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Reconstruction of hepatic organoid by hepatic stem cells

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Abstract Recent advances in culture methods, stem cell research, and tissue engineering provide clues for making tissues in vitro that are functionally and structurally similar to hepatic tissues. To reconstruct hepatic organoids, two approaches to establish the methods have been proposed: the use of cells and the combination of cells and a scaffold (called *tissue engineering*). Recently, the coculture of hepatic cells (mature hepatocytes, small hepatocytes, hepatoblasts) and hepatic nonparenchymal cells has been reported to form hepatic organoids that possess differentiated hepatic functions. On the other hand, hepatocytes in a roller bottle were shown to form specific structures, consisting of biliary epithelial cells, connective tissue, mature hepatocytes, and endothelial cells. In this review, the studies of hepatic tissue formation in vitro will be summarized.

Key words Hepatic organoid · Stem/progenitor cells · Small hepatocytes · Reconstruction · Extracellular matrix · Hepatic nonparenchymal cells · Differentiation/maturation · Proliferation

Introduction

The liver is one of the most structurally and functionally complicated organs in mammals. However, it can be simply considered to be an aggregate of small histologic units, lobules, which, in cross-section, are organized like a polyhedral prism. It is roughly hexagonal, and at the angles of the hexagon are interlobular portal canals containing connective tissue stroma and the portal triads. A central vein runs in the center of a lobule. Each lobule consists of mature hepatocytes (MHs) and sinusoidal lining cells, such as endothelial cells, stellate (Ito) cells, Kupffer cells, and Pit cells. Portal triads include three different canals: a portal vein, a hepatic artery, and bile ducts. Of these cells, MHs play the most important role in hepatic functions, which are the: (1) metabolism of food, (2) production of plasma proteins, (3) synthesis and secretion of bile, (4) drug metabolism and detoxification, and (5) storage of vitamins and iron, and so on. Other cells work to help the MHs perform their functions as efficiently as they can, and for immunological protection.

Many researchers have attempted to resolve the switching mechanisms of hepatic growth and differentiation and to determine how to freely manipulate the growth and differentiation of cultured hepatocytes. If such methods can be established, it will be possible to reconstruct hepatic tissues ex vivo or in vitro. Recent advances in culture methods, stem cell research, and tissue engineering have provided clues as to how to make hepatic organoids functionally and structurally similar to hepatic tissues. To reconstruct a hepatic organoid, various experiments have been performed, and they have generally employed two approaches (Fig. 1): (1) the use of cells, and (2) a combination of cells and a scaffold (*tissue engineering*). Concerning tissue engineering, much literature is available[1] and one can refer to some excellent reviews.[2–5] This article is a review of recent studies of in-vitro hepatic tissue formation by cells and the contribution of stem/progenitor cells, especially small hepatocytes, to this formation.

Cell shape and differentiation

It has been considered to be very difficult to maintain differentiated hepatic functions and to get primary hepatocytes to proliferate. Under conventional conditions, which involve plating cells on a substratum of dried rat tail collagen in the presence of epidermal growth factor (EGF) and insulin, culture of freshly isolated hepatocytes is accompanied by an extensive and fairly rapid loss of liver-specific gene transcription.[6,7]

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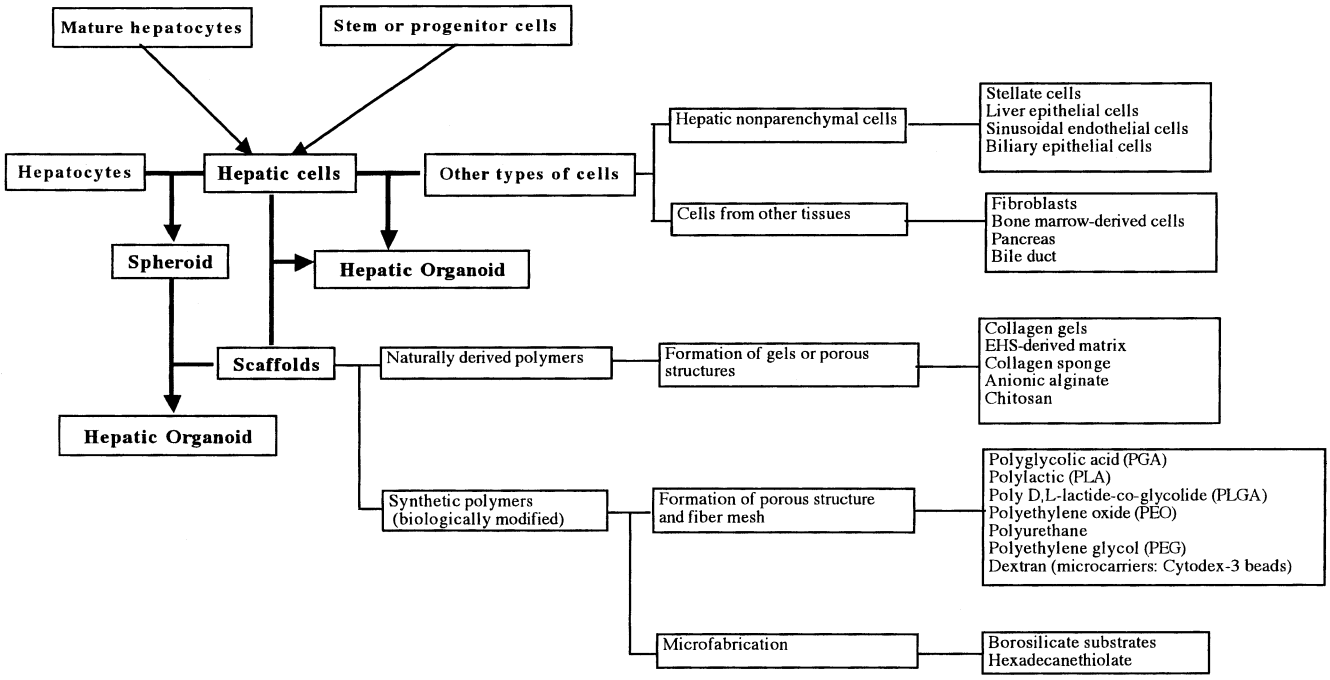

Fig. 1. Methods for the reconstruction of hepatic organoid: the source of cells and scaffolds. *EHS*, Engelbreth-Holm-Swarm; Cytodex-3 beads (Amersham)

Therefore, for the purpose of maintaining the differentiated functions, investigators have added various substances to the culture medium: for example, phenobarbital,[8] dimethylsulfoxide (DMSO),[9–15] and nicotinamide.[16–18] Furthermore, MHs were cocultured with nonparenchymal cells (NPCs).[19] In addition, various substrata have been used as beds for the cells, e.g., biomatrices,[20] proteoglycans,[21] collagen gel,[22,23] and Engelbreth-Holm-Swarm (EHS) gel (Matrigel; Becton Dickinson Labware).[6,24–26] These modifications of the culture conditions have improved the longevity of primary cells maintaining differentiated hepatic functions. During the study of hepatic differentiation, researchers have recognized the relationship between the cell shape and differentiated hepatic functions. The importance of cell shape was shown in a study by Landry et al.,[27] who demonstrated that isolated liver cells could survive for 5 to 6 weeks even in the absence of an adhesive surface for attachment and retain a considerable degree of liver-specific functions by aggregating into "spheroids," which approximate the organization of the normal liver. The formation of aggregates/spheroids resulted in the maintenance of highly differentiated functions for several weeks.[28–30] Cells grown in such configurations are more cuboidal than those grown on plastic or on matrix proteins coated on plastic, and may have the capacity to form more extensive cell–cell contacts with their neighbors than flattened cells can. Furthermore, Clayton et al.[31] demonstrated that transcription of liver-specific genes was better maintained in liver slices than in dispersed cells, confirming that the three-dimensional organization is important for differentiation. Recently, by the use of a method for the fabrication of a rubber stamp and the creation of patterned substrata, Singhvi et al.[32] showed that limiting the degree of heptocyte extension provided control over the growth of the cell and albumin secretion. Thus, the achievement of three-dimensionality is essential for hepatocytes to display their ability.[33]

Growth of hepatocytes

In the adult liver, most hepatocytes are quiescent and fully differentiated. However, once some hepatocytes are lost, the liver immediately responds and the replication of neighboring hepatocytes compensates for the loss. We can practically see the dramatic recovery of the mass after a two-thirds surgical resection of the liver in rodents. In spite of hepatic growth ability in vivo, it took a long time to establish experimental conditions in which MHs could continue to grow in vitro. The use of a high concentration of nicotinamide, which is an aqueous vitamin, finally brought about cell proliferation on a culture dish.[16–18] We then showed that nutrient-rich medium, especially with amino acids, was important for primary hepatocytes to grow, and that the proliferating cells possessed some differentiated functions.[34] In

addition, the expression of tryptophan 2,3-dioxygenase (TO),[35] serine dehydratase (SDH),[35] connexin32 (Cx32), and Cx26,[14,36] which are thought to be highly differentiated functions, could be induced by 2% DMSO in cells cultured for more than 10 days. Furthermore, liver-enriched transcription factors, such as hepatocyte nuclear factor 4 (HNF4), CCAAT/enhancer binding protein α (C/EBPα), and C/EBPβ, which are expressed in MHs, were reexpressed in the DMSO-induced cells.[37] Nakamura et al.[38] reported a reciprocal relationship between growth activity and differentiated functions in cultured hepatocytes, and that the growth activity in the DMSO-induced differentiated cells was almost completely suppressed. Using a combination of these culture methods, a growth-differentiation-growth protocol for primary hepatocytes was introduced.[39] Although the survival of primary hepatocytes with differentiated hepatic functions can be prolonged, a limitation of the number of cell divisions may exist in primary hepatocytes isolated from the adult rodent liver. Thus, for making a hepatic organoid in culture, we need to use cells that possess high growth potential and a certain degree of hepatic function, that is, hepatic stem/progenitor cells.

Small hepatocytes

Until now, many cells have been introduced as hepatic stem/progenitor cells.[40–45] Most of these cells have the potential to differentiate into hepatocytes only in vivo when the cells are transplanted into syngeneic animals. In addition, some of the cells can expresses albumin and/or cytokeratin 8/18, and a small number of them can express TO and/or tyrosine aminotransferase. Thus, the induction of hepatic differentiation/maturation of the stem/progenitor cells has not succeeded well in culture.

Small hepatocytes (SHs) have been identified as proliferating cells with hepatic characteristics. We first found a remarkable increase of small mononucleated cells within primary hepatocytes cultured in medium supplemented with 10 mM nicotinamide and EGF.[46] The population of SHs in the adult rat liver is estimated to be 1.5%–2.0% of all hepatocytes, and the number of the cells decreases with age.[47] The cells could also be isolated from the human liver, and their clonal expansion was demonstrated in culture.[48] When isolated SHs are cultured in a collagen-coated dish, the first cell division is observed from day 2 to day 3, and they then proliferate to form a colony. The average number of cells in a colony reaches about 30 at 10 days after plating.[49] In our culture conditions, SHs are cocultured with NPCs, such as stellate (Ito) cells, liver epithelial cells, Kupffer cells, and sinusoidal endothelial cells. Three to four percent of the plated cells can form SH colonies.[50] Although nicotinamide and fetal bovine serum (FBS) are essential for the development of the colonies in the early culture, the cells can grow without FBS and EGF after they form relatively large colonies. Hepatocyte growth factor (HGF), transforming growth factor (TGF)-α, and fibroblast growth factor, which can stimulate the proliferation of MHs, also have the potential to induce colony formation. Although the exclusion of hepatic NPCs at the time of plating results in the suppression of colony formation, the conditioned medium from Swiss 3T3 cells or the addition of pleiotrophin to the culture medium can stimulate the growth of purified SHs.[51] On the other hand, we observed that the proliferation of SHs was remarkably promoted when 1% DMSO was added to the culture medium. The growth was especially enhanced when the cells were cultured on collagen gels (Fig. 2). The area of a colony cultured on collagen gel with 1% DMSO was more than fivefold larger than that of a colony cultured on a collagen-coated dish at 30 days after plating (Table 1). In these culture conditions, the growth of NPCs was apparently inhibited. Furthermore, when the SH colonies were separated from the culture dishes and transferred to a new dish, the SHs rapidly proliferated, but the growth of other NPCs, which attached to the isolated colonies, was suppressed. It is of interest that isolated colonies can be cryopreserved for more than 1 year and that the thawed cells can proliferate.[52] During cryopreservation, many NPCs were killed, so that relatively purified colonies of SHs could expand after replating. These results suggest that SHs may require a certain factor(s) produced by co-cultured cells in the early culture period. However, once SHs start to proliferate and form a colony, the cells may grow without exogenous growth factor(s) and the colony can continue expanding.

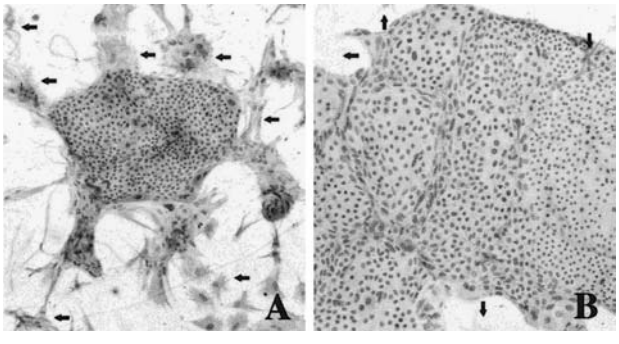

Fig. 2A,B. The growth of small hepatocyte (SH) colonies on collagen gels. The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with (**B**) or without (**A**) 1% dimethylsulfoxide (DMSO) on day 20. The cells were fixed, and immunocytochemistry for vimentin was carried out.[50] A few nonparenchymal cells (NPCs; *arrows*) are observed in **B**, whereas many NPCs (*arrows*) surround the small hepatocyte colony (**A**)

Table 1. Growth of small hepatocytes on collagen gels

Substratum	DMSO (%)	CK8-positive area (%)		
		Day 10	Day 20	Day 30
Rat-tail collagen coat	(+)	6.53 ± 0.15	11.70 ± 0.26	13.42 ± 1.41
Collagen gel	(−)	12.20 ± 3.46	28.40 ± 3.29	42.76 ± 6.55
	(+)	14.00 ± 1.00	39.25 ± 2.30	68.06 ± 6.25

The cells isolated from an adult rat liver were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 10 mM nicotinamide, 1 mM ascorbic acid 2-phosphatase, and 10 ng/ml epidermal growth factor (EGF);[50] 3×10^5 cells were plated on 35-mm dishes and 1% dimethylsulfoxide (DMSO) was added to the medium from day 4. The cells were fixed and immunocytochemistry for CK8 was carried out. The areas of CK8-positive cells on dishes were calculated. The numbers show percentages (CK8-positive area/culture area of dish × 100)

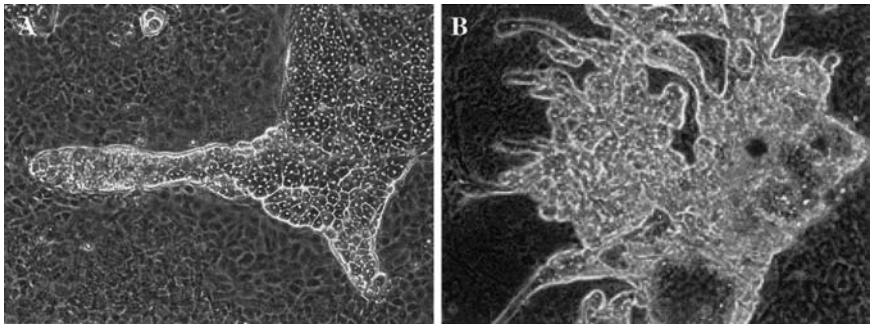

Fig. 3A,B. Phase-contrast photographs of cells cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 10 mM nicotinamide, 1 mM ascorbic acid 2-phosphate, 10 ng/ml epidermal growth factor (EGF), and antibiotics. From day 4, 1% DMSO was added to the medium. **A** At 31 days after plating, the piled-up cells elongate from a SH colony and form a liver plate-like structure; **B** 500 μg of Matrigel (Becton Dickinson Labware) was added to the cells on day 28, and this photograph was taken on day 58. Many plate-like structures protrude from the colony

Hepatic organoid formation

We previously showed that SHs in a colony sometimes change shape.[50] Most cells not completely surrounded by NPCs remained small and flat, whereas some SHs surrounded by NPCs gradually changed shape and looked as if they were piling up on the colony. In our culture conditions, about one fourth of the colonies possessed piled-up cells at 30 days after plating.[53] When NPCs rapidly proliferate and invade under colonies of proliferating SHs, maturation of SHs is stimulated with the accumulation of extracellular matrix (ECM). The enlarged cytoplasm of the cells is rich in mitochondria, rough endoplasmic reticulum, peroxisomes, and glycogen. Some cells possess two nuclei, but the appearance of multiple nuclei and the formation of giant cells, features which are often observed in primary hepatocytes stimulated by EGF, do not occur. Thereafer, alteration of the cellular morphology is attributed to the reconstruction of hepatic tissues, which may mimic hepatic plate formation (Fig. 3A). Between the cells, bile canaliculi (BC) are formed, and BC membrane proteins, such as dipeptidylpeptidase IV, ectoATPase, and multidrug-resistant-related protein 2 localize along the structures. In addition, these canals can move actively (Sudo et al., submitted for publication). On the other hand, proliferating SHs express HNF4, whereas the mature large/piled-up hepatocytes express C/EBPα and HNF6 in their nuclei, as well as HNF4.[54] Changes of cell shape may result in the sequential expression of liver-enriched transcription factors.

Accumulation of ECM may result in morphological changes and the maturation of SHs. Similarly, the overlay of Matrigel can induce rapid morphological changes in SHs; piled-up cells slowly proliferate, form plate-like structures, and the plates gradually elongate (Fig. 3B). By contrast, it is well known that DNA synthesis of MHs on Matrigel is inhibited and that the cells form spheroids.[2,6,25,26] Therefore, the formation of a liver plate-like structure has been observed neither in primary hepatocytes nor in differentiated hepatoma cell lines, using Matrigel. However, fetal rat hepatocytes cultured in medium supplemented with TGF-β and EGF were reported to form three-dimensionally elongated, cord-like structures.[55] In addition, Michalopoulos et al.[56] showed that MHs isolated from an adult rat liver could form plate-like structures within Matrigel after they were cultured on collagen-coated polystyrene

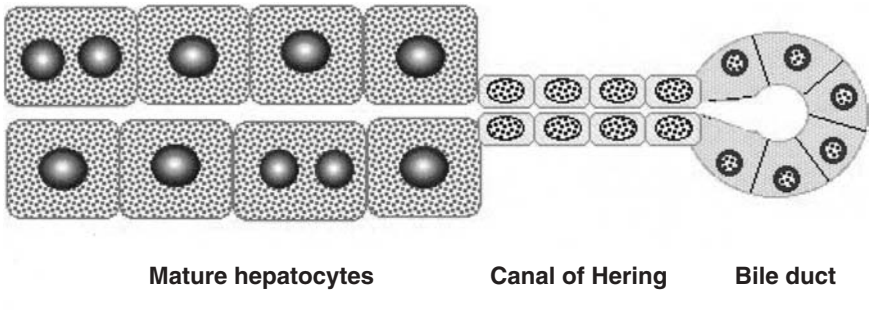

Fig. 4. Illustration of minimum hepatic unit. The unit consists of mature hepatocytes (MHs), cells in canals of Hering, and bile duct cells

beads in a roller bottle. In their experiment, the epithelial cells on beads had characteristics of small MHs and, after the cells on beads were implanted in Matrigel, the elongation of plate-like protrusions was observed. The protrusions consisted of hepatocytes showing the characteristics of well-differentiated hepatocytes compared with the cells before implantation.

Recently, Michalopoulos et al.[57] also reported that primary rat hepatocytes cultured in a collagen-coated pleated-surface roller bottle formed a characteristic tissue architecture composed of a superficial layer of biliary epithelial cells (BECs), an intermediate layer of connective tissue and hepatocytes, and a basal layer of endothelial cells. EGF, HGF, and dexamethasone were essential to form the complete histological structures. However, the cells in the NPC fraction after low-gravity centrifugation (we usually use this fraction as an SH-rich fraction) could not form a specific structure under their culture conditions. Although about half of the primary heptocytes died in the early culture period and a quite small number of BECs (less than 0.05%) were involved in the plated cells, the specific structure consisted of MHs and stromal cells, which were superficially covered with BECs. Concerning the origin of the BECs, Michalopoulos et al.[57] suggested a close relationship with the appearance of immature SH precursors in the early culture. These small cells expressed cytokeratin 19 (CK 19), which is a marker of BECs, and the appearance of CK 19 explains the redifferentiation of the immature hepatocytes that happen to be on the surface of the tissue, as Block et al.[58] showed that MHs lost their differentiated functions to become hepatoblasts. Furthermore, primary rodent hepatocytes and immortalized mouse hepatocytes showed the formation of bile duct-like structures, the cells of which expressed CK19, when the cells were cultured in medium supplemented with fibroblast-conditioned medium or with both EGF and insulin.[59] On the other hand, Michalopoulos et al.[57] suggested that the existence of MHs might be very important for the formation of hepatic organoids and that the expansion of the mesenchymal stromal cells might be dependent on the presence of unknown factors provided by hepatocytes. Similarly, additional factors derived from hepatocytes were reported to be more effective for the tubulogenesis of isolated human BECs sandwiched between collagen gels than the combination of EGF and HGF.[60] These results suggest that the interactions of hepatocytes and NPCs are very important for reconstructing hepatic tissues. Thus, as the mechanisms of the differentiation/maturation of hepatocytes and the formation of hepatic organoids are not well understood, further intensive studies are necessary.

Perspectives

Cell culture is a closed system. As the technology of hepatocyte culture progresses, the expression of differentiated hepatic functions of the cells has much improved compared with that seen a decade ago. Although researchers paid attention to the capacity for albumin secretion, ammonia metabolism, urea synthesis, and so on in those days, we must now be aware of the excretion of bile pigment, bile salts, or potentially toxic waste products, especially when considering the use of the hepatic tissues for an artificial liver system. It will be necessary to design a ductular component that is actually bound to the hepatic tissues.

Ignoring their angioarchitecture, liver lobules may be simplified as an aggregate of hepatic plates and bile ducts. As shown in Fig. 4, the small hepatic tissues, which consist of MHs, cells in the canals of Hering, and BECs, are thought to be the minimal units of hepatic function, based on bile secretion. Secreted bile can pass through BC and pour into bile ducts. Cells in the canal of Hering combine BC with bile ducts. As described above, BC can be reformed between the differentiated hepatocytes of the plate-like structures, and cystic structures are formed in the tips of the plates.[50] When bilirubin is added to the culture medium, its accumulation is observed in the cystic regions (Sudo et al., submitted for publication). Otherwise, bile ductules reformed by BECs may have the possibility to combine with the hepatic tissues reconstructed by hepatocytes and NPCs. As for the cells in the canal of Hering, bone marrow-

derived cells have been shown to differentiate into such cells.[61] Although we have no proof as yet, the coculture of bone marrow cells with hepatocytes and NPCs may result in the creation of hepatic tissues with a drainage system for bile.

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