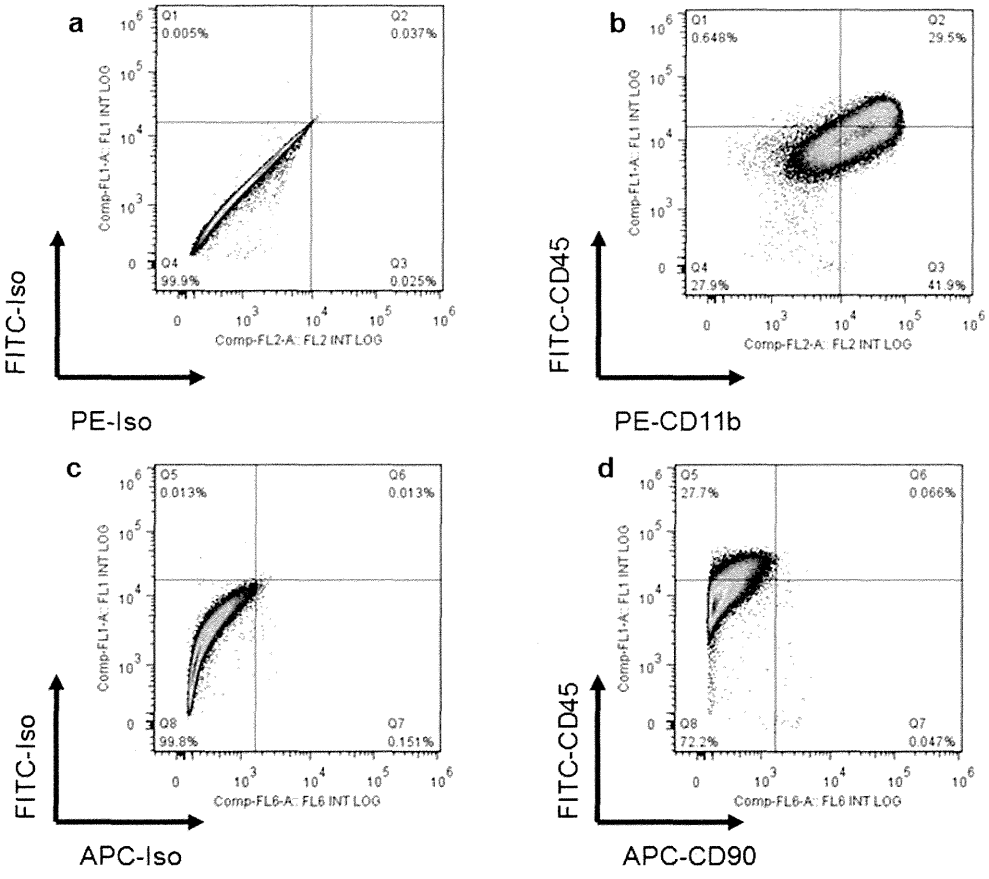

Fig. 3 Cellular characterization of cultured BMCs by flow cytometric analysis. Representative flow cytometric analyses for CD11b, CD45 and CD90 antigens in bone-marrow-derived cultured cells. **a** Isotype (*Iso*) control for phycoreythrin (*PE*)- and fluorescein isothiocyanate (*FITC*)-conjugated antibodies. **b** Expression of CD11b (*x-axis*) and CD45 (*y-axis*). **c** Isotype control for allophycocyanin (*APC*)- and fluorescein isothiocyanate (*FITC*)-conjugated antibodies. **d** Expression of CD90 (*x-axis*) and CD45 (*y-axis*)