tetrachloride (CCl_4) and then green fluorescence protein (GFP)-labeled BMC from GFP-transgenic mice were transplanted *via* the tail vein. Transplanted GFP-positive cells appeared in the peri-portal area of hepatic lobules, where they trans-differentiated into a hepatoblast phenotype followed by hepatocytes producing albumin. We named this model "GFP/CCl_4 model" [6, 7]. Analysis of this model has shown that BMC transplantation improves liver function, liver fibrosis, and survival rate [6, 8]. We also examined the molecular signatures that arise after BMC transplantation in the GFP/CCl_4 model using a cDNA microarray. Fibroblast growth factor (FGF), HOX type transcription factor and helix-loop-helix (HLH) transcription factors might be important for regulating the early trans-differentiation of BMC into hepatocytes in the GFP/CCl_4 model. The expression of hepatocyte nuclear factor (HNF) 4 and glucose-6-phosphate isomerase becomes elevated later [9]. These processes will be useful to understand the molecular mechanism of BMC trans-differentiation into hepatocytes, but they are limited to monitoring liver regeneration after BMC transplantation. Under clinical conditions, serum marker proteins will be useful to monitor the condition of recipients after BMC transplantation because blood sampling is convenient and simple. The present study performed proteomic analyses of sera from recipient mice with liver cirrhosis after BMC transplantation.

2 Materials and methods

2.1 GFP/CCl_4 model

C57BL6/Tg14 (act-EGFP) OsbY01 mice (GFP-Tg mice) expressing GFP in various tissues and cells were provided by Masaru Okabe (Genome Research Center, Osaka University, Osaka, Japan) [10]. Female C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). All procedures including surgical steps proceeded in accordance with Yamaguchi University guidelines for experiments involving animals and recombinant DNA. GFP-Tg mice were sacrificed by cervical dislocation and the limbs were removed. GFP-positive BMC were flushed from the medullary cavities of the tibias and femurs using a 25G needle with PBS culture solution. Six-week-old C57BL/6 female mice were injected under anesthesia with 1 mL/kg CCl_4 into the peritoneum twice each week for 4 wk to establish liver cirrhosis. One day after the last injection, 1.0×10^5 GFP-positive BMC were injected *via* the tail vein [6].

2.2 Serum sample preparation

We separated the mice with liver cirrhosis into one group that underwent BMC transplantation (BMC group, $n = 6$) and a control group that was not transplanted (control group, $n = 6$). We sacrificed both groups and collected blood samples at 48 h after BMC transplantation. The supernatants were collected by centrifugation at 3000 rpm for 15 min. Albumin or IgG was removed from the supernatants using the aurum serum protein mini kit (BioRad Laboratories, Hercules, CA, USA), and the supernatants were concentrated by centrifugation at 5000*g* using Ultrafree-MC PLCC centrifugal filter unit 5 kDa membranes (Millipore, Billerica, MA, USA) to 25% of the original volume. The concentrated supernatants (protein samples) were stored at −80°C. We independently made another model with which to observe the time course of changes in levels of serum proteins before, 48 h, 1 and 4 wk after BMC transplantation (BMC group, $n = 3$; control group, $n = 3$).

2.3 2-DE

Serum samples (50 μg) were applied to immobiline dry strips (pH 3–10, 7 cm; Amersham Pharmacia Biotechnology, Uppsala, Sweden) in a total volume of 125 μL containing 8 M urea, 2% CHAPS, 0.5% IPG buffer (Amersham Pharmacia Biotechnology) and 0.56% 2-mercaptoethanol (2ME). After rehydration for 14 h, proteins were separated by isoelectrofocusing (IEF) at 20°C and at 50 μA/strip with the following linear voltage increases: 500 V for 1 h, 1000 V for 1 h, and 8000 V for 2 h. The strips were then equilibrated twice in 50 mM Tris containing 6 M urea, 30% glycerol and 2% SDS for 10 min and then 2ME was added, followed by iodoacetamide. The second dimension proceeded on 12.5% SDS-polyacrylamide gels (SDS-PAGE; 24.5 cm × 11 cm) at 600 V and 20 mA for 30 min followed by 600 V and 50 mA for 70 min in a multiphor horizontal electrophoresis unit (Amersham Pharmacia Biotechnology) as described [11, 12]. Six independent experiments confirmed the experimental reproducibility. The second-dimensional separation of paired samples proceeded simultaneously on the same gel.

2.4 Silver staining

Separated protein spots were fixed with 40% ethanol and 10% acetic acid for 30 min and then visualized using the silver staining kit, protein (Amersham Pharmacia Biotechnology). The proteins were sensitized with 30% ethanol, 6.8% sodium acetate, and 0.2% sodium thiosulfate containing glutaraldehyde as an enhancer of silver stain for 30 min, and then washed three times with distilled water. Proteins were immersed in 0.25% silver nitrate containing 0.0148% formaldehyde for 20 min, and washed twice with distilled water. After development to the desired intensity with 2.5% sodium carbonate and 0.0074% formaldehyde, the reaction was terminated in 1.46% EDTA–$Na_2 \cdot 2H_2O$.

2.5 Image analysis

The positions of protein spots in samples from transplanted and control groups were recorded using an Agfa ARCUS 1200™ image scanner (Agfa-Gevaert N.V., Mortsel, Belgium) and analyzed using GELLAB II+ software version 2.01 (Scanalytics, Fairfax, VA, USA).

2.6 Protein identification by MS

Spots with increased intensity in the BMC group were excised from the gels and identified using an oMALDI-Qq-TOF MS/MS QSTAR® Pulsar *i* (Applied Biosystems, Foster City, CA, USA) by Hitachi Science Systems (Hitachinaka, Japan) [13, 14]. Proteins were first identified by PMF using MALDI-TOF MS, and by MS/MS when necessary.

2.7 Liver sample preparation

Liver samples from the BMC and control groups were homogenized in 20 mM Tris-HCl (pH 7.5) containing 50 mM NaCl, 1 mM EDTA, 1% triton X, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, and 1 mM PMSF (lysis buffer). The homogenate was separated by centrifugation at 10 000 rpm for 15 min to yield a supernatant that was stored at −80°C.

2.8 Immunoblot analysis

Liver and serum samples (10 μg each) were resolved by SDS-PAGE at 15 mA/gel and then fractionated proteins were electrophoretically transferred onto PVDF membranes (Immobilon; Millipore Corporation, Bedford, MA, USA) and blocked overnight at 4°C with TBS containing 5% skim milk. The membrane was then washed and immersed in goat anti-apolipoprotein A1 antibody (1:4000) (ROCKLAND, Philadelphia, PA, USA) for 1 h. After four washes with TBS containing 0.05% Tween-20, the membrane was incubated for 1 h with horseradish peroxidase-conjugated secondary antibody (1:2000) and visualized using the chemiluminescent ECL reagent (Amersham Pharmacia Biotechnology). The proteins transferred to PVDF membranes were stained with 0.1% CBB R-250 overnight to check the quantity of loading control. The membranes were destained with 30% methanol and 10% acetic acid for 30 min, and then with 7% acetic acid for 12 h.

2.9 Immunohistochemistry

Paraffin-embedded blocks of liver samples from both groups and 3 μm sections were examined by standard immunohistochemistry using the avidin-biotin-peroxidase complex (ABC) [15]. Sections that were de-waxed in xylene and dehydrated in alcohol were heated in a microwave oven for 6 min to activate antigens and then endogenous peroxidase activity was blocked by immersing the sections for 30 min in 0.3% hydrogen peroxidase in methanol. The sections were washed with distilled water, incubated in PBS containing rabbit serum for 20 min to block nonspecific binding and then incubated with goat anti-apolipoprotein A1 antibody (1:50) (ROCKLAND) and goat anti-proliferating cell nuclear antigen (anti-PCNA) antibody (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C. The sections were incubated with biotin-conjugated secondary antibody in PBS for 150 min at room temperature, and reacted with ABC for 30 min. Positive reactions were developed for about 3 min using PBS containing hydrogen peroxidase and 3,3'-diaminobenzidine (DAB).

3 Results

3.1 Serum proteomics of GFP/CCl_4 model mice

We performed a development of silver staining for a relatively long time to analyze a small amount of proteins in the sample. Silver staining revealed that 2-DE separated about 700 serum protein spots and screening showed that six of them were increased in the BMC groups compared with the controls by focusing on the area under the molecular weight of 48 kDa where the separation of protein spots was fine (Fig. 1). MS of these spots identified five proteins as apoA1, apoC3, vitamin D-binding protein (DBP), alpha-1-antitrypsin (AT), and proteasome subunit alpha type 1 (Fig. 2). ApoA1 was identified in two spots since the No. 5 spot in Fig. 2 was a fragment of apoA1. We also analyzed the spot intensity of six paired samples (BMC/Control group) and found that AT, apoA1 and apoC3 were increased in all of them. Table 1 shows the mean ratios of spot intensity in both groups. The ratios of apoA1, DBP, and proteasome subunit alpha type 1 were significantly higher in the BMC than in the control group ($p < 0.05$).

3.2 ApoA1 protein levels are elevated at 48 h and 1 wk in serum and liver of recipient cirrhotic mice after BMC transplantation

We confirmed the results of the proteomic analysis by immunoblotting and analyzed apoA1 expression using an anti-apoA1 antibody. We could not analyze the other proteins because adequate antibodies were not available to us. Immunoblotting showed that the levels of serum apoA1 were increased in the BMC group compared with the control group at 48 h after BMC transplantation (Fig. 3A). Both immunoblotting and immunohistochemistry showed that apoA1-expression in the liver was also increased at 48 h after BMC transplantation (Fig. 3B, C-1 and C-2). In the same time, PCNA-positive cells were increased in the liver after BMC transplantation (Fig. 3D-2) compared with control group (Fig. 3D-1). Figure 4 shows changes in serum and liver apoA1 expression after BMC transplantation compared with controls. Serum and liver apoA1 levels were elevated after compared with before BMC transplantation and remained elevated for 1 wk thereafter.

4 Discussion

To identify serum marker proteins of recipient GFP/CCl_4 model mice with liver cirrhosis after BMC transplantation is important. We initially analyzed the expression profiles of

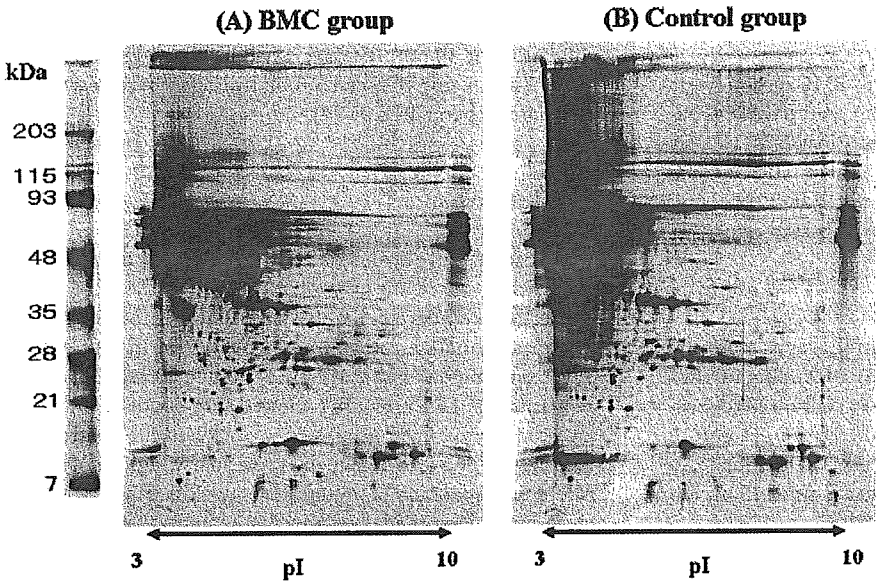


Figure 1. Profiles of serum proteins from GFP/CCl_4 mice resolved by 2-DE. Serum proteins were separated by IEF (pH 3–10) and SDS-PAGE. About 700 spots each were visualized by silver staining gels from both (A) BMC and (B) control groups.

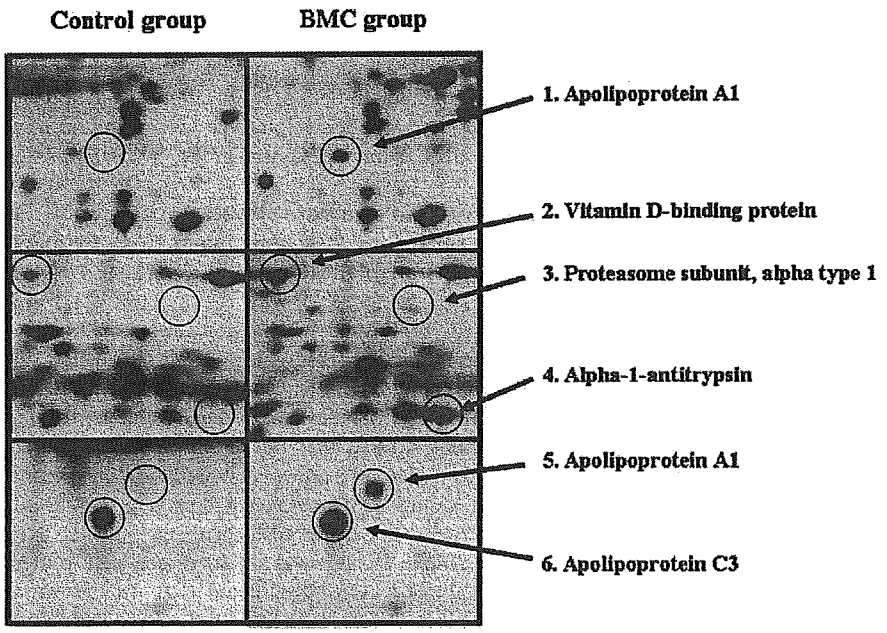


Figure 2. Protein spots increased in BMC group. Intensity of six spots increased in BMC compared with control group. MS identified apolipoprotein A1, vitamin D binding protein, proteasome subunits alpha type 1, alpha-1-antitrypsin and apolipoprotein C3. Spots are numbered from 1 to 6.

serum proteins in GFP/CCl_4 model mice at 48 h after BMC transplantation using 2-DE. MS revealed that serum levels of apoA1, apoC3, DBP, AT, and proteasome subunit alpha type 1 were increased in the BMC group compared with the control group (Fig. 2 and Table 1). We then confirmed the expression of apoA1 using immunoblotting and immunohistochemical analysis. We also investigated changes in apoA1 expression before, 48 h, 1 and 4 wk after BMC transplantation and found that serum levels were increased at 48 h and 1 wk after BMC transplantation. Figures 3B and C-2, 4B show that liver apoA1 expression also increased at the early stage (from 48 h to 1 wk) after BMC transplantation. Our previous analysis of the GFP/CCl_4 model using a DNA chip revealed that FGF, HOX, and HLH transcription factors are up-regulated in the liver of recipient mice at the early stage after BMC transplantation [9]. In acute liver injury generated by a single administration of CCl_4, the mRNA expression of apoA1 increases during liver regeneration in rat models [16]. The early elevation of apoA1 expression in sera at 48 h and 1 wk after BMC transplantation might be related to an early regenerative effect in recipient mice after BMC transplantation. As shown in Fig. 3, apoA1 expression

Table 1. Protein spots that increased in BMC group

Spot no.[a]	Protein	Accession no.	Number of pairs[b]	Ratio, BMC/control[c]
1	Apolipoprotein A-1	gi|109571	5/6	2.76 ± 0.84
2	Vitamin D-binding protein	gi|139642	5/6	1.89 ± 0.30*
3	Proteasome subunit, alpha type 1	gi|33563282	5/6	3.88 ± 1.09*
4	Alpha-1-antitrypsin	gi|192094	6/6	9.09 ± 4.54
5	Apolipoprotein A-1	gi|109571	6/6	2.81 ± 0.66*
6	Apolipoprotein C-3	gi|15421856	6/6	2.53 ± 0.75

a) These numbers correspond with the numbers in Fig. 2.
b) These numbers indicate the number of pairs in which protein expression increased more in BMC group than in control group among six pairs.
c) This shows the ratio of the spot intensity in BMC group to that in control group. (mean ± SE)
* $p < 0.05$ (*t*-test).

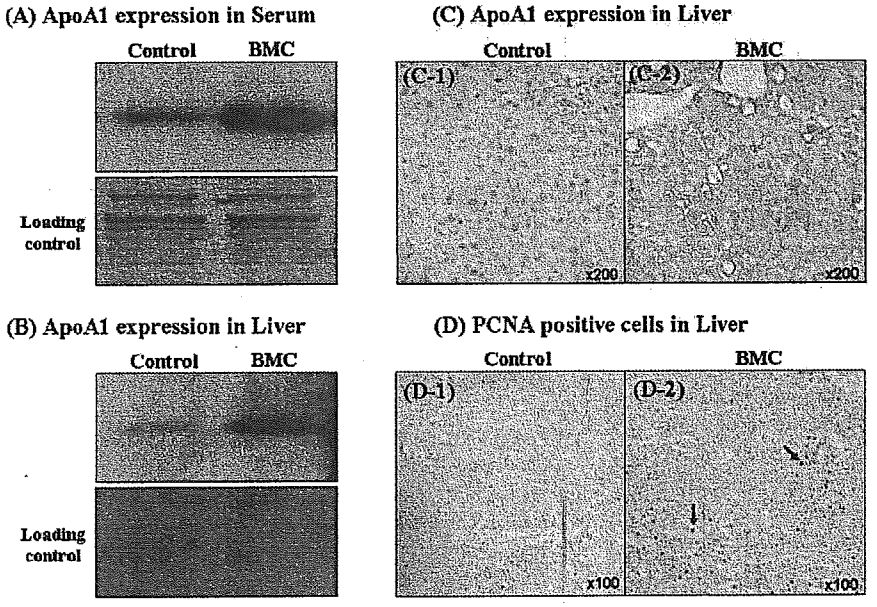


Figure 3. Apolipoprotein A1 expression in serum and liver after BMC transplantation. Immunoblot analysis of apoA1 expression in (A) serum and (B) liver at 48 h after BMC transplantation. CBB staining was performed on the PVDF membranes as loading controls. BMC: bone marrow cells transplantation group, control: control group (no transplanted group). (C) Immunohistochemistry of anti-apolipoprotein A1 antibodies in liver section of (C-1) control and (C-2) BMC groups. Apolipoprotein A1 is expressed in hepatocytes. ApoA1 expression increased in BMC than in control group (x 200). (D) Immunohistochemistry of anti-PCNA antibodies in liver section of (D-1) control and (D-1) at 48 h after BMC groups. PCNA-positive cells were increased at liver after BMC transplantation. Arrows indicate positive cells (x 100).

was increased in both serum and liver in recipient mice. PCNA is a maker of cell proliferation. Figure 3D-2 revealed that BMC transplantation will promote cell proliferation in the liver of recipient mice. These results might show that apoA1 expression was related with liver regeneration of cirrhotic liver by BMC transplantation. ApoA1 is a main component of high-density lipoprotein (HDL) that is involved in the transport and metabolism of cholesterol [17]. Hepatocyte growth factor (HGF) facilitates liver regeneration in rats with cirrhosis after partial hepatectomy and stimulates hepatocyte lipid synthesis and lipoprotein secretion [18, 19]. Stery *et al.* [20] demonstrated using 2-DE and MS that levels of proteins involved in lipid metabolism such as adipose differentiation related protein and enoyl coenzyme A hydratase 1 are up-

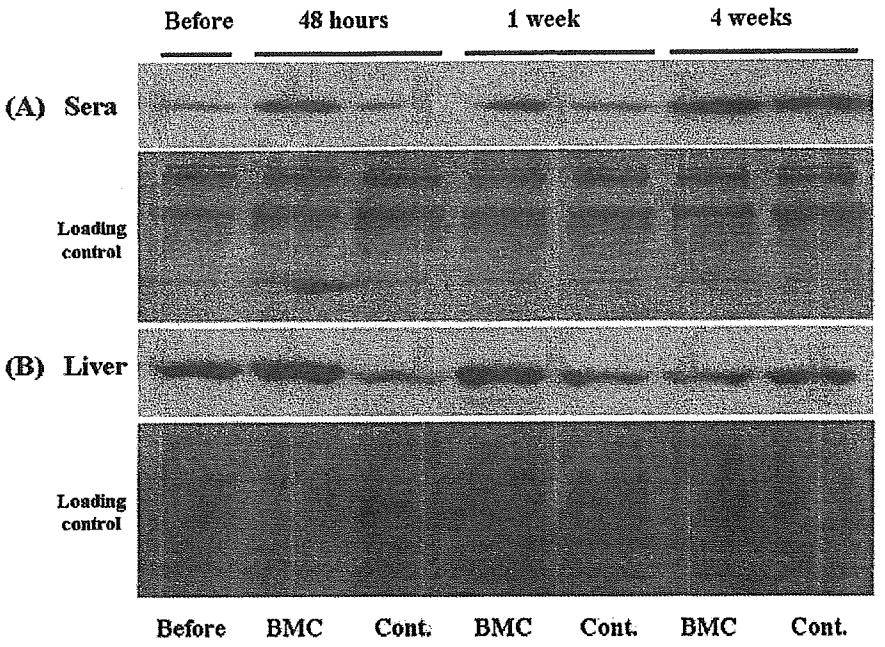


Figure 4. Time course of serum and liver apoA1 expression in recipient cirrhotic mice after BMC transplantation. Immunoblots of samples before, 48 h, 1 and 4 wk after BMC transplantation. ApoA1 expression in (A) serum and (B) liver after BMC transplantation. Serum and liver apoA1 levels are elevated at 48 h and 1 wk after BMC transplantation, but do not differ from controls at 4 wk. Loading controls were revealed by CBB staining. BMC: BMC group, cont.: control group.

regulated in the liver at 6 and 12 h after partial hepatectomy. These results showed that lipid metabolism plays an important role during the early stage of liver regeneration. The present study found that both serum and liver apoA1 levels were increased in transplanted mice with liver cirrhosis. These results indicated that lipid metabolism is activated soon after BMC transplantation. However, the precise mechanism of BMC trans-differentiation into hepatocytes in the GFP/CCl_4 model is still obscure. Our serum proteomic analysis found that lipid metabolism, especially apoA1 induction, became activated in recipient cirrhotic mice after BMC transplantation. This information will be useful for monitoring clinical BMC transplant recipients because we can analyze apoA1 expression of sera easily in clinical recipients. Proteomic analyses of serum samples from GFP/CCl_4 model mice revealed that apoA1, apoC3, DBP, AT, and proteasome subunit alpha type 1 are elevated at 48 h after BMC transplantation in cirrhotic mice. Next analysis with immunoblotting and immunohistochemical analyses found that apoA1 was increased from 48 h to 1 wk after BMC transplantation. These results suggest that apoA1 will serve as a useful marker of BMC trans-differentiation into hepatocytes. Now we started the research project of cell therapy using autologous BMC to repair liver cirrhosis patients. In this research, the finding of this proteomics analysis will be useful to monitor the liver regeneration after BMC transplantation.

We thank Dr. Masaru Okabe (Genome Research Center, Osaka University) for providing the GFP transgenic mice. This study was supported by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No. 13470121, 13770262, 15790348, 16390211, and 16590597) and for translational research from the Ministry of Health, Labor and Welfare (H-trans-5, H17-Special-015).

5 References

[1] Petersen, B. E., Bowen, W. C., Patrene, K. D., Mars, W. M. *et al.*, *Science* 1999, *284*, 1168–1170.

[2] Theise, N. D., Nimmakayalu, M., Gardner, R., Illei, P. B. *et al.*, *Hepatology* 2000, *32*, 11–16.

[3] Alison, M. R., Poulsom, R., Jeffery, R., Dhillon, A. P. *et al.*, *Nature* 2000, *406*, 257.

[4] Krause, D. S., Theise, N. D., Collector, M. I., Henegariu, O. *et al.*, *Cell* 2001, *105*, 369–377.

[5] Jang, Y. Y., Collector, M. I., Baylin, S. B., Diehl, A. M., Sharkis, S., *J. Nat. Cell Biol.* 2004, *6*, 532–539.

[6] Terai, S., Sakaida, I., Yamamoto, N., Omori, K. *et al.*, *J. Biochem. (Tokyo)* 2003, *134*, 551–558.

[7] Terai, S., Sakaida, I., Nishina, H., Okita, K., *Hepatobiliary Pancreat. Surg.* 2005, *12*, 203–207.

[8] Sakaida, I., Terai, S., Yamamoto, N., Aoyama, K. *et al.*, *Hepatology* 2004, *40*, 1304–1311.

[9] Omori, K., Terai, S., Ishikawa T., Aoyama, K. *et al.*, *FEBS Lett.* 2004, *578*, 10–20.

[10] Okabe, M., Ikawa, M., Kominami, K., Nakanishi, T., Nishimune, Y., *FEBS Lett.* 1997, *407*, 313–319.

[11] Yokoyama, Y., Kuramitsu, Y., Takashima, M., Iizuka, N. *et al.*, *Proteomics* 2004, *4*, 2111–2116.

[12] Takashima, M., Kuramitsu, Y., Yokoyama, Y., Iizuka, N. *et al.*, *Proteomics* 2003, *3*, 2487–2493.

[13] Shevchenko, A., Wilm, M., Vorm, O., Mann, M., *Anal. Chem.* 1996, *68*, 850–858.

[14] Yanagida, M., Miura, Y., Yagasaki, K., Taoka, M. *et al.*, *Electrophoresis* 2000, *21*, 1890–1898.

[15] Kitoh, T., Yanai, H., Saitoh, Y., Nakamura, Y. *et al.*, *J. Gastroenterol.* 2004, *39*, 434–440.

[16] Pandura, A., Castrillon, L., Gonzalez, L., Shafritz, D. A., *Biochim. Biophys. Acta* 1993, *1167*, 37–42.

[17] Gotto, A. M., Jr., Pownall, H. J., Havel, R., *J. Methods Enzymol.* 1986, *128*, 3–41.

[18] Kaibori, M., Kwon, A. H., Nakagawa, M., Wei, T. *et al.*, *J. Hepatol.* 1997, *27*, 381–390.

[19] Kaibori, M., Kwon, A. H., Oda, M., Kamiyama, Y. *et al.*, *Hepatology* 1998, *27*, 1354–1361.

[20] Stery, C. W., Winters, M. S., Markiewski, M. M., Lambris, J. D., *Proteomics* 2005, *5*, 318–325.

Med Mol Morphol (2005) 38:197–202
DOI 10.1007/s00795-005-0298-z

SPECIAL REVIEW SERIES: New ultrastructural discoveries in the pathophysiology of liver diseases

Isao Sakaida · Shuji Terai · Hiroshi Nishina
Kiwamu Okita

Development of cell therapy using autologous bone marrow cells for liver cirrhosis

Received: April 13, 2005 / Accepted: May 9, 2005

Abstract The plasticity of bone marrow has been confirmed by the autopsy of a female recipient of bone marrow cell transplantation from a male donor. To establish new clinical cell therapies using autologous bone marrow cells for patients with liver failure, we developed a new in vivo model named the green fluorescent protein (GFP)/carbon tetrachloride (CCl_4) model. Using the GFP/CCl_4 model, we found that transplanted Liv8-negative cells efficiently repopulated into cirrhotic liver tissue and differentiated into albumin-producing hepatocytes under persistent liver damage induced by carbon tetrachloride. Moreover, bone marrow cell transplantation into mice with liver cirrhosis improved liver function and liver fibrosis with the strong expression of matrix metalloproteinases (MMPs), especially MMP-9 activity, resulting in an improved survival rate. Results from the GFP/CCl_4 model showed that cell therapy using autologous bone marrow cells has the potential to become an effective treatment for patients with liver failure. A summary of findings from the GFP/CCl_4 model is described.

Key words Bone marrow cell (BMC) · Liver cirrhosis · GFP (green fluorescent protein) · Liver fibrosis · Stem cell · Liv8

Introduction

Currently, liver transplantation is one of the most effective therapies to cure patients with liver disease. However, transplantation has many problems, such as lack of donors, operative damage, rejection, and high costs. Cell transplantation therapy should be a minimally invasive procedure with fewer potential complications.

Regenerative medicine using stem cells is an attractive treatment for patients with severe liver disease. The capacity of bone marrow cells (BMCs) to differentiate into hepatocytes and intestinal cells was confirmed through the detection of the Y chromosome in an autopsy analysis of human female recipients of BMCs from male donors.[1–4]

We developed a new in vivo model named the green fluorescent protein (GFP)/carbon tetrachloride (CCl_4) model,[5,6] used to monitor the differentiation of BMCs into functional hepatocytes. In this article, the newest findings from the GFP/CCl_4 model are described.

Candidate cells for cell therapy

A somatic human stem cell that could be propagated in large quantities while retaining its ability to differentiate into different cell types could serve as a highly valuable resource for the development of cellular therapy in liver diseases.

If we limit the definition of stem cells to their ability to self renew and reconstitute a given tissue in vivo, hepatocytes fulfill both criteria. However, hepatocyte transplantation has very rarely produced therapeutic effects in human clinical trials, mainly because their numbers are too low to achieve a biological effect.[7,8] Under certain conditions, when hepatocyte replication is blocked, bipotent oval cells profilerate and participate in liver regeneration. However, the fact that they have been shown to generate hepatocellular carcinoma and cholangiocarcinoma cells in rodents is a concern for their use for cell therapy.

As a result, bone marrow cells are now being considered. The capacity of bone marrow cells (BMCs) to differentiate into hepatocytes was found using Y-chromosome detection in an autopsy analysis of human female recipients of BMCs from male donors as described previously.[1,2] BMC transplantation itself is an established treatment for hematological diseases. These results suggest that bone marrow is an

I. Sakaida (✉) · S. Terai · K. Okita
Department of Gastroenterology & Hepatology, Yamaguchi University School of Medicine, 1-1-1 Minami Kogushi, Ube 755-8505, Japan
Tel. +81-836-22-2239; Fax +81-836-22-2240
e-mail: sakaida@yamaguchi-u.ac.jp

H. Nishina
Department of Developmental and Regenerative Biology, Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan

attractive cell source for regenerative medicine, because obtaining BMCs is easier than other tissue-specific stem cells. In the field of cardiovascular diseases, clinical studies have been performed to evaluate the use of BMCs in regenerating the myocardium and vessels of limb ischemia.[9–12] Although various theories explain the existence of pluripotent stem cells in BMCs, the exact composition of stem cells among BMCs remains unclear. The following cell types are known to exist in bone marrow: hematopoietic stem cells (HSC),[13–14] side population cells (SP),[15] and mesenchymal stem cells (MSC).[16] Although past studies used the existing antibodies and techniques, there have been no studies based on the findings associated with natural liver development.

Hematopoietic stem cells (HSCs) have been shown to adopt the phenotype of the recipient cells with fusion.[17,18] This fusion event has been demonstrated to occur between resident hepatocytes and myelomonocytes,[19,20] and also in normal mice using the Cre-cox system.[21] However, using the same approach, Harris et al. recently demonstrated that epithelial cells can develop from bone marrow cells without cell fusion.[22] Also, recent publications have suggested that bone marrow-derived hepatocytes may originate from the mesenchymal compartment, rather than the hematopoietic compartment.[23,24]

The GFP/CCl_4 model

To investigate whether BMCs can be used to repair liver damage, the GFP/CCl_4 medel has been developed.[5,6] In this model (Fig. 1), 0.5ml/kg carbon tetrachloride (CCl_4) is administered twice weekly to C57BL/6 female mice to induce liver cirrhosis, and then green fluorescent protein (GFP)-positive BMCs obtained from GFP-Tg mice [C57BL6/Tg14 (act-EGFP) OsbY01 mice][25] are transplanted through the tail vein (donor and recipient mice are of the same strain). In this model, 1×10^5 GFP-positive BMCs were transplanted without culture. By analyzing the GFP-positive BMCs in the recipient mice, repopulation and differentiation of BMCs under continuous liver injury were evaluated. Immunostaining using anti-GFP antibodies[26] showed that GFP-positive BMCs migrated into the marginal area of the hepatic lobule starting 1 day after BMC transplantation, and with time, the distribution of GFP-positive BMCs expanded[5,6] while forming a hepatic cord toward the central vein. The use of Liv2, a hepatoblast-specific antibody,[27] also showed that BMCs first differentiate into Liv2-positive hepatoblasts and then differentiate into albumin-positive hepatocytes. Furthermore, the level of serum albumin significantly increases with time in recipient mice.

These findings suggest that the GFP/CCl_4 model can be used to elucidate the process of differentiation of BMC into hepatocytes. On the other hand, GFP-positive cells were not detected in the liver tissue of control mice (no damage) following BMC transplantation. Persistent liver damage induced by CCl_4 injection is important for producing a specific differentiation "niche" to activate the plasticity of BMCs and their subsequent differentiation into hepatocytes. Oval cells were thought to be one of the types of HSCs derived from the canal of Hering following severe liver damage.[28,29]

Based on the findings of Petersen et al. that, under some conditions, oval cells are derived from bone marrow cells,[30]

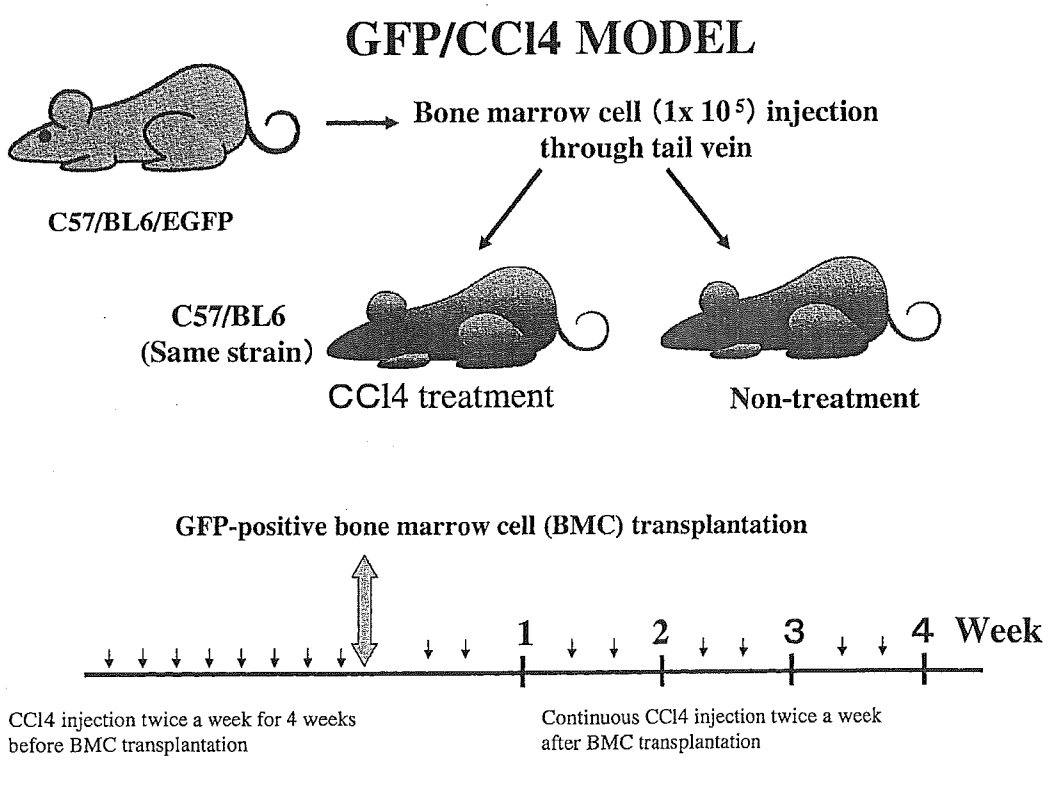


Fig. 1. Experimental protocol for the green fluorescent protein/carbon tetrachloride (GFP/CCl_4) model

we also analyzed the activation of oval cells using a specific oval cell marker, A6 antibody. A6-positive cells were detected at the periportal region 1 week after BMC transplantation in the GFP/CCl_4 model, but A6-positive oval cells did not increase in the 4 weeks after BMC transplantation in the GFP/CCl_4 model. We could not detect A6-positive cells that also express GFP in the liver after BMC transplantation. These results suggest that some signals that activate oval cells are induced by BMC transplantation into CCl_4-induced cirrhotic livers, but that oval cells might not be derived from transplanted BMCs. BMCs transplanted into the GFP/CCl_4 model differentiated into hepatoblast phenotypes, then differentiated into albumin-producing hepatocytes in the "differentiation niche" created by persistent CCl_4 injection.

Effect of BMC transplantation on liver fibrosis, liver function, and survival rate

Transplanted BMCs differentiated into albumin-producing hepatocytes, leading to an increase in the serum albumin level. Interestingly, an improvement in liver fibrosis after BMC transplantation was seen.[31,32] Although the exact mechanism of fibrolysis remains unclear,[33] transplanted BMCs migrate along with the fibers with the strong expression of matrix metalloproteinase (MMP)-9, resulting in the resolution of fibrosis (Figs. 2, 3). The degradation of the extracellular matrix presumably leads to improved liver function resulting in better survival in mice following BMC transplantation.

To clarify which fraction of BMCs is responsible for this improvement of liver function and resolution of liver fibrosis, the Liv8 antibody was developed.[34] Liv8 antibody detects hematopoietic cells. The mouse fetal liver at 11.5 functions as a definitive hematopoietic organ, and Liv8-positve cells of the fetal liver at E11.5 include C-*kit*-positive immature hematopoietic cells and CD-45-positive lymphoid cells. These results indicate that Liv8-positive BMCs include almost all immature and mature hematopoietic cells.

We also analyzed differences in liver fibrosis following transplantation of Liv8-positive or Liv8-negative BMCs. Our results showed that Liv8-negative BMC transplantation improved liver function (e.g., serum albumin level) and fibrosis more that Liv8-positive BMC transplantation. These results show that subpopulations of Liv-8 negative cells (nonhematopietic cells) will be useful for curing liver cirrhosis.

Our double-fluorescence data may also indicate that transplanted BMCs seem to become stellate cells, in agreement with a recent report,[35] although the number was very small in our experimental model. This result seems to be contradictory to our result for the resolution of liver fibrosis by BMC transplantation, because differentiated stellate cells may produce collagens.[36] Our preliminary results indicated a reduced mRNA expression of type I procollagen, transforming growth factor-beta (TGF-β1), and no change of hepatocyte growth factor (HGF) mRNA expression in the liver 1 week after BMC transplantation compared with the CC14-alone-treated liver. Migrated BMCs seemed to reduce the fine network pattern of activated stellate cells. Thus, transplanted BMCs may affect activated stellate cells to reduce their number; e.g., by leading them to apoptosis. However, further studies are necessary to determine the exact relationship between BMCs and resident stellate cells.

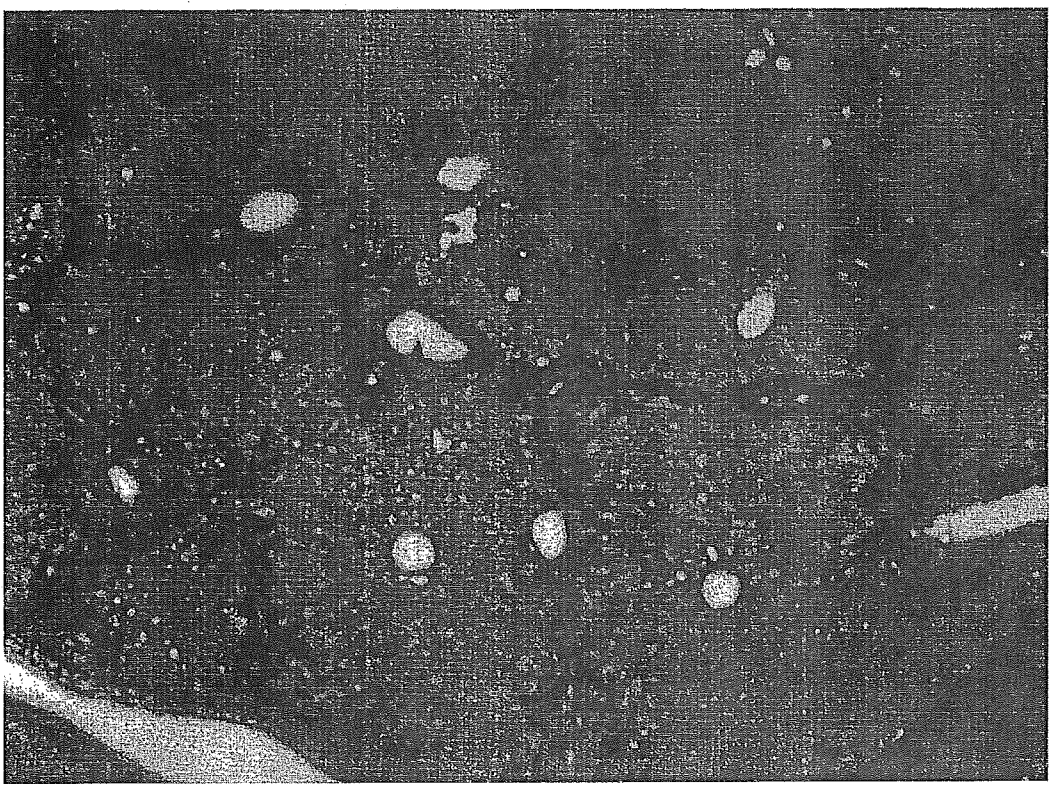


Fig. 2. GFP and Sirius red staining. Migrated bone marrow cells are seen along with the fibers

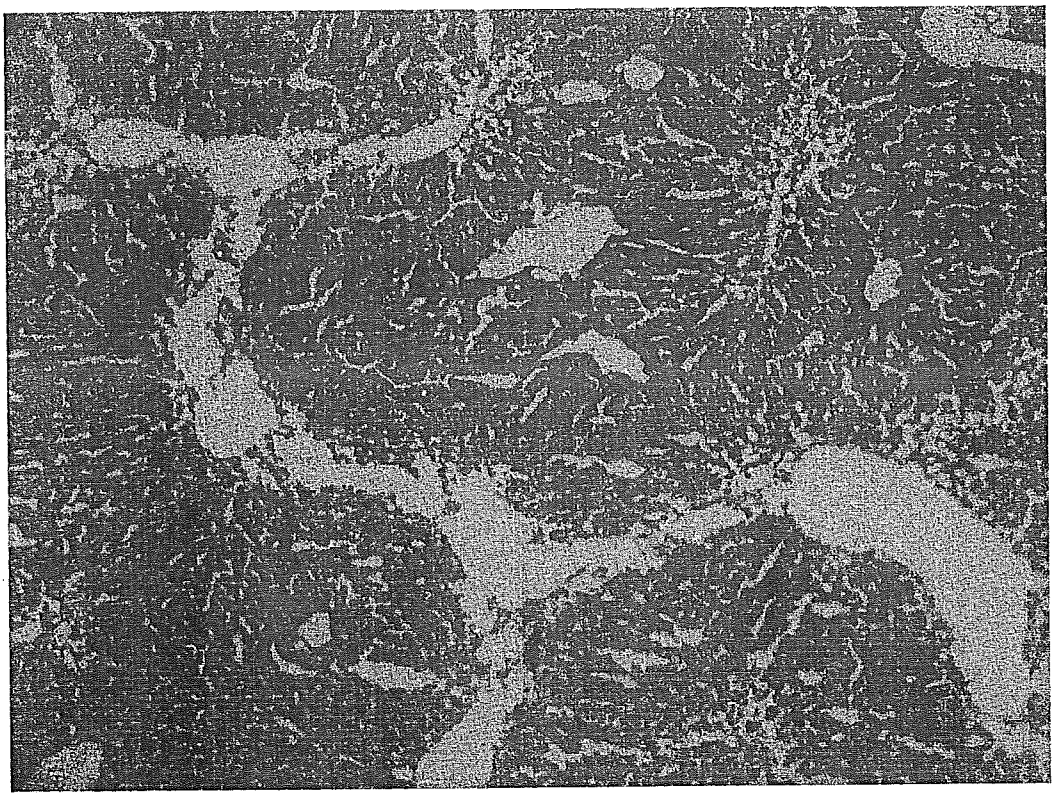


Fig. 3. In situ zymography. Migrated bone marrow cells are expressing MMP-9 and resolving the gelatin (extracellular matrix), leading to the resolution of liver fibrosis

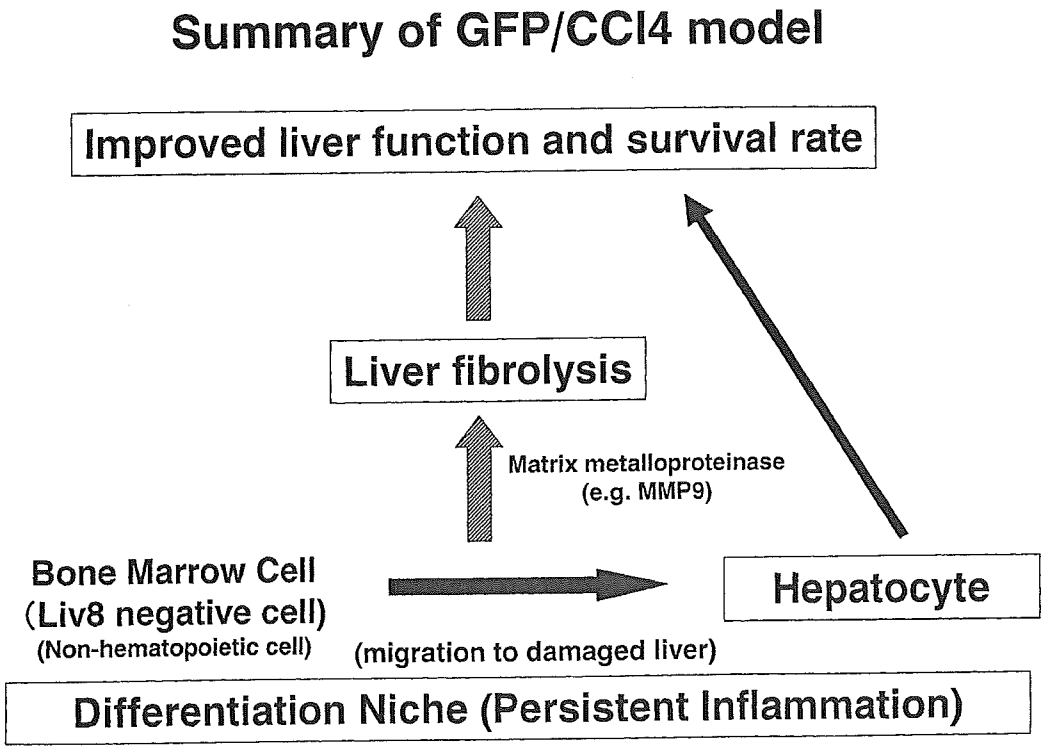


Fig. 4. Summary of GFP/CCl_4 model

BMC transplantation into liver cirrhotic mice has two effects: BMC differentiation into albumin, producing hepatocytes with the resolution of liver fibrosis. These effects of BMC transplantation accelerate the improvement of liver function and the survival rate (Fig. 4).

Molecular mechanisms of BMC differentiation into hepatocytes

The differentiation of BMCs into hepatocytes in the fumarylacetoacetate hydrolase (FAH) model was thought to show the importance of cell fusion in the differentiation of HSC into hepatocytes.[37,38] However, other groups have

reported little evidence of in vivo cell fusion during the differentiation of BMCs into other cell lineages.[39] We analyzed the cell fusion rate using cultured Neo-resistant Embryonic Stem (ES) cells and GFP-positive BMCs under the same culture conditions as Terada et al.[17] (cell fusion rate of $1/10^5$–10^6) and found similar cell fusion rates in our in vitro assay. Mouse hepatocytes have ploidy values of 2N, 4N, 8N, or 16N. Cell fusion of diploid (2N) BMCs with hepatocytes produces cells with ploidy values of 4N, 6N, 10N, or 18N. It seems that the variety of ploidy values would make it very difficult to analyze cell fusion.

We analyzed the DNA ploidy patterns of isolated primary hepatocytes in persistent CCl_4-damaged mice with and without BMC transplantation at 4 weeks. We were able to isolate about 1.2×10^8 hepatocytes from recipient mice at 4 weeks using a two-step collagenase method and analyzed the DNA ploidy patterns with a fluorescence-activated cell sorter (FACS). We found 2N, 4N, 8N, and 16N DNA bands. Comparisons of these DNA ploidy patterns showed that the 2N and 4N bands were similar, but the peaks representing the 8N and 16N bands were slightly different. These results suggest that cell fusion could have occurred in the GFP/CCl_4 model, but further examination is necessary. Although we could not neglect the possibility that cell fusion had occurred in our model, BMC seemed to differentiate into Liv2-positive hepatoblasts and functional hepatocytes, mainly without cell fusion. Also, we analyzed the mechanism of this plasticity using DNA chips, which are recently developed tools of genetic analysis.[40] Although it is possible to obtain vast amounts of genetic data using DNA chips, interpretation of the factors involved in gene expression requires the application of a statistical technique such as a self-organizing map (SOM) to visualize the vast amounts of complicated and multidimensional data.[41]

In this analysis, we derived a specific equation to extract genes that regulate the differentiation of BMCs into hepatocytes. Genes related to morphology were dramatically activated at an early stage, whereas genes associated with hepatocyte differentiation were upregulated at a later stage in the GFP/CCl_4 model. In the early stage after BMC transplantation, we found that genes such as FGF and c-*kit*, as well as HOX and HLH transcription factors, might have been important. In later stages, genes associated with metabolic function, such as hepatocyte nuclear factor 4 (HNF4) and glucose-6-phosohatase (G6Pase) isomerase, were induced, suggesting that at 4 weeks after BMCs transplantation, transplanted BMCs began to assume some of the metabolic functions of hepatocytes.[42] Although many details remain unconfirmed, the Microarray-SOM analysis for the GFP/CCl_4 model confirmed the idea that BMCs differentiated into immature cells and then differentiated into mature hepatocytes. This information will be useful for understanding the mechanism of plasticity of BMCs in the GFP/CCl_4 model.

Summary

As shown in Fig. 4, transplanted GFP-positive BMCs (especially the Liv8-negative cell population, without culturing) migrated into the periportal regions of the cirrhotic liver. The transplanted GFP-positive BMCs differentiated into Liv2-positive hepatoblasts and then differentiated into albumin-producing hepatocytes. The differentiation "niche" induced by persistent liver damage resulting from continuous CCl_4 injection seems to be an essential factor. Microarry-SOM analysis showed that at an early stage after BMC transplantation the genes related to morphology were activated. Then, later, genes associated with liver metabolism were activated. Finally, BMC transplantation improved liver function, liver fibrosis, and the survival rate. These findings strongly support the development of a new cell therapy using autologous BMCs to treat liver cirrhosis patients, because BMC transplantation itself is an established treatment for hematological diseases. Based on the results obtained in basic research using the GFP/CCl_4 model, human trials are now undergoing.

References

1. Alison MR, Poulsom R, Jeffery R, Dhillon AP, Quaglia A, Jacob J, Novelli M, Prentice G, Williamson J, Wright NA (2000) Hepatocytes from non-hepatic adult stem cells. Nature (Lond) 406:257
2. Theise ND, Nimmakayalu M, Gardner R, Illei PB, Morgan G, Teperman L, Henegariu O, Krause DS (2000) Liver from bone marrow in humans. Hepatology 32:11–16
3. Korbling M, Katz RL, Khanna A, Ruifrok AC, Rondon G, Albitar M, Champlin RE, Estrov Z (2000) Hepatocytes and epithelial cells of donor origin in recipients of peripheral-blood stem cells. N Engl J Med 346:738–746
4. Okamoto R, Yajima T, Yamazaki M, Kanai T, Mukai M, Okamoto S, Ikeda Y, Hibi T, Inazawa J, Watanabe M (2002) Damaged epithelia regenerated by bone marrow-derived cells in the human gastrointestinal tract. Nat Med 8:1011–1017
5. Terai S, Yamaoto N, Omori K, Sakaida I, Okita K (2002) A new cell therapy using bone marrow cells to repair damaged liver. J Gastroenterol 37(suppl XIV):162–163
6. Terai S, Sakaida I, Yamamoto N, Omori K, Watanabe T, Ohata S, Katada T, Miyamoto K, Shinoda K, Nishina H, Okita K (2003) An in vivo model for monitoring trans-differentiation of bone marrow cells into functional hepatocytes. J Biochem (Tokyo) 134:551–558
7. Fox IJ, Chowdhury JR, Kaufman SS, Goertzen TC, Chowdhury NR, Warkentin PI, Dorko K, Sauter BV, Strom SC (1998) Treatment of the Crigler–Najjar syndrome type I with hepatocyte transplantation. N Engl J Med 338:1422–1426
8. Muraca M, Gerunda G, Neri D, Vilei MT, Granato A, Feltracco P, Meroni M, Giron G, Burlina AB (2002) Hepatocyte transplantation as a treatment for glycogen storage disease type 1a. Lancet 359:317–318
9. Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, Schumichen C, Nienaber CA, Freund M, Steinhoff G (2003) Autologous bone-marrow stem-cell transplantation for myocardial regeneration. Lancet 361:45–46
10. Wexler SA, Donaldson C, Denning-Kendall P, Rice C, Bradley B, Hows JM (2003) Adult bone marrow is a rich source of human mesenchymal "stem" cells but umbilical cord and mobilized adult blood are not. Br J Haematol 121:368–374
11. Kobayashi T, Hamano K, Li TS, Nishida M, Ikenaga S, Hirata K, Zempo N, Esato K (2002) Therapeutic angiogenesis induced by

local autologous bone marrow cell implantation. Ann Thorac Surg 73:1210–1215
12. Tateishi-Yuyama E, Matsubara H, Murohara T, Ikeda U, Shintani S, Masaki H, Amano K, Kishimoto Y, Yoshimoto K, Akashi H, Shimada K, Iwasaka T, Imaizumi T (2002) Therapeutic Angiogenesis using Cell Transplantation (TACT) Study Investigators. Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. Lancet 360:427–435
13. Krause DS, Theise ND, Collector MI, Henegariu O, Hwang S, Gardner R, Neutzel S, Sharkis SJ (2001) Multi-organ, multilineage engraftment by a single bone marrow-derived stem cell. Cell 105:369–377
14. Lagasse E, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L, Wang X, Finegold M, Weissman IL, Grompe M (2000) Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. Nat Med 6:1229–1234
15. Uchida N, Fujisaki T, Eaves AC, Eaves CJ (2001) Transplantable hematopoietic stem cells in human fetal liver have a CD34(+) side population (SP) phenotype. J Clin Invest 108:1071–1077
16. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, Moorman MA, Simonetti DW, Craig S, Marshak DR (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147
17. Terada N, Hamazaki T, Oka M, Hoki M, Mastalerz DM, Nakano Y, Meyer EM, Morel L, Petersen BE, Scott EW (2002) Bone marrow cells adopt the phenotype of other cells by spontaneous cell fusion. Nature (Lond) 416:542–545
18. Ying QL, Nichols J, Evans EP, Smith AG (2002) Changing potency by spontaneous fusion. Nature (Lond) 416:545–548
19. Camargo FD, Finegold M, Goodell MA (2004) Hematopoietic myelomonocytic cells are the major source of hepatocyte fusion partners. J Clin Invest 113:1266–1270
20. Willenbring H, Bailey AS, Foster M, Akkari Y, Dorrell C, Olson S, Finegold M, Fleming WH, Grompe M (2004) Myelomonocytic cells are sufficient for therapeutic cell fusion in liver. Nat Med 10:744–748
21. Alvarez-Dolado M, Pardal R, Garcia-Verdugo JM, Fike JR, Lee HO, Pfeffer K, Lois C, Morrison SJ, Alvarez-Buylla A (2003) Fusion of bone-marrow-dervied cells with Purkinje neurons, cardiomyocytes and hepatocytes. Nature (Lond) 425:968–973
22. Harris RG, Herzog EL, Bruscia EM, Grove JE, Van Arnam JS, Krause DS (2004) Lack of a fusion requirement for development of bone marrow-dervied epithelia. Science 305:90–93
23. Lee KD, Kuo TK, Whang-Peng J, Chung YF, Lin CT, Chou SH, Chen JR, Chen YP, Lee OK (2004) In vitro hepatic differentiation of human mesenchymal stem cells. Hepatology 40:1275–1284
24. Jiang Y, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM (2002) Pluripotency of mesenchymal stem cells derived from adult marrow. Nature (Lond) 418:41–49
25. Okabe M, Ikawa M, Kominami K, Nakanishi T, Nishimune Y (1997) "Green mice" as a source of ubiquitous green cells. FEBS Lett 407:313–319
26. Shinoda K, Mori S, Ohtsuki T, Osawa Y (1992) An aromatase-associated cytoplasmic inclusion, the "stigmoid body," in the rat brain: I. Distribution in the forebrain. J Comp Neurol 322:360–376
27. Watanabe T, Nakagawa K, Ohata S, Kitagawa D, Nishitai G, Seo J, Tanemura S, Shimizu N, Kishimoto H, Wada T, Aoki J, Arai H, Iwatsubo T, Mochita M, Watanabe T, Satake M, Ito Y, Matsuyama T, Mak TW, Penninger JM, Nishina H, Katada T (2002) SEK1/MKK4-mediated SAPK/JNK signaling participates in embryonic hepatoblast proliferation via a pathway different from NF-kappaB-induced anti-apoptosis. Dev Biol 250:332–347
28. Grisham JW, Thorgeirsson SS (1997) Liver stem cells. Academic Press, Manchester, pp 233–282
29. Petersen BE, Zajac VF, Michalopoulos GK (1998) Hepatic oval cell activation in response to injury following chemically induced periportal or pericentral damage in rats. Hepatology 27:1030–1038
30. Petersen BE, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP (1999) Bone marrow as a potential source of hepatic oval cells. Science 284:1168–1170
31. Sakaida I, Terai S, Yamamoto N, Okita K (2002) Transplantation of bone marrow cell reverses CCl_4-induced liver fibrosis. Hepatology 36:295A
32. Sakaida I, Terai S, Yamamoto N, Aoyama K, Ishikawa T, Nishina H, Okita K (2004) Transplantation of bone marrow cells reduces CC14-induced liver fibrosis in mice. Hepatology 40:1304–1311
33. Azouz A, Razzaque MS, El-Hallak M, Taguchi T (2004) Immunoinflammatory responses and fibrogenesis. Med Electron Microsc 37:141–148
34. Yamamoto N, Terai S, Ohata S, Watanabe T, Omori K, Shinoda K, Miyamoto K, Katada T, Sakaida I, Nishina H, Okita K (2004) A subpopulation of bone marrow cells depleted by a novel antibody, anti-Liv8, is useful for cell therapy to repair damaged liver. Biochem Biophys Res Commun 313:1110–1118
35. Forbes SJ, Russo FP, Rey V, Burra P, Rugge M, Wright NA, Alison MR (2004) A significant proportion of myofibroblasts are of bone marrow origin in human liver fibrosis. Gastroenterology 126:955–963
36. Senoo H (2004) Structure and function of hepatic stellate cells. Med Electron Microsc 37:3–15
37. Wang X, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Olson S, Grompe M (2003) Cell fusion is the principal source of bone-marrow-dervied hepatocytes. Nature (Lond) 422:897–901
38. Vassilopoulos G, Wang PR, Russell DW (2003) Transplanted bone marrow regenerates liver by cell fusion. Nature (Lond) 422:901–904
39. Ianus A, Holz GG, Theise ND, Hussain MA (2003) In vivo derivation of glucose-competent pancreatic endocrine cells from bone marrow without evidence of cell fusion. J Clin Invest 111:843–850
40. Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270:467–470
41. Xiao L, Wang K, Teng Y, Zhang J (2003) Component plane presentation integrated self-organizing map for microarray data analysis. FEBS Lett 538:117–124
42. Omori K, Terai S, Ishikawa T, Aoyama K, Sakaida I, Nishina H, Shinoda K, Uchimura S, Hamamoto Y, Okita K (2004) Molecular signature associated with plasticity of bone marrow cell under persistent liver damage by self-organizing-map-based gene expression. FEBS Lett 578:10–20

最新医学・第60巻・第4号　(2005年4月号 別刷)

特集　肝疾患研究の新たな展開

自己骨髄細胞を用いた肝臓再生療法

坂井田　功　　寺井崇二　　沖田　極

最新医学社

自己骨髄細胞を用いた肝臓再生療法

坂井田　功**　寺井崇二*　沖田　極***

要　旨

進行した非代償性肝硬変症に対する肝再生治療として，自己骨髄細胞を用いた肝臓再生療法を開発した．基礎的検討では GFP/CCl_4 モデルにおいて，持続炎症の存在する肝硬変状態において，末梢静脈より投与した骨髄細胞は効率良く肝臓に浸潤し，アルブミンを産生する肝細胞へ分化した．また，この分化過程において肝線維化を溶解する効果があることが明らかになった．さらに，これをもとに臨床研究を展開した．

はじめに

我が国においては，慢性肝不全患者（肝硬変）に対してすでに生体肝移植が行われているが，ドナーの不足や外科的侵襲など克服すべき課題は多い．こうした肝不全（肝硬変）に対する新しい治療法としての「自己骨髄細胞を用いた肝臓再生療法」の研究を紹介する．

骨髄細胞の肝細胞への分化の可塑性の発見

肝臓は，肝細胞，星細胞（伊東細胞），Kupffer 細胞，Pit 細胞，内皮細胞より構成されており，代謝，タンパク質合成，解毒など多機能をつかさどっている臓器である．2000年に骨髄細胞の肝細胞への分化の可塑性について，男性ドナーから骨髄移植を受けた女性レシピエントの剖検例において，骨髄細胞が肝細胞に分化することが報告された[1]．これは女性レシピエントの肝細胞内にY染色体陽性細胞の存在を証明し，骨髄細胞の肝細胞への分化の可塑性を報告したものである．また骨髄細胞の腸管への分化についても報告され，骨髄中にはさまざまな臓器の細胞に分化する細胞が存在することが明らかになった[2]．

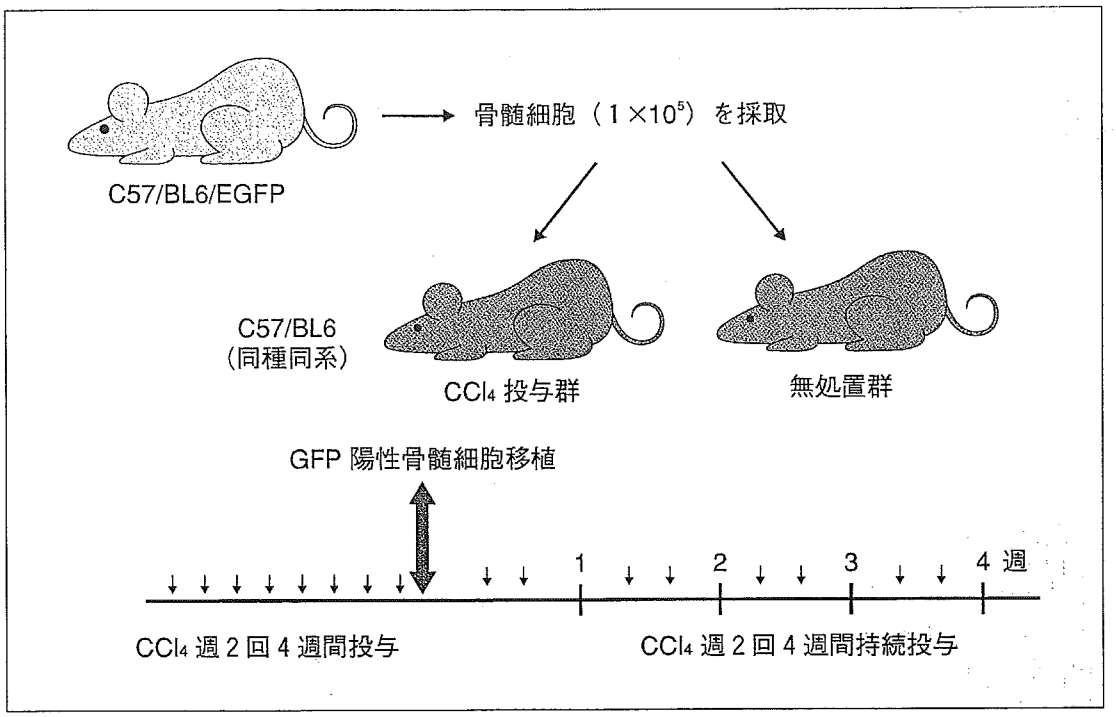
骨髄細胞における分化の可塑性

我々は自己骨髄細胞を用いた肝臓再生療法の研究を推進するに当たり，どのような状態において骨髄細胞は肝細胞に分化するかという問題を解決したいと考えた．この骨髄細胞の分化に関与する微小環境“Niche”は，さまざまな炎症，免疫的なシグナル，転写因子，細胞外マトリックスなどが関与して制御されていると考えられる．血球幹細胞が肝細胞に分化し，その結果肝臓の機能を代償したことについては，チロシン血漿突然変異マウス（FAH 欠損マウス）に対し，β-ガラクトシダーゼ（β-gal）陽性の血球幹細胞の投与により，移植したβ-gal 陽性細胞が肝臓に細胞塊を作って定着し，肝臓の機能がうまく代償

* 山口大学医学部 消化器病態内科学　** 同 助教授　*** 同 教授

キーワード：骨髄細胞，肝再生，肝線維化，マトリックスメタロプロテアーゼ

図1 GFP/CCl_4 モデル



略語：巻末の「今月の略語」参照

されることが報告された[3]．しかし，このモデルは非常に特殊なチロシン血症モデルであり，我々が日常の現場で見る肝炎，肝硬変モデルと病態は異なる．一方で，我々が実際に再生療法を行い救命したいと考える患者は，ウイルス肝炎による持続肝障害が続いている肝硬変患者（非代償性肝硬変症）が対象になる．このため，レシピエントモデルを四塩化炭素（CCl_4）の持続投与によって作った肝硬変マウスとし，GFP トランスジェニックマウス[4]から採取した GFP 陽性骨髄細胞を尾静脈から投与して肝細胞への分化の有無を評価した（GFP/CCl_4 モデル）．すなわち，週2回の CCl_4 投与を4週間行い，この持続肝障害により肝硬変状態にしたレシピエントマウスに，GFP 陽性の骨髄細胞（非培養，ヘテロな細胞集団）を尾静脈より投与した（図1）．その後も CCl_4 をさらに4週間投与し，GFP 陽性骨髄細胞の肝への定着・分化について評価した．肝障害がない状態では骨髄細胞は肝臓には浸潤・定着しないが，CCl_4 での持続肝障害状態で投与した骨髄細胞は投与後1日目から門脈周囲に定着し，持続肝障害下の Niche において骨髄細胞から肝芽細胞に分化し，最終的には機能的な肝細胞索様構造を作ってアルブミン産生の肝細胞へ分化することが明らかになった[5]．実際に，血液中のアルブミン値も上昇し肝機能を改善していた．骨髄細胞は A6 陽性 Oval 細胞の表現型はとらないことが明らかになり，GFP/CCl_4 モデルにおいては投与した骨髄細胞が Oval 細胞の表現型を経ることはなく，発生段階と同様に肝芽細胞の表現型を経ながら肝細胞に分化することが明らかになった[6]．GFP/CCl_4 モデルにおける骨髄細胞の分化転換に炎症性シグナルが重要なことは，骨髄細胞投与後に CCl_4 を中止するとほとんど骨髄細胞が肝内に見られないことから判明した．

肝線維化に対する効果

肝内に浸潤した骨髄細胞は過剰に産生・沈着した線維に沿って存在する（図2）．さら

図2　GFP/sirius red 染色：骨髄細胞投与2週後のマウス肝臓

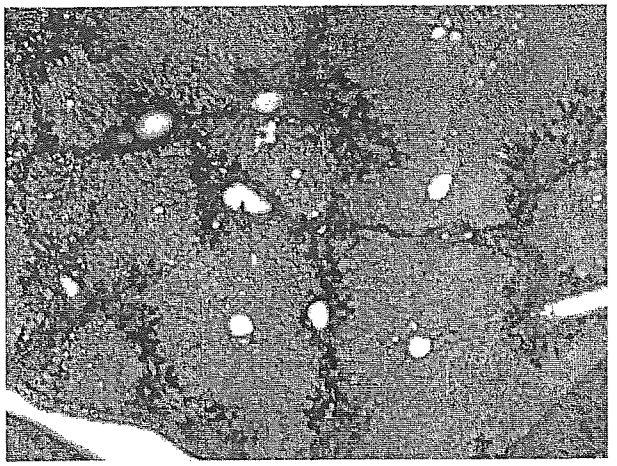
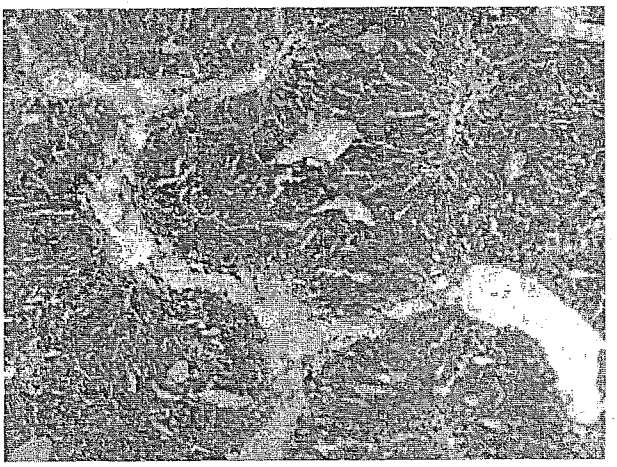


図3　*in situ* zymography：骨髄細胞投与2週後のマウス肝臓



に週を追うごとに既存の線維の中に浸潤していく像が見られる．この過程で，細胞外マトリックスを溶解するマトリックスメタロプロテアーゼ（MMPs，特に MMP-9）の発現が増加することが判明し，MMP-9 産生細胞が骨髄細胞であることは MMP-9 の mRNA，タンパク質の発現が GFP 陽性の細胞であることから証明され，さらにこの MMP-9 が実際にタンパク質分解酵素としての酵素活性を持つことを，*in situ* zymography で証明できた（図3）[7]．骨髄細胞を投与後も CCl_4 を持続投与し，壊死・炎症が持続したにもかかわらず，骨髄細胞投与後4週目の肝線維化は1週目よりも改善（減少）した．慢性肝炎などで持続炎症があると，肝内の星細胞（伊東細胞）が活性化されてコラーゲンなどの細胞外マトリックスを産生し，活性化初期に MMPs の発現は認めるものの，時間がたつとこれを阻害する TIMPs が発現し，線維は溶解せずに蓄積の方向に向かう．インターフェロン（IFN）でウイルスが完全排除されて壊死・炎症がなくなると，星細胞は不活性化されてコラーゲンや TIMPs の産生を停止し，相対的に MMPs の活性が高まり既存の線維は溶解する．今回の実験結果では，壊死・炎症が持続しているにもかかわらず線維化が減少しているという，日常のこれまでの臨床からは考えられない結果となった．すなわち，これまでの肝線維化をできるだけ抑制して肝再生を促すといった受身的なものから，積極的に肝再生を引き起こしその過程で肝線維化も減少させることができるという理想的な結果を得ることができた（図4）．

臨床への応用

骨髄細胞移植を用いた下肢および血管再生療法はすでに臨床応用が行われており[8)9)]，血液疾患の治療に骨髄移植は過去 20 年以上の経験があり，骨髄採取そのものについては確立されている．我々の基礎的な検討では，骨髄細胞の投与により肝機能の改善が確認され，また肝線維化・生存率も改善していた．これらの結果は，肝不全（非代償性肝硬変症）などに対する「自己骨髄細胞を用いた肝臓再生療法」の臨床開発の可能性を強く示す結果であった．我々は 2003 年 11 月 14 日より，（自己骨髄細胞を用いた肝臓再生療法）第Ⅰ相臨床研究を開始し，その安全性を検証している．対象患者（非代償性肝硬変症）は，総ビリルビンは 3 mg/dL 以下，血小板は5万以上，肝細胞癌合併例については治療によりコントロール良好な症例であること，また食道・胃静脈瘤合併例においても破裂の危険性のない症例とした．さらに骨髄採取に伴い全

図4　骨髄細胞移植による肝機能改善のメカニズム

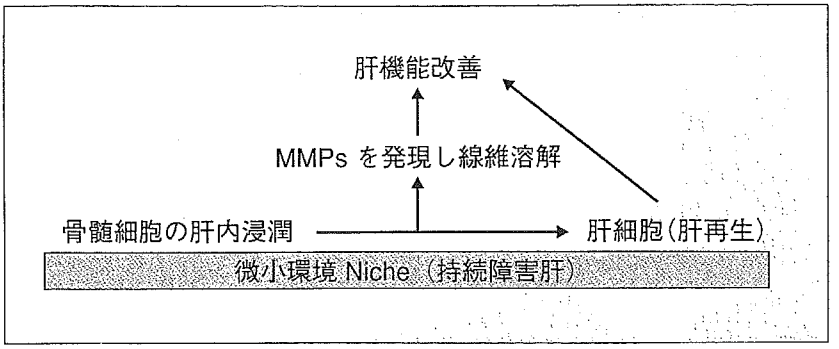
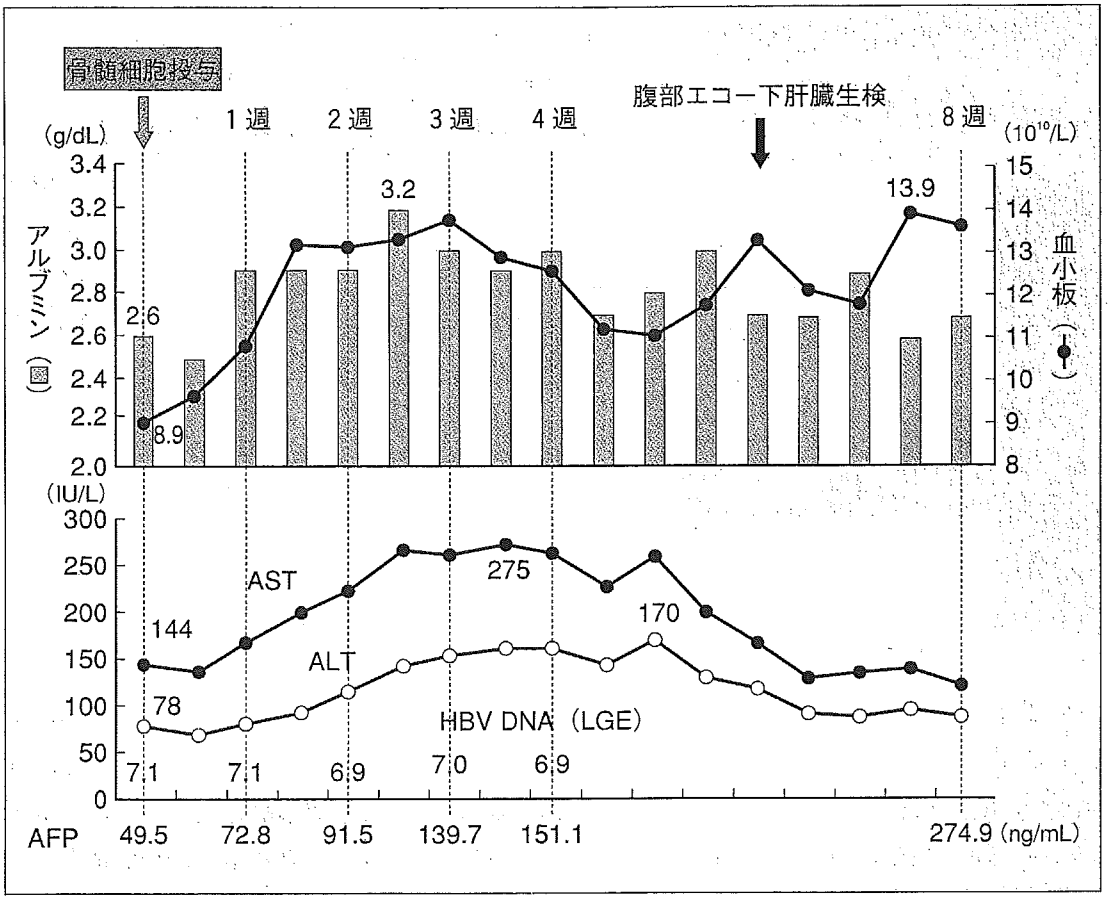


図5　自己骨髄細胞移植患者の臨床経過（文献[10]より引用改変）



略語：巻末の「今月の略語」参照

身麻酔をかけるので，心肺機能に問題のない患者とした．

症例：臨床経過

B型肝炎ウイルス（HBV）陽性非代償性肝硬変症の69歳男性に対し，全身麻酔下にて自己骨髄細胞を400mL採取し，洗浄後有核細胞の 4×10^9 個を採取した．採取した自己骨髄細胞を約1時間かけて末梢静脈から投与し，肝再生の誘導の有無について評価した．術中特に大きな合併症もなく，術後血液検査にて骨髄細胞の肝再生に与える影響について評価した．その結果，基礎研究の結果より予測されたように，骨髄細胞投与後より血清アルブミン値は術前2.5g/dLであったものが最大3.2g/dLまで改善し，また腹部エコーにて腹水の減少を確認し利尿薬の投与量の減量に成功した．また図5に経過を示すように，

α-フェトプロテイン（AFP）も骨髄細胞投与により増加した（L3 分画は基準以下）．その他，血小板の値も改善した．また，骨髄細胞投与後に施行した肝生検において HNF4, AFP などの誘導も確認され，肝再生が誘導された可能性が考えられた．

今後の課題

持続肝障害下において投与した骨髄細胞が効率良く肝臓に遊走・定着し，幼弱化しながら肝芽細胞になり肝細胞になることが明らかになったが，① 障害肝に骨髄細胞を引き寄せるメカニズム，② 移植した細胞の運命，③ 移植した骨髄細胞の肝内への浸潤・分化に伴う既存の肝構成細胞への影響などについて解明していかなければならない．また臨床においては，現在までの 6 例の検討では重篤な副作用の発生はない．今後は慎重に症例を重ねて第Ⅰ相臨床研究を進めていき，肝臓領域における新たな再生療法としての「自己骨髄細胞を用いた肝臓再生療法」の開発の可能性を評価していきたい．

謝 辞

本研究は，厚生労働省厚生科学研究費（基礎研究成果の臨床応用推進事業）の補助を得て推進されている．

文　献

1) Theise N D, et al: Liver from bone marrow in humans. Hepatology 32: 11–16, 2000.
2) Okamoto R, et al: Damaged epithelia regenerated by bone marrow–derived cells in the human gastrointestinal tract. Nat Med 8: 1011–1017, 2002.
3) Lagasse E, et al: Purified hematopoietic stem cells can differentiate into hepatocytes *in vivo*. Nat Med 6: 1229–1234, 2000.
4) Okabe, M, et al: 'Green mice' as a source of ubiquitous green cells. FEBS Lett 407: 313–319, 1997.
5) Terai S, et al: An *in vivo* model for monitoring trans–differentiation of bone marrow cells into functional hepatocytes. J Biochem (Tokyo) 134: 551–558, 2003.
6) Yamamoto N, et al: A subpopulation of bone marrow cells depleted by a novel antibody, anti–Liv8, is useful for cell therapy to repair damaged liver. Biochem Biophys Res Commun 313: 1110–1118, 2004.
7) Sakaida I, et al: Transplantation of bone marrow cells reduces CCl_4–induced liver fibrosis in mice. Hepatology 40: 1304–1311, 2004.
8) Hamano K, et al: Therapeutic angiogenesis induced by local autologous bone marrow cell implantation. Ann Thorac Surg 73: 1210–1215, 2002.
9) Stamm C, et al: Autologous bone–marrow stem–cell transplantation for myocardial regeneration. Lancet 361: 45–46, 2003.
10) 寺井崇二, 他: 自己骨髄細胞を用いた肝硬変症に対する肝臓再生療法．治療学 38: 1158–1159, 2004.

Liver Regeneration Therapy by Autologous Bone Marrow Cell Transplantation

Isao Sakaida, Shuji Terai, Kiwamu Okita
Department of Gastroenterology & Hepatology, Yamaguchi University, School of Medicine

医学と医療の最前線

骨髄幹細胞移植による肝疾患の治療

沖田　極　寺井　崇二　坂井田　功

日本内科学会雑誌　第94巻　第4号別刷

2005年4月10日

骨髄幹細胞移植による肝疾患の治療

沖田　極　寺井　崇二　坂井田　功

要　旨

骨髄には造血幹細胞が存在し白血病患者に対する骨髄移植によって患者の体内で一生に渡り血球細胞を供給することが可能である．骨髄移植は既に20年以上の実績を積んだ体性幹細胞を用いた再生医療である．最近になり男女間での骨髄移植の結果，骨髄由来の肝細胞，小腸細胞への分化が確認され，骨髄中に多分化能を持つ“骨髄由来幹細胞”の存在が考えられるようになった．すでに循環器領域では虚血性疾患に対して臨床応用として血管新生を誘導するために自己の骨髄細胞を患部に自家移植する試みがすでに行われてきた．我々は自己骨髄細胞を用いた肝臓再生療法を開発するため，どんな状況で骨髄細胞移植が有効であるかを検討し，持続肝障害が続く状態において骨髄細胞が肝再生誘導，肝線維化制御に役立つことを見出した．さらにそれらの基礎研究の結果を基盤とし，国内最初の臨床研究：自己骨髄細胞を用いた肝臓再生療法のPhaseI研究を平成15年11月より開始し現在進行中である．

〔日内会誌　94：769～774，2005〕

Key words：骨髄細胞，分化ニッチ，肝幹細胞，肝再生，肝線維化制御，肝不全，細胞療法

はじめに

現在国内において肝炎ウイルス（B，C型）を原因とした肝疾患は増え続き，さらに肝細胞がんによる癌死は年間の癌死の第3位を占めるようになってきた．肝癌患者の背景にある病変が肝硬変症であるためいかに肝不全を制御するかが重要な問題になっている．肝不全患者に対して生体肝移植が行われているがドナーの不足，外科侵襲，経済的な問題が大きい．今後増加すると予測される肝不全患者に対する，肝移植に代わる次世代の肝臓再生療法の開発は重要と考えられる．我々は，あらたに肝不全に対する治療として『自己骨髄細胞を用いた肝臓再生療法』の開発を目指し基礎研究，臨床研究を進めてきたのでここに概説する．

おきた　きわむ，てらい　しゅうじ，さかいだ　いさお：
山口大学先端分子応用医科学講座消化器病態内科学

1．肝臓に存在する肝幹細胞と，骨髄細胞の肝細胞への分化の可塑性の発見

肝臓は代謝，蛋白合成，解毒など司る多機能な臓器である．肝臓についての発生過程においてはその分化細胞系譜として，胎児期にまず肝芽細胞が発生し，その後肝細胞，肝内胆管の前駆細胞に分かれて，成熟肝細胞，胆管細胞に発生分化すると考える（図1）[1~3]．また胎児期の肝臓は造血臓器として働く特徴がある．一方肝臓に存在する体性幹細胞そのものについては以前より研究が行われきているが，重篤な肝障害に伴い発生する卵円形のOval細胞は肝幹細胞の一つと考えられてきた[4]．このOval細胞については肝細胞以外に，膵臓，胆管，小腸細胞に分化する可能性が知られている．また肝幹細胞の分離同定が試みられ，ラットよりsmall hepatocyte[5]，Long-Evans Cinnamon（LEC）ratより肝幹細胞