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BREAKTHROUGHS AND VIEWS

Hepatic Stem Cells: From Bone Marrow Cells to Hepatocytes

Toshihiro Mitaka[1]
Department of Pathology, Cancer Research Institute, Sapporo Medical University School of Medicine, Chuo-Ku, S-1, W-17, Sapporo 060-8556, Japan

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Hematopoiesis and the hepatic environment are known to have a close relationship at the time of hepatic development and systemic diseases. Recently, transplanted cells isolated from bone marrow of rodents and humans have been shown to differentiate into oval cells, which are considered to be hepatic stem cells, and hepatocytes in the liver. Then, purified hematopoietic stem cells were shown to have the ability to replace original liver cells in mice with hereditary tyrosinemia. In this review the interactions between hepatic stem cells are summarized and a hypothesis of hepatic differentiation will be proposed.

Key Words: **hepatic stem cell; hematopoietic stem cell; oval cell; small hepatocyte; differentiation; maturation.**

Are there stem cells in the livers in adult animals? This question has been debated for about half a century. Although many researchers around the world have tried to identify and characterize such cells in the liver, convincing results have not yet been reported. Some candidates for hepatic stem or progenitor cells such as oval cells and small hepatocytes have been proposed. However, if the stem cells are defined as undifferentiated, multipotent cells, and the progenitor cells as nonhepatocyte epithelial cells generating a dual lineage (biliary and hepatocytic) with hepatocyte markers, it can be said that no pure population of either type of cell has been isolated from normal, injured or preneoplastic adult liver. Now, however, it appears that we close to answering the above question, owing to the great advances of hematopoietic cell research in the 1990s, especially identification of surface markers of hematopoietic stem cells, the development of cell sorting technology, and clinical experience with bone marrow transplants. This progress has indeed contributed to the successful identification of certain types of hepatic stem cells. Petersen *et al.* (1) first proved that bone marrow cells transplanted into lethally irradiated rats migrated to the liver and then differentiated into oval cells and hepatocytes. Thereafter, similar results were reported for mice (2) and human patients (3, 4). Very recently, Lagasse *et al.* (5) showed that purified hematopoietic stem cells (HSCs) could differentiate into hepatocytes and replace parts of the liver in mice with hereditary tyrosinemia with new mature hepatocytes from the stem cells.

It seems obvious from historical studies about hepatic development that most hepatocytes and bile duct cells in the adult liver in animals originally derive from hepatoblasts resident in the fetal liver. Thus, hepatoblasts are considered to be bipotential stem/progenitor cells of epithelial cells in the liver. However, we can never find cells morphologically and phenotypically similar to hepatoblasts in the normal adult liver, even in the regenerating liver. Thus, we have to return to the primary question: “Are there stem cells in the liver in adult animals?”

Now most cell types related to the hepatic lineage from bone marrow cells to mature hepatocytes may appear on the stage. Therefore, the next steps of hepatic stem cell research are to elucidate the mechanisms of differentiation/maturation of each cell and to clarify the roles of the various cells in the hepatic lineage.

Abbreviations used: HSC, hematopoietic stem cell; 2AAF, 2-acetylaminofluorene; PH, partial hepatectomy; DPPIV, dipeptidyl peptidase IV; ECM, extracellular matrix; NPC, nonparenchymal cell; HNF, hepatocyte nuclear factor.

[1] Fax: +81-11-615-3099. E-mail: tmitaka@sapmed.ac.jp.

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CANDIDATES OF HEPATIC STEM CELLS

In certain conditions in the liver treated with hepatotoxins and hepatocarcinogens, or infected with hepatic virus, epithelial cells different from mature hepatocytes have been histologically found and designated as follows.

(a) Oval Cells

When liver damage is so severe that hepatocytes are largely killed or that their proliferation is prevented by exposure to hepatotoxins or carcinogens, liver progenitor cells, so-called oval cells, appear in the periportal areas of liver lobules. Oval cells are small cells with scant cytoplasm and ovoid nuclei, and they are thought to have the ability to proliferate clonogenically and a bipotential capacity to differentiate into both hepatocytes and bile duct cells. To induce oval cells in the rat liver, the combination of 2-acetylaminofluorene (2AAF) treatment and two-thirds partial hepatectomy (PH)/ CCl_4 is often used. The continuous administration of a low concentration of 2AAF suppress the proliferation of hepatocytes and at the middle point of the treatment (usually after 7 to 10 days) the animals are subjected to a massive loss of hepatocytes by administration of CCl_4 or PH. The lack of response of hepatocytes to growth signals results in the rapid growth of oval cells. The oval cells initially appear next to biliary ductules and then some migrate into the hepatic parenchyma. The origin of oval cells have been questioned, although most researchers tend to believe that the cells may come from the canals of Hering, which is a transitional zone between the periportal hepatocytes and the biliary cells lining the smallest terminal bile ducts. Recent studies clearly showed that one of the original cells was derived from bone marrow cells. Furthermore, detailed tracing examination revealed that oval cells first change into basophilic small hepatocytes and then differentiate into mature hepatocytes.

(b) Bone Marrow-Derived Cells

The accumulation of data concerning oval cells has revealed that a number of surface makers are shared between hematopoietic stem cells and oval cells, including c-*kit*, CD34, and Thy-1 in rodents, and c-*kit* and CD34 in humans. In 1999 Petersen *et al.* (1) first showed that a population of rat bone marrow cells might give rise to oval cells in the liver and have the potential to further differentiate into hepatocytes and/or bile duct cells. To prove their results, they used three different protocols combined with 2-AAF/CCl_4 treatment: (1) Bone marrow cells obtained from male rats were transplanted into lethally irradiated female recipients and the fate of Y chromosome-positive cells in the livers of the recipients was investigated; (2) marrow from dipeptidyl peptidase IV (DPPIV)-positive male rats was transplanted into DPPIV-deficient female ones; and (3) whole liver transplantation with Lewis rats that expressed the L21-6 antigen as recipients was performed using Brown-Norway rats that did not express the antigen as allogeneic donors to confirm that an extrahepatic source could repopulate the transplanted liver. A similar approach using a mouse marrow transplant model without overt liver damage was carried out and some hepatocytes were shown to express the markers of transplanted cells (2). Then, in a human female liver receiving bone marrow transplantation from a male donor and in male patients after liver transplantation from a female donor, some hepatocytes in the recipients possessed Y-chromosomes in their nuclei (3, 4). In these studies, the number of cells which underwent the transition appeared to be relatively small. However, very recently, Laggasse *et al.* (5) reported that only a small number of purified HSCs could rescue the fumarylacetoacetate hydrolase (FAH)-deficient mouse (a model animal of hereditary tyrosinemia type I), which show a progressive liver failure. At 7 months after transplantation, more than 30% of hepatocytes were replaced with donor-derived cells.

These results remind us of a familiar association between hematopoiesis and the liver. Embryonic HSCs first appear in the aorta–gonad–mesonephros region, and migrate into the fetal liver. Most hematopoietic cells leave the liver to the bone marrow and spleen before birth. However, some HSCs can still persist in the rodent liver into adulthood (6). In addition, the adult liver can again become a site for hematopoiesis when patients suffer from some diseases, for example, myeloproliferative disorders such as myelofibrosis and severe structural damage to the marrow secondary to infiltration by tumor cells. To my knowledge, hematopoietic stem cells, which have the potential to differentiate into hepatocytes and/or bile duct cells, may not differentiate into hepatocytes or bile duct cells in any other tissues or organs except the liver. The microenvironment in which the liver provides the stem cells may be important for hepatic differentiation even in adulthood, as well as in the fetus.

(c) Small Hepatocytes

Small hepatocytes have been identified as proliferating cells with hepatic characteristics. We first found a remarkable increase of small mononucleate cells within primary hepatocytes cultured in medium supplemented with 10 mM nicotinamide and EGF (7). A small hepatocyte can proliferate and form a colony. The growth speed is slow and 5 to 6 divisions occur within 10 days. The population of small hepatocytes in the adult rat liver is estimated to be 1.5–2.0% of hepato-

cytes, and the number of the cells decreases with age. The cells can also be isolated from the human liver and their clonal expansion has been demonstrated in culture. Although the cells can continue growing without losing hepatic characteristics for several months, the immortalization of the cells is so difficult that cell lines have not yet been established. On the other hand, clusters of small hepatocytes *in vivo* are observed in rodents with severe liver diseases resulting from treatment with hepatotoxins such as dipin, D-galactosamine, retrorsine and so on. Small hepatocytes also appear in human livers suffering from acute viral hepatitis and that have recovered from fulminant hepatitis. Furthermore, pathologists often observe small cell clusters in liver specimens from long-term chronic hepatitis and liver cirrhosis. Although the morphological and histological appearance of the "small cells" observed in *in vitro* and *in vivo* may be very similar, their equivalence has not been proved. As specific antibodies to small hepatocytes have not been found, the precise origin or location within the liver cannot be defined either.

DIFFERENTIATION/MATURATION OF HEPATIC STEM CELLS

The relationships between the cells of hepatic lineage mentioned above are shown in Fig. 1. In the late period of the fetal liver, hepatoblasts differentiate into small hepatocytes and the cells located in the periportal region may differentiate into bile duct cells. At the commitment of either differentiation of the liver cells, hematopoietic cells may play an important role because oncostatin M produced by CD45-positive hematopoietic cells in the fetal liver can induce the differentiation of hepatoblasts, from fetal to postnatal phenotypes, and make the microenvironment unfavorable for hematopoiesis (8). Therefore, most hematopoietic cells leave the liver for the bone marrow and spleen. However, some HSCs still remain in the liver (6). On the other hand, the cells in the canals of Hering are known to be c-*kit*$^+$ and/or CD34$^+$ (9). These results invite the speculation that some HSCs resident in or circulating to the liver may integrate into the canals of Hering and differentiate into hepatocytes or bile duct cells. Most hepatic cells may be small hepatocytes in the neonatal period and then actively divide with liver growth.

Small hepatocytes *in vitro* can maintain the ability of rapid proliferation unless hepatic nonparenchymal cells (NPCs) such as stellate cells grow and attach to them (10). When NPCs rapidly proliferate and invade under colonies of proliferating small hepatocytes, maturation of small hepatocytes is stimulated with accumulation of an extracellular matrix (ECM). The cells enlarge their cytoplasm that is rich in mitochondria, rough endoplasmic reticulum, peroxisomes and glycogen. Some cells possess two nuclei. Thereafter, alteration of the cellular morphology is attributed to the reconstruction of hepatic tissues, which may mimic hepatic plate formation (Fig. 2). Between the cells bile canaliculi are formed, and these move actively. On the other hand, proliferating small hepatocytes express hepatocyte nuclear factor 4 (HNF4), whereas the matured large hepatocytes express CCAAT/enhancer binding protein α and HNF6 in their nuclei as well as HNF4 (unpublished observation). Changes of cell shape may result in the sequential expression of liver-enriched transcription factors. Although it is unclear which ECMs can induce the maturation, these results also fuel speculation that, in pre- and postnatal periods, the arrangement of plates in liver lobules may be performed by the invasion of stellate and sinusoidal endothelial cells. Plates several thick become 1 or 2 cells thick accompanying the maturation of hepatic cells. When NPCs invade and secrete ECMs with forming sinusoids, hepatic cells are becoming mature. In adulthood, most dead hepatocytes may be compensated for by new hepatocytes formed when neighboring mature ones divide, and dead bile duct cells may be replaced the same way as hepatocytes. Some cells in the canals of Hering may contribute to the supplementation of hepatocytes and bile duct cells. When the cells in the canals of Hering die, the loss may be compensated for by circulating or resident HSCs. This may explain why a very small number of bone marrow-derived hepatocytes in liver plates was observed in male patients without lethal irradiation or any liver damage, to whom a female liver was transplanted.

At the time of an emergency of hepatic functions in the adult mammal liver, responses to the growth signals of each cell may be dependent on the degree of the damage: if the growth responses of mature hepatocytes are suppressed and/or the continuous loss of hepatocytes finally consumes the mature hepatocytes that can proliferate, the growth of small hepatocytes is activated and their clonal expansion occurs. In the case of severe damage to liver cells accompanying hepatic dysfunction, when, for example, most hepatocytes are dead and/or exposed to specific hepatotoxins, oval cells rapidly proliferate and differentiate into small hepatocytes and then mature hepatocytes to compensate for the loss. In this case, bone marrow cells will become a major source of oval cells.

FUTURE PROSPECTS

Although the mechanisms of fetal hepatic development have gradually been clarified, we do not have enough clues as to how the differentiation and/or maturation of hepatic stem cells occur in adulthood, from HSCs to oval/canal of Hering's cells or bile duct cells

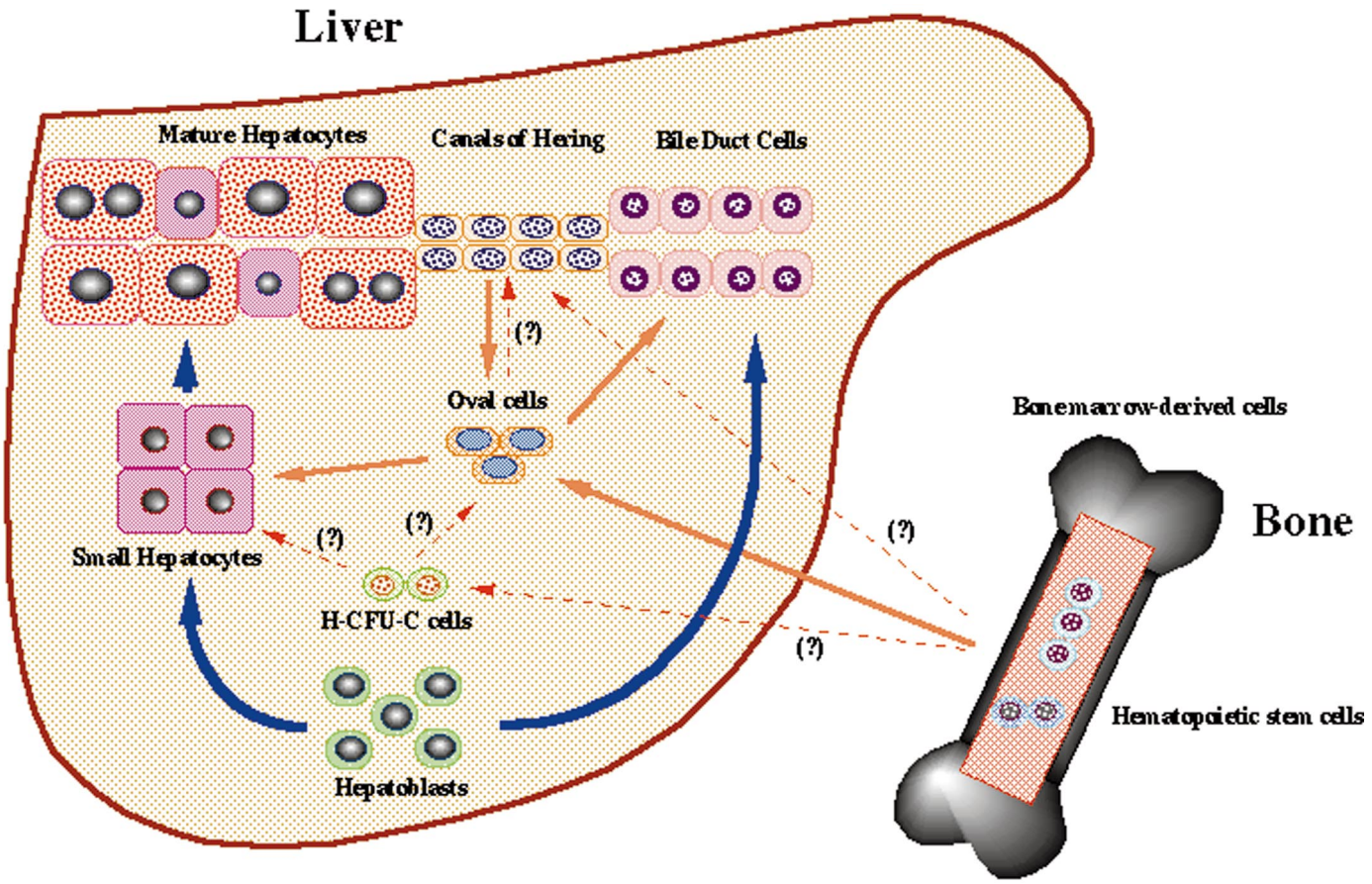

FIG. 1. Illustration of the interaction between hepatic stem cells. Thick blue arrows show the major stream of hepatic development and thin red arrows represent emergent routes. Dotted arrows are other routes for which convincing results are not yet available. H-CFU-C (c-*kit*$^-$/CD45$^-$/TER119$^-$) cells, which were isolated from murine fetal liver cells and express hepatic and bile ductular markers, were recently introduced as hepatic stem cells (11).

and from oval/canal of Hering's cells to hepatocytes or bile duct cells. We now know that, when hepatic stem cells are transplanted to the conditioned liver that possesses a genetic alteration, or to which a certain treatment has already been applied, the cells can be integrated into hepatic plates several months later. The cells then change their phenotype to that of mature and/or bile duct cells, and the liver sometimes replaces original hepatocytes with the descendants of transplanted ones. Although the entrance and the exit are so clear, the inside of the liver is a black box. To clarify the mechanisms of hepatic differentiation/maturation, we must establish new culture systems in which the proliferation and differentiation/maturation of hepatic stem cells can be freely manipulated and the interaction between hepatic stem cells and other types of liver cells such as stellate cells, sinusoidal endothelial cells, and Kupffer cells, can be investigated. Such investigations using hepatic stem cells will contribute to the construction of an artificial liver and the formation of hepatic tissues *ex vivo* as well as to cell transplantation therapy.

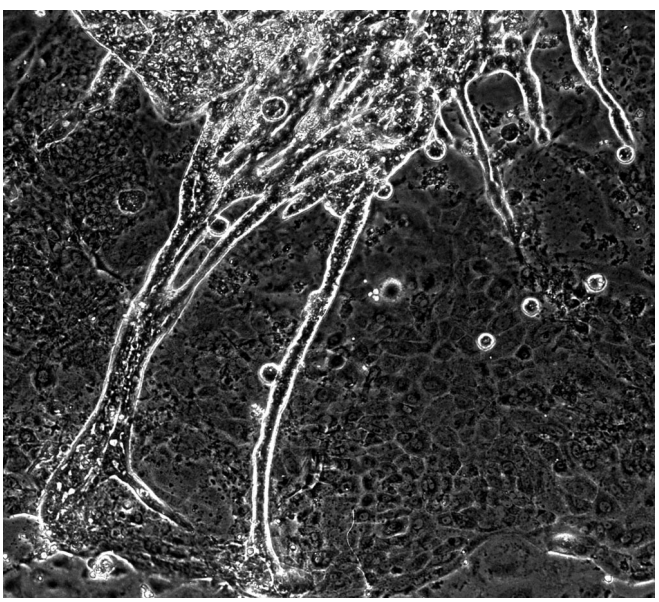

FIG. 2. A small hepatocyte colony possessing piled-up cells on the colony at day 84. The piled-up cells proliferate like forming hepatic plates, which consist of mature hepatocytes.

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