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

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

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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

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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), 0.5 ml/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]

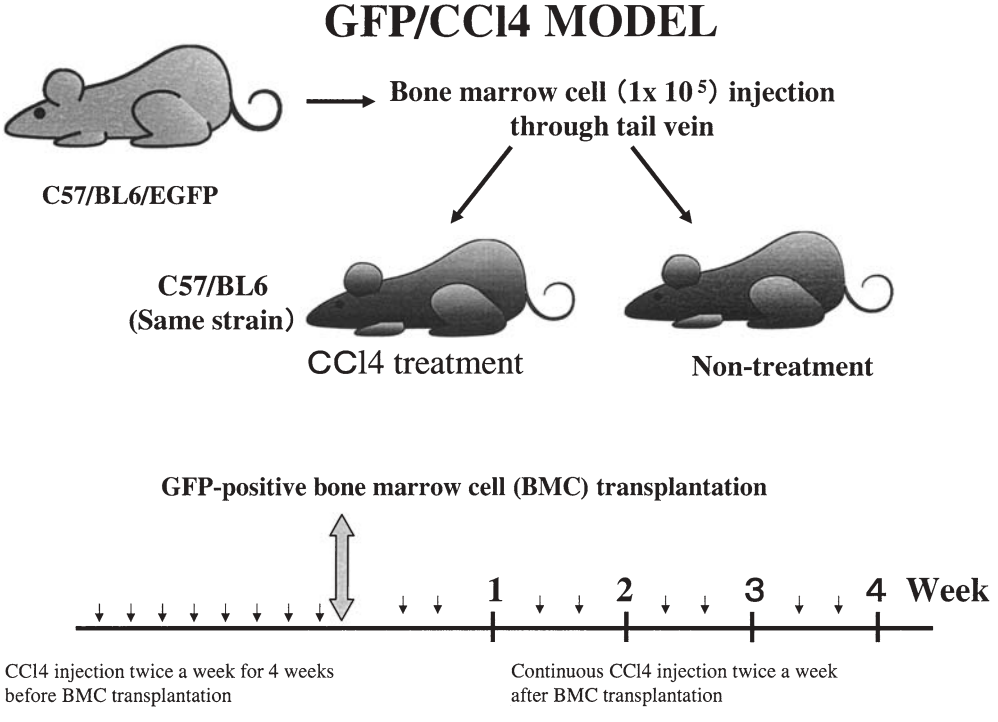

Fig. 1. Experimental protocol for the green fluorescent protein/carbon tetrachloride (GFP/CCl_4) model

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.

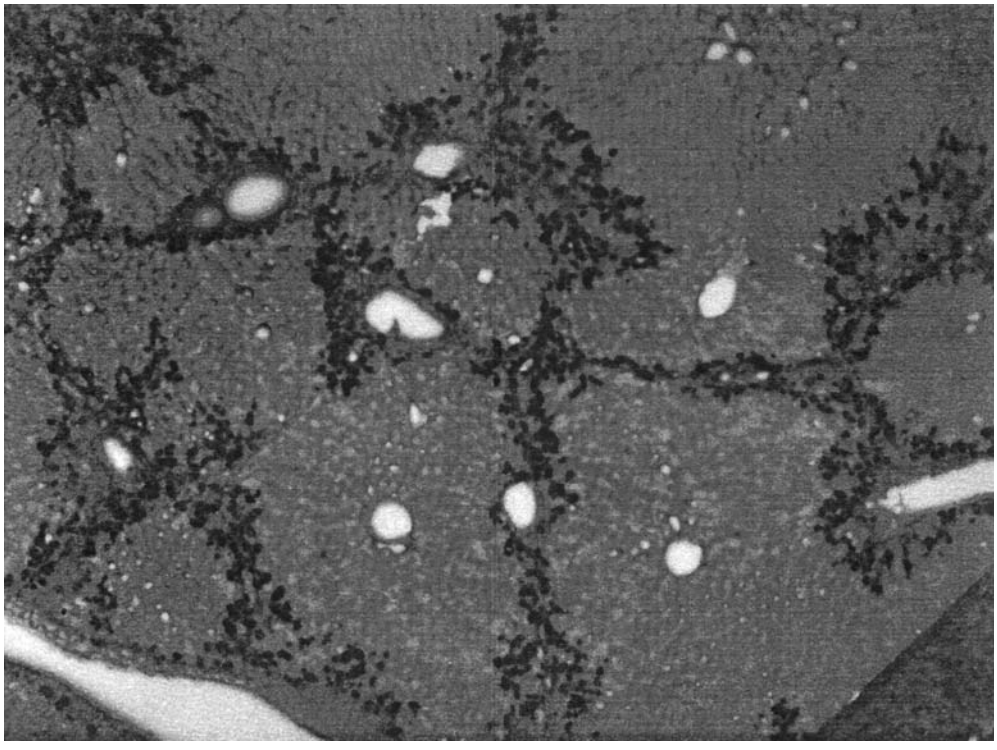

Fig. 2. GFP and Sirius red staining. Migrated bone marrow cells are seen along with the fibers

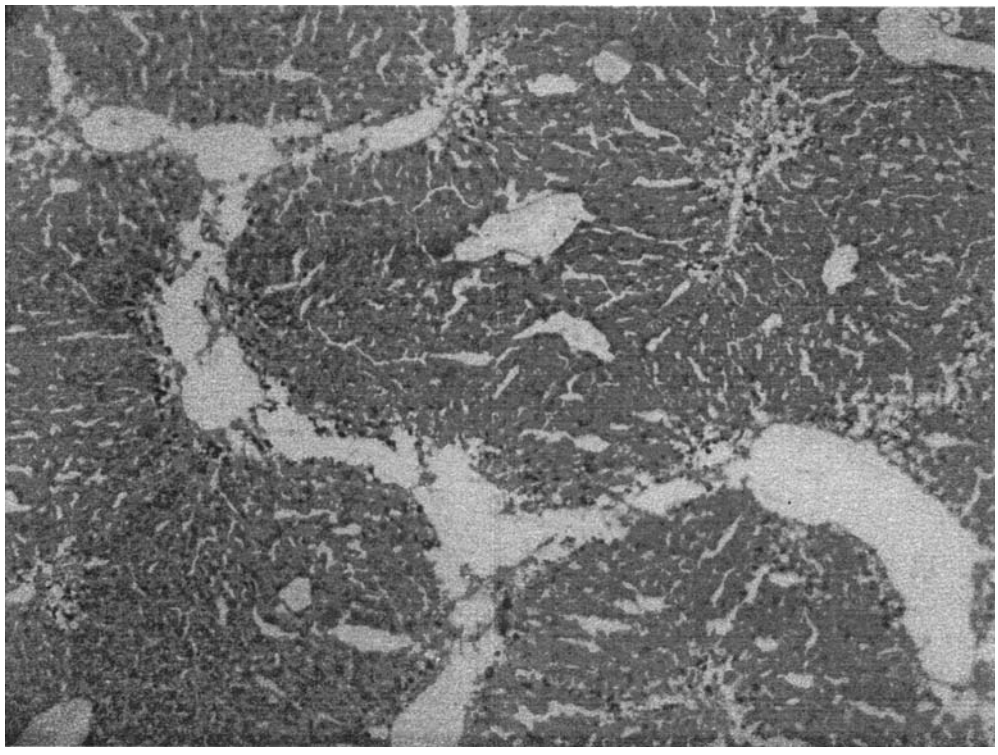

Fig. 3. In situ zymography. Migrated bone marrow cells are expressing MMP-9 and resolving the gelatin (extracellular matrix), leading to the resolution of liver fibrosis

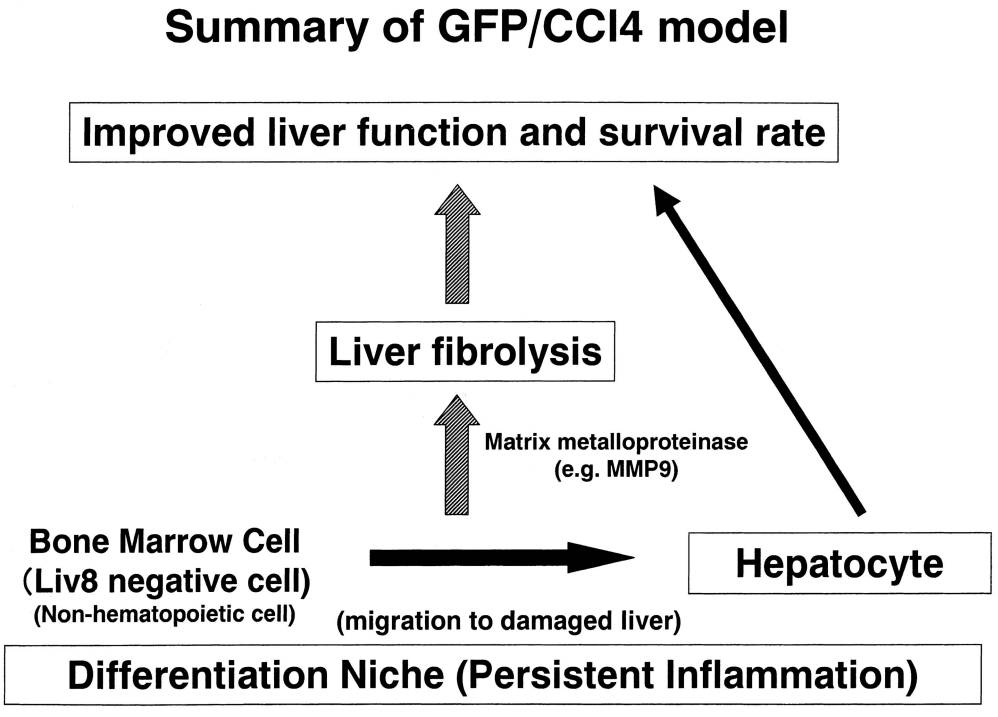

Fig. 4. Summary of GFP/CCl_4 model

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.

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