width of the BC. The fluorescent intensity in intercellular spaces was detected after FD treatment [Fig. 1(B)]. The fluorescein localized in the restricted areas of intercellular spaces that represented BC networks. Under phase-contrast microscopy, the merged images showed translucent belts that coincided with fluorescent networks [Fig. 1(C)]. These results suggested that the translucent belts observed under phase-contrast microscopy corresponded exactly to the width of the BC. Therefore, BC movements were analyzed based on variations in the translucent areas under phase-contrast microscopy. In order to quantify the translucent areas, thresholding was applied to phase-contrast micrographs to coincide with the translucent belts [Fig. 1(D)]. This thresholding was feasible because the thresholding images [red area, Fig. 1(D)] corresponded to the fluorescent images [green area, Fig. 1B)].

BC Movements in the Piled-Up Colonies

Time-lapse microscopy revealed that BC movements occurred in the piled-up cells. Figure 2 shows an SH colony composed of the piled-up cells that form the BC. In the region indicated with rectangle *a* in Fig. 2(A), the left BC contracted in the early period of the experiment [left arrowheads, Figs. 2(B) and (D)]. The right BC contracted next [right arrowheads, Figs. 2(D) and (F)]. On the other hand, BC in rectangle *b* remained wide or dilated while the BC in rectangle *a* contracted [arrowheads, Figs. 2(C), (E), (G), (I)]. In addition, the cystic structure that formed in the tip of the colony [asterisk, Fig. 2(A)] dilated following the BC contraction in rectangle *a* and the dilation in rectangle *b* (data not shown). These sequential movements of BC suggested a flow of the fluid toward the cystic structure.

Some BC contractions were synchronized (Fig. 3). BC in the top rectangle [rectangle, Fig. 3(A)] contracted synchronously when the BC in the lower rectangle [rectangle, Fig. 3(A)] contracted (arrowheads, Figs. 3(C–F)). To clarify this phenomenon, the BC movements were analyzed using the thresholding methods. The thresholding was applied to each image of the time-lapse series and ROIs were set on each image [a, b, Fig. 3(B)]. Variations of the normalized thresholding areas within each ROI are graphically shown in Fig. 3(G). The BC contraction that was observed in the time-lapse images was consistent with the variations of the normalized thresholding area in the graph [between 82 min and 107 min, Fig. 3(G)]. The maximum amplitude of each graph [amp, Fig. 3(G)], which indicates the degree of the BC movement in each ROI, was calculated by subtracting the minimum value [min, Fig. 3(G)] from the maximum value [max, Fig. 3(G)] in the experimental period.

In the present experiment we analyzed 68 ROIs of BC in 17 colonies. Some BC dramatically contracted or dilated, others showed small morphological changes. Each BC had its own motility, which was represented by a maximum amplitude. To show the motility of the BC, the maximum

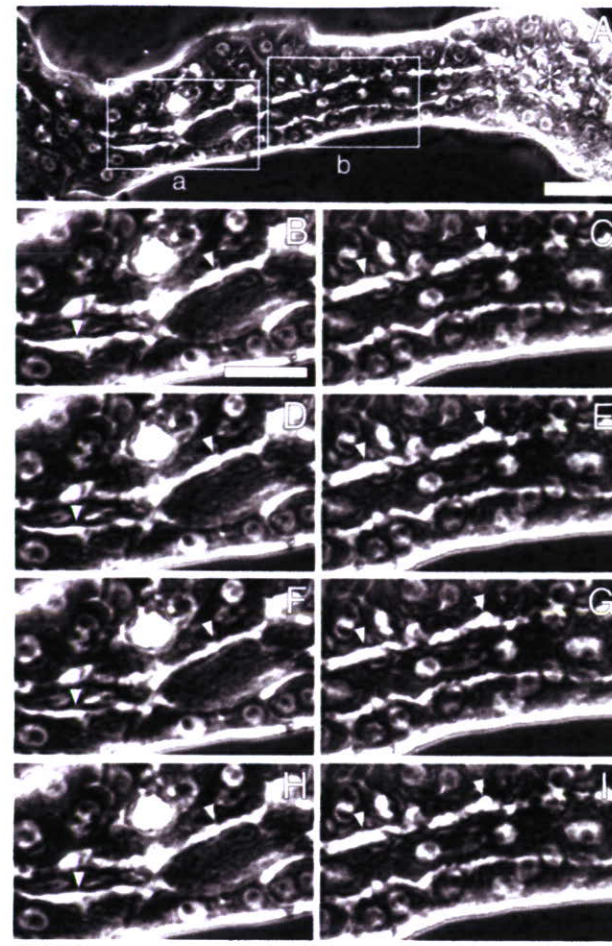

FIGURE 2. Phase-contrast micrographs of the cells that form BC. A: Parallel BC developed in the piled-up cells at day 27. B–I: Enlarged time-lapse images of the same part indicated by the rectangle *a* (B, D, F, H) and *b* (C, E, G, I) in A. The total number of the time-lapse images was 2160. The frame numbers are 75 (B, C), 79 (D, E), 82 (F, G), and 100 (H, I), respectively. BC contractions were detected in the sequential images (arrowheads). Scale bars, 50 μm (A) and 30 μm (B).

amplitude of each BC movement was calculated and represented in the form of a histogram (Fig. 4). The maximum amplitude had a wide range, varying from 0.2 to 1.8, and no frequency at the range of 0.0–0.2. These results indicate that the BC of each colony had various kinds of motility, and all BC had motility of at least 0.2 in the experimental period. In addition, the most probable motility was in the range of 0.4–0.6 (Fig. 4), which suggests the area was increased or decreased by 40–60% when compared with the initial BC area.

Coordination of the BC Movements

Analysis of the time-lapse images showed that coordination of BC contractions and dilations appeared to be present. We classified BC movements according to the speed of movement: BC movements within 10 min (fast)

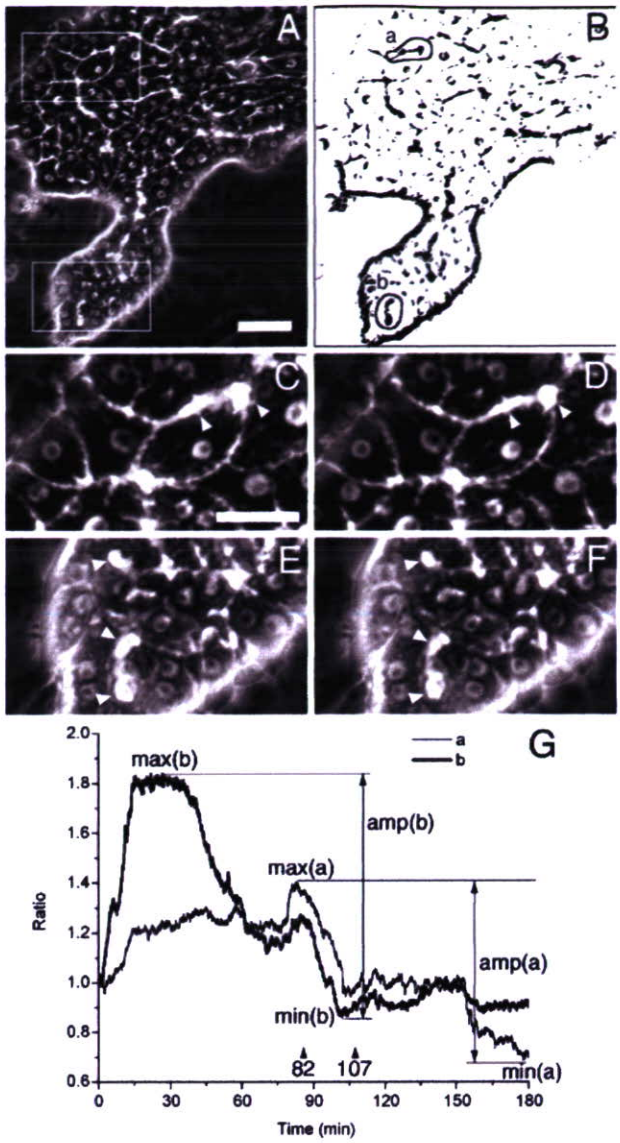

FIGURE 3. Phase-contrast micrographs and graphical representation of BC movements. A: Phase-contrast micrograph of the piled-up cells at day 23. B: Thresholding was applied to A. ROIs were set on the image (a, b). C–F: Enlarged images of the same parts indicated by rectangles in A (top rectangle: C–D, lower rectangle: E–F). Images correspond to the times of 82 min (C, E) and 107 min (D, F), indicated by arrowheads in G. G: Graphical representation of BC movements in two ROIs (a, b, B). The thresholding area within the ROIs of each time was calculated and the values were normalized by dividing each value by the initial value. Maximum amplitude of each graph is shown by vertical arrows designated amp (a) and amp (b). Scale Bars, 50 μm (A) and 30 μm (C).

and those that continued for more than 30 min (slow). We also classified BC movements according to whether the movements were coordinated or not. Visual analysis of the movie showed that all colonies had BC movements, and 94% of those were coordinated (Table 1).

By further observation of time-lapse images, we found that BC movements could be classified into three types.

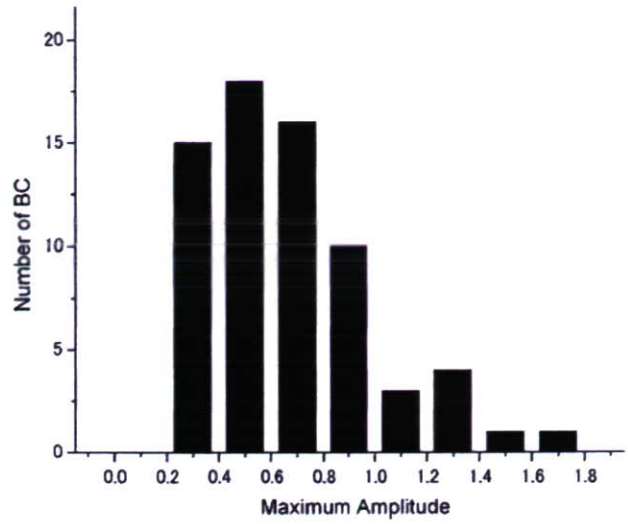

FIGURE 4. Maximum amplitude histogram of the total BC in ROI. In this experiment, 68 regions of BC (4 ROIs/colony and 17 colonies) were analyzed and the maximum amplitude of the graph in the experimental period (3 h) was measured. Note that there is a peak at the maximum amplitude of 0.4–0.6. There is no frequency at the maximum amplitude of 0.0–0.2.

Therefore, we show three typical types of BC movement in Fig. 5 although 17 colonies were analyzed. Figure 5 shows BC movements in three colonies in which different types of movement occurred. BC dramatically contracted and dilated as shown in Figs. 5(C), (F), and (I), and coordination of movements was observed. The coordination was observed in the contractions of both fast and slow types and the observation periods are indicated by the closed box [p1–9, Figs. 5(C), (F), (I)]. For quantitative analysis of the coordination, we calculated correlation coefficients during periods p1–9, and the values are shown in Table 2. Thus, Fig. 5 shows the BC movements and the values in Table 2 show the degree of the coordination corresponding to Fig. 5. In this study, BC movements with an absolute correlation coefficient value of more than 0.66, the average value, were presumed to be coordinated. At least one correlation coefficient between the ROIs was more than 0.66 in all periods (asterisks, Table 2), which indicates that BC movements occurring in neighboring areas were coordinated. (1) Figures 5(A–C) show the BC movements which occurred throughout the colony. There were three contractions in the experimental period [arrowheads, Fig. 5(C)]. Figure 5(C) and Table 2 show that in the contraction in period p1 [p1, Fig. 5(C)], the values of the correlation coefficients between #1–#4, #2–#3, #2–#4, and #3–#4 were 0.67, 0.93, 0.96, and 0.97, respectively (p1, Table 2). Similarly, in the contractions at periods p2 and p4 the correlation coefficients between each ROI were more than 0.86 (p2, p4, Table 2). Thus, although the BC in region #1 was about 10 cells distant from that in region #4, BC around the areas including #1, #2, #3, and #4 were synchronously moving. (2)

TABLE 1. Motilities of the BC networks in SH colonies

	Fast		Slow		
	Contraction	Dilatation	Contraction	Dilatation	Either
Movement	10 (59%)	3 (18%)	11 (65%)	16 (94%)	17 (100%)
Coordination	9 (53%)	3 (18%)	8 (47%)	9 (53%)	16 (94%)

Note. Each value indicates the number of colonies in which BC movements (upper) or coordinated movements (lower) occurred in the experimental period. Percentages are in brackets. BC movements of 17 colonies were classified into two types depending on the rate of movement, BC movements within 10 min (fast) and those of more than 30 min (slow).

Figures 5(D–F) show the BC movements, speed of which was relatively fast, and cellular contraction can be observed. The correlation coefficients at periods p5 and p6 showed negative values, proving the BC dilations (#4) were followed by a BC contraction (#1) in the neighboring area (p5–6, Table 2). (3) Figures 5(G–I) show the BC movements, the speed of which was relatively slow. The correlation coefficient between #1–#3 at the period p8 was positive, while that at the period p9 was negative (p8–9, Table 2). These results indicated that BC both in #1 and #3 dilated at period p8, although the BC in #1 contracted at period p9 while BC in #3 dilated.

CB Treatment

CB inhibits polymerization of actin by binding to the barbed ends of actin filaments.[2,4,6] The agent has been widely used to study the role of actin in biological processes.[4] Numerous actin filaments have been observed around the BC in hepatocytes, which suggests that they play an important role in BC contraction.[29] Therefore, the role of actin in BC contraction was studied using CB.

As shown in Fig. 6, the morphology of BC in piled-up cells treated with CB dramatically changed. The BC started to dilate soon after the addition of CB. When the cells were treated with 1.0 μg/ml CB, BC became progressively dilated for 1 h, and then maintained that width for more than 2 h [Figs. 6(A–C)]. On the other hand, when the cells were treated with 5.0 μg/ml CB, BC became progressively dilated throughout the experimental period, and cystic structures were observed [arrowheads, Fig. 6(F) inset]. Cystic dilation of BC was noticeable near the edges of the colony. Remarkable dilations of the BC network were observed at 60 min after addition of CB [Fig. 6(E)], and canalicular structures changed into cystic ones after 180 min [Fig. 6(F)]. The motility of BC was reduced, and no contraction was observed when the cells were treated with CB at concentrations of more than 1.0 μg/ml. In contrast, when the cells were treated with low concentration (0.1 μg/ml) of CB, no remarkable dilatations of BC were observed (data not shown).

As shown in Fig. 7, actin filaments were abundant around BC in the cells treated with each concentration of CB. The actin filaments around the BC revealed narrow spaces between them in cells without CB treatment [arrowheads, Fig. 7(A)]. On the other hand, when the cells were treated with 1.0 μg/ml CB, the actin filaments around the BC were wider [arrowheads, Fig. 7(B)]. In addition, when the cells were treated with a high concentration (5.0 μg/ml) of CB, the actin filaments showed indented distribution around the dilated BC [Fig. 7(C)]. Some actin filaments were also distributed in the cytoplasm [asterisks, Fig. 7(C)], and increased intensity of positivity was heterogeneously observed along the BC [arrowheads, Fig. 7(C)].

Immunocytochemistry for Cx 32

Immunocytochemistry for Cx 32 was carried out to determine whether the piled-up cells had the ability to communicate via gap junctions. Immunocytochemistry for actin was also carried out to visualize cell–cell borders. Actin in the piled-up cells was intensively stained around the BC [inset, Fig. 8(C)]. Cortical actin was observed in those SHs that form no BC between the cells [inset, Fig. 8(G)]. Dotted expression of Cx 32 was distributed on basolateral membranes that coincided with the translucent belts formed in the piled-up cells, although the expression had heterogeneity [Figs. 8(A–D)]. By contrast, no Cx 32 was expressed in the cytoplasm of the flat cells and no localization was observed [Figs. 8(E–H)].

Secretion of Microinjected Dye in Piled-Up Cells

CMFDA was microinjected into one of the piled-up cells in the colony [asterisk, Fig. 9(A)]. It was metabolized within the injected cell and the metabolite was excreted into BC by a transporter such as MRP2. As previously reported, MRP2 is uniformly expressed on BC membranes of the piled-up cells.[35] As shown in Fig. 9(C), soon after the injection bright fluorescence was observed in the cytoplasm of the cell and a line of fluorescence extended in the direction of the tip of the colony. Two and half minutes later, the dye was clearly detected in four cells distant from the injected cell [arrowhead,

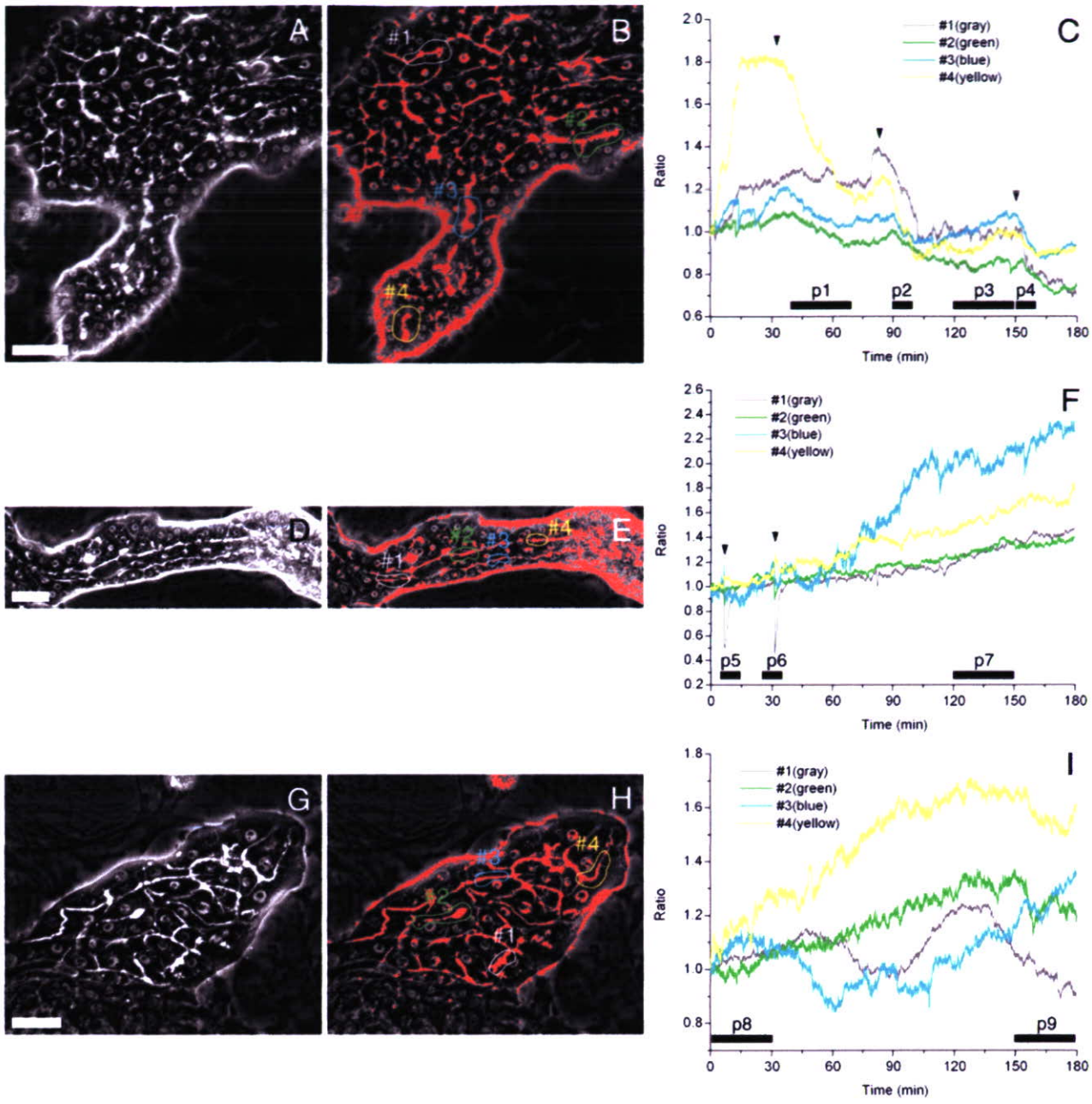

FIGURE 5. Corresponding phase-contrast (A, D, G), thresholding images (B, E, H) and graphs (C, F, I). Thresholding was applied to phase-contrast images of the piled-up cells at A: day 23, D: day 27, and G: day23, and B, E, H: the merged images of each phase-contrast and thresholding image are shown. C, F, I: The graphs indicate the variations of the thresholding area in each ROI set on the thresholding image (#1–4, B, E, H), normalized by division by the value at the initial time in the experimental period. Closed boxes (p1–9, C, F, I) indicate observation periods in which correlation coefficients were calculated. Arrowheads indicate contractions. Scale bars, 50 μm (A, D, G).

Fig. 9(E)]. The intensity was observed within BC branching toward the tip of the colony. In the piled-up cells, Cx 32 was expressed along the BC. Therefore, the dye might have been transferred through gap junctions although cells adjacent to the injected one were only faintly stained. The fluorescence intensity increased in the route of the transferred dye for several minutes and then gradually decreased.

DISCUSSION

Coordination of BC Contraction

The BC form a pathway to transport bile secreted by hepatocytes. Transportation of bile is very important because disorders of bile transportation are associated with cholestasis. Therefore, motility of BC is necessary for the

TABLE 2. Correlation coefficients between BC movements in different ROIs

		#1	#2	#3	#4
p1	#1	1.00			
	#2	0.65	1.00		
	#3	0.61	0.93*	1.00	
	#4	0.67*	0.96*	0.97*	1.00
p2	#1	1.00			
	#2	0.93*	1.00		
	#3	0.88*	0.92*	1.00	
	#4	0.96*	0.95*	0.94*	1.00
p3	#1	1.00			
	#2	0.12	1.00		
	#3	−0.15	0.41	1.00	
	#4	−0.17	0.47	0.92*	1.00
p4	#1	1.00			
	#2	0.90*	1.00		
	#3	0.95*	0.88*	1.00	
	#4	0.96*	0.86*	0.95*	1.00
p5	#1	1.00			
	#2	0.43	1.00		
	#3	−0.19	−0.47	1.00	
	#4	−0.67*	−0.16	−0.24	1.00
p6	#1	1.00			
	#2	0.95*	1.00		
	#3	0.35	0.26	1.00	
	#4	−0.89*	−0.92*	−0.13	1.00
p7	#1	1.00			
	#2	0.83*	1.00		
	#3	0.02	0.04	1.00	
	#4	0.85*	0.86*	0.06	1.00
p8	#1	1.00			
	#2	0.50	1.00		
	#3	0.61	0.06	1.00	
	#4	0.74*	0.70*	0.40	1.00
p9	#1	1.00			
	#2	0.41	1.00		
	#3	−0.75*	−0.27	1.00	
	#4	0.69*	0.57	−0.40	1.00

Note. Correlation coefficients between pairs of thresholding areas in each ROI (#1–4, Figs. 5B, E, H) during periods indicated by a closed box (p1–9, Figs. 5C, F, I) were calculated. Coefficients whose absolute values were more than 0.66 are presumed to be coordinated (asterisks).

physiological function. In addition, coordination of BC movements is sequential and synchronous, and therefore essential for transporting bile into bile ducts. BC contractions were first observed in hepatocyte couplets, intact pairs of hepatocytes isolated from rat livers.[26] Subsequent studies using couplets demonstrated BC contractions,[27,34] the Ca^{2+}-induced BC contractions,[43] and some agents to increase the BC contractions.[8,22,45] Although hepatocyte couplets are useful models for analysis of BC contractions, coordination of adjacent hepatocytes along BC could not be investigated because BC in couplets form a closed sphere, whereas the BC *in vivo* form a tubular structure. BC movements and the coordination of these movements have not been investigated in studies that investigated BC formation using the collagen gel sandwich method[12,38] and hepatocyte spheroid culture.[10,11] Therefore, this BC network made it possible to analyze the coordination of the SHs required for tissue-level function.

In the present experiment we analyzed BC movements to determine whether they were coordinated. Time-lapse microscopy revealed sequential contraction along the BC (Fig. 2) and coordination of BC contraction (Fig. 5). The degree of the coordination was quantified to calculate the correlation coefficient. Although each BC had its own motility (Fig. 4), the values of the correlation coefficients during the observed periods (p1–9, Fig. 5) were more than 0.66, which suggests coordination of the BC contraction (Fig. 5, Table 2). These results suggest that individual cells forming BC act in a coordinated manner to achieve an integrated function for the transportation of bile.

Intercellular Communication for the Coordination

Various intracellular processes in the liver are known to be coordinated by second messengers such as cAMP, cytosolic Ca^{2+}, and inositol trisphosphate.[3,7,31,39,44] An organ-level response to hormonal stimuli may be produced by integration of signals among hepatocytes within the liver plate.[23,24,40] Nathanson *et al.*[25] reported that the organization of second messenger signals across the hepatic lobule played an important role in hormonal regulation of bile secretion in the perfused liver. Serriere *et al.*[33] reported that unidirectional agonist-induced intercellular Ca^{2+} waves via gap junctions might drive canalicular peristalsis and increase bile flow.

To reconstruct hepatic tissues, proliferation and interaction of SHs and NPCs are essential. It has been reported that hepatocytes can maintain viability for extended periods when the cells form three-dimensional aggregates like spheroids.[10,11] Although the hepatocytes in spheroids could express differentiated functions and form BC-like structures, apical domains of these cells were gradually lost with time in culture.[1] On the other hand, after SHs proliferate and form colonies within a couple of weeks, matured SHs appear on the colonies, in which BC networks are well developed.

In the present experiment we investigated the cellular ability for the intercellular communication to immunostain gap junctional protein Cx 32. The piled-up cells expressed Cx 32 in the basolateral membranes along BC networks although no expression of Cx 32 in the basolateral membranes was observed in the proliferating SHs that were not mature and formed no BC (Fig. 8). Cx 32 was expressed heterogeneously along BC networks (Fig. 8), which suggested the heterogeneity of BC movements. BC networks started to form in piled-up colonies. As these piled-up areas developed in the colony, the networks gradually expanded. Thus, considering the temporal formation of the BC, BC networks in piled-up colonies might be heterogenous. In

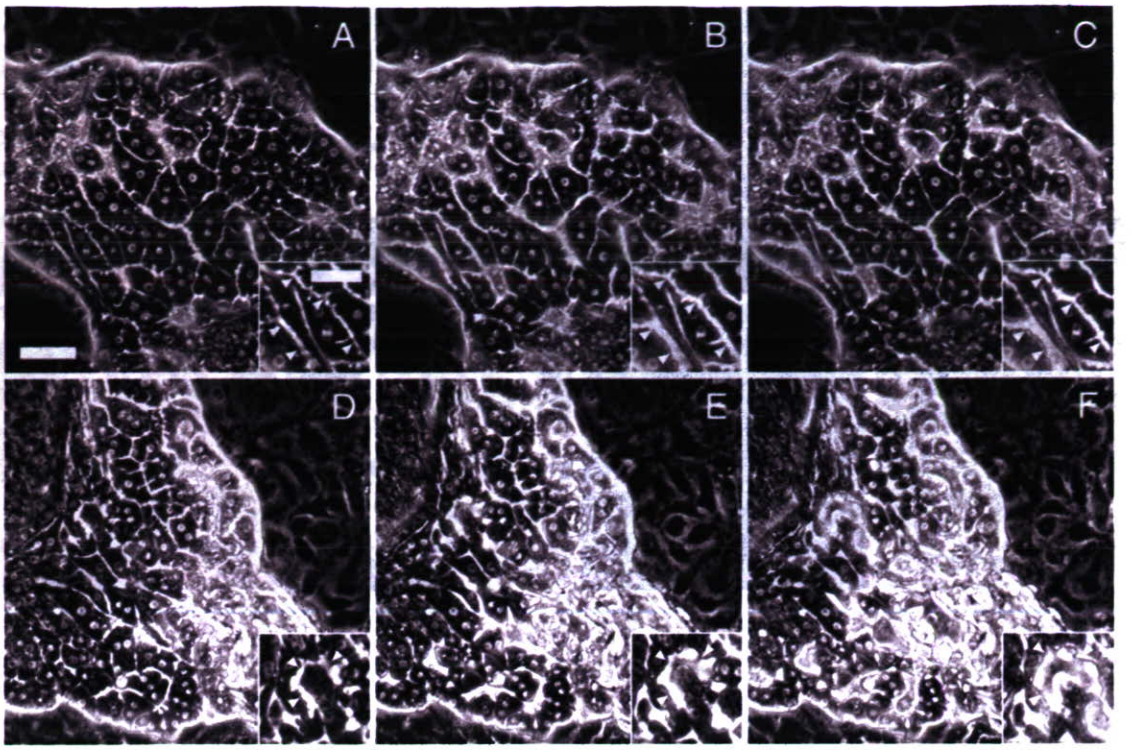

FIGURE 6. Time series phase-contrast images of the cells reconstructing BC networks at day 43 under administration of CB (1.0 μg/ml: A–C, and 5.0 μg/ml: D–F). Corresponding images of the cells after administration of CB shows dilation of BC networks. A, D: immediately after administration, B, E: after 60 min, C, F: after 180 min. Insets in each figure show magnified images of corresponding BC (arrowheads, A–F insets). Scale bars, 50 μm (A), and 30 μm (A inset).

addition, analysis of the BC movements revealed that even within a colony there were regions where the BC could act in a coordinated fashion, which was also consistent with the heterogenous expression of Cx 32. Furthermore, treatment with A23187, a Ca^{2+} ionophore, transiently induced coordinated BC contraction (data not shown). Thus, coordination of neighboring and apposing SHs may integrate the action of the whole BC network by second messengers such as Ca^{2+} and inositol trisphosphate via gap junctions.

The Role of Actin in BC Contraction

The role of actin in BC contraction has been investigated using CB. When CB is administered to rats, dilated BC are visualized in the liver.[46] The dilation is also observed in

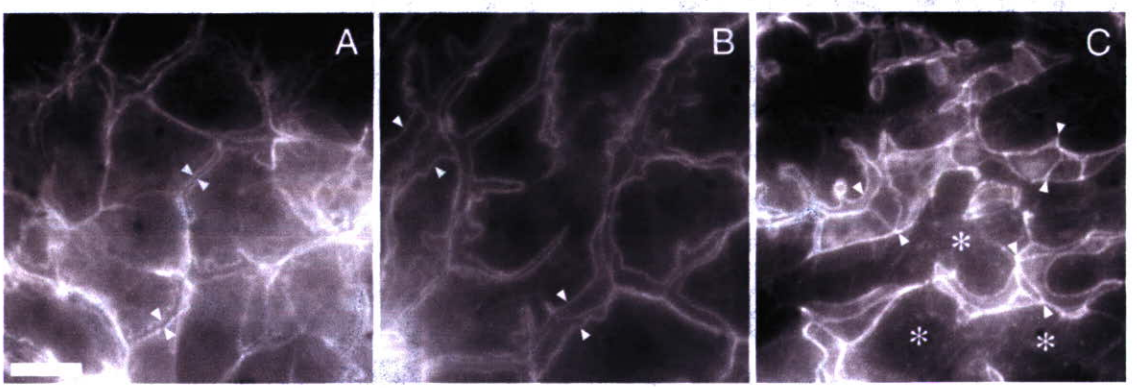

FIGURE 7. The localization of actin filaments in the piled-up cells reconstructing BC networks at day 43. A: the control cells show intensive expression of actin filaments around BC (arrowheads). B: administration of 1.0 μg/ml CB causes wide spaces between actin filaments coinciding with dilation of BC (arrowheads). C: the actin filaments of the cells treated with 5.0 μg/ml CB show indented distribution around dilated BC (arrowheads). In addition, dotted intensities in the cytoplasm (asterisks) and heterogeneity of the intensity around BC are observed.

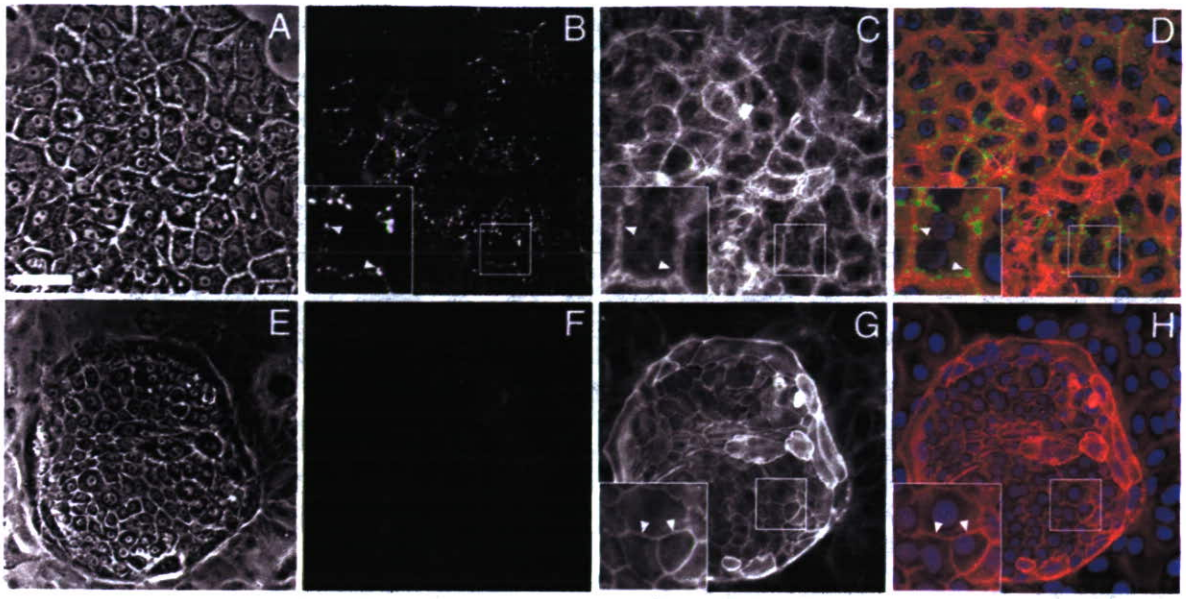

FIGURE 8. The localization of Cx 32 protein in the flat cells and piled-up cells reconstructing BC. Triple immunostaining for Cx 32 (green), actin (red), and DAPI (blue) was carried out. Phase-contrast micrographs of the cells at day 27 (A) and day 26 (E). Corresponding images of Cx 32 (B, F), actin (C, G), and the merged images of Cx 32, actin and DAPI (D, H). Dotted expression of Cx 32 was observed along the BC (B–D insets), whereas no expression of Cx 32 was observed in the flat cells (F). Scale bar, 50 μm.

hepatocyte couplets[28] and hepatocyte spheroids.[1,47] In the present experiment the morphology of BC networks treated with CB dramatically changed to induce marked dilations of BC. This result showed morphological phenomena similar to those observed when 5.0 μg/ml CB was infused through portal vein and saccular dilatations of BC were induced in the liver.[46] Immunocytochemical staining also revealed that CB treatment caused wide spaces between actin filaments

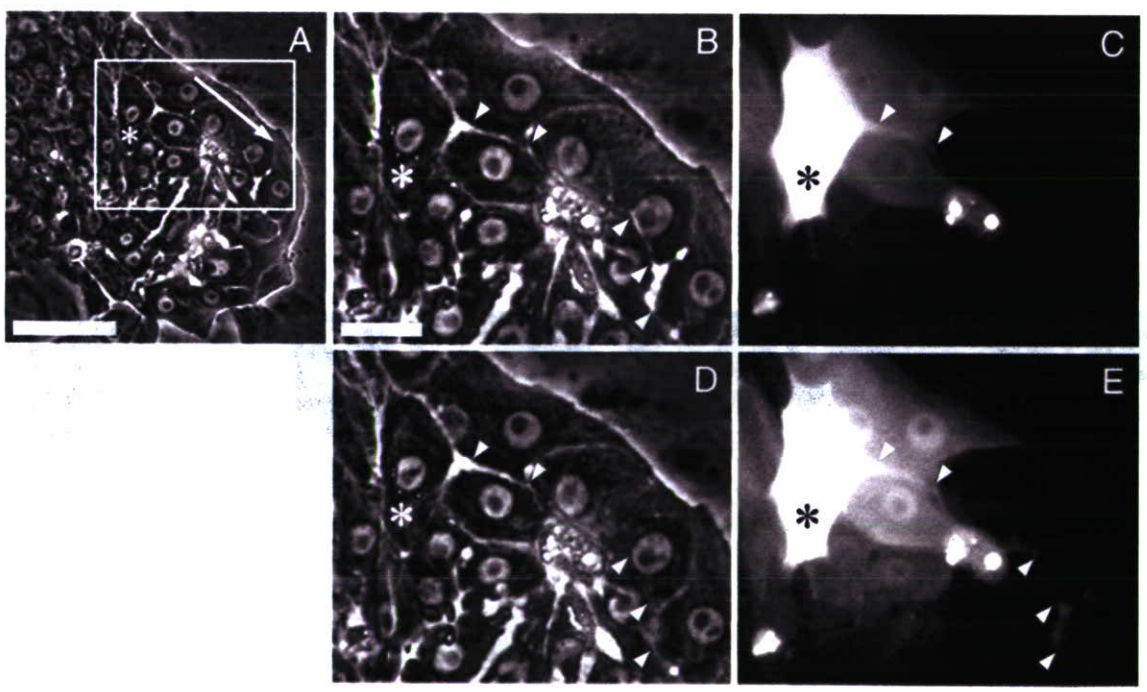

FIGURE 9. Fluorescent images of microinjected dye in the piled-up cells reconstructing BC. CMFDA was microinjected in a cell (asterisk, A) and the fluorescent image was immediately photographed (C). One time-lapse image (2.5 min after C) is shown in (E). Phase-contrast photographs corresponding to the fluorescent images C and E are shown in B and D, respectively. The arrow shows the observed direction of fluorescein transportation. The arrowheads show the fluorescent line secreted from an injected cell. The transported fluorescein within BC is elongated to the tip of the colony (arrowheads, C, E). Scale bars, 50 μm (A) and 20 μm (B).

around BC. The pericanalicular actin filaments were present around dilated BC. These results are consistent with the dilations of BC observed under phase-contrast microscopy.

These results may indicate the involvement of actin filaments in the mechanism of BC contraction. Actin filaments can interact with myosin, which is regulated by Ca^{2+}-calmodulin-dependent phosphorylation. In hepatocytes, myosin is abundant around BC, and the colocalization of actin and myosin was reported in a model of an isolated BC compartment.[41] In addition, ML-9, a myosin light-chain inhibitor, inhibited BC contraction in hepatocyte couplets.[42] Thus, BC contraction may be achieved by coordination of the actin-myosin system, which is regulated by second messengers via gap junctions.

BC Movements and the Flow Within BC

Although the triggers of the BC contractions are still unclear, some possibilities surfaced in the present experiment. (1) The BC contractions could have occurred when the contents of BC were regurgitated into cytoplasm, observed as diacytotic vacuoles. Kawahara *et al.*[9] reported that such regurgitation, called diacytosis,[13] was observed during BC contractions. In this study, similar diacytotic vacuoles were often observed when BC formed in SH colonies contracted. Some small vacuoles were budding off from the BC and this process was simultaneously seen in several parts of BC networks. We previously reported that the tight junctional protein ZO-1 was well expressed along the BC and that accumulated bilirubin did not leak from BC even a few days after the treatment.[35] The results suggested that the reconstructed BC was tightly sealed by the junctions. Therefore, when the pressure in the canalicular lumen was rapidly increased by contractions, biliary content was directed into hepatic cytoplasm and showed diacytosis. (2) The cytokinesis of the cells consisting of BC networks seems to be a trigger for BC contraction. Analysis of the time-lapse movie revealed that BC contractions were sometimes synchronized with the timing of cytokinesis. When the cells that form BC are dividing, the BC may be transiently deformed and the luminal pressure near the region may increase. The increase of the pressure may induce the contraction.

Luminal contents of the BC network need to move because hepatocytes may secrete bile-like substances into motile BC. In this culture the BC networks were closed. Therefore, luminal contents gathered at certain regions and formed cyst-like structures. These often formed at the peripheral regions of the piled-up colonies because BC networks were gradually developed coinciding with the development of the piled-up colonies. The formation of cystic structures may facilitate BC contractions and the canalicular flow. In fact visual analysis of the time-lapse movie showed the flow of luminal contents directed to the cystic structure. Quantified measurement of BC contractions also confirmed this phenomenon [Figs. 5(D–F)]. BC contractions in the root parts of the piled-up colonies caused dilation of BC in the peripheral regions of the colony [Fig. 5(E)], which indicates negative correlation, confirmed by the values of correlation coefficients (p5, Table 2).

BC movements revealed various characteristics. All BC moved, although various speeds of contraction and dilation were observed (Table 1). Analysis of correlation coefficients revealed that BC had both positive and negative correlations. These results indicate that some BC synchronously contract or dilate, and some BC dilate as a result of the contraction of neighboring BC. Although BC is thought to act as a peristaltic pump, both types of movement can transfer their contents. When the cells were treated with CB, dilations of BC were observed particularly in the peripheral regions of the piled-up colonies (Fig. 6). In addition, microinjection studies revealed that the dye secreted from injected cells had a tendency to flow toward the peripheral regions of the piled-up colonies (Fig. 9). These results suggest that secreted bile may be transferred to the peripheral regions by the two types of contractions.

Reconstructing well-assembled hepatic tissues is valuable for tissue engineering of the liver because these tissues can maintain hepatic functions during long-term culture and can be useful for bioartificial livers or tissue-engineered organ transplantation. Although the functions of individual cells have been well analyzed, multicellular functions such as coordination of BC movements have not been investigated. In the present experiment we analyzed BC movements in the organoids and verified coordination of the movements. Therefore, hepatic organoids reconstructed by SHs have potential for the tissue engineering of the liver, and this culture system is a useful model to investigate how the cells are integrated into the tissue.

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Review article

Hepatocyte transplantation for total liver repopulation

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Abstract
Hepatocyte transplantation (HT) is an attractive therapeutic alternative to liver transplantation. A number of experiments have shown the feasibility of total liver parenchymal cell replacement by transplanted hepatocytes. In this review, we would like to highlight researches and clinical reports of HT for liver repopulation. Cellular source of clinical HT should be safety. Immortalized cells, hepatic stem cells, and other stem cells have been used for an experimental model for HT. The exact mechanism of the cell engraftment after HT has not been completely understood, although there were some markers to detect and investigate transplanted cells. In order to achieve liver repopulation following HT, a mild hepatic damage may need to facilitate cell engraftment and replace the host liver by transplanted cells. Hormonal factor may use for the same purpose. Despite the results of preclinical studies promising clinical benefits for cell therapy, the clinical experience of HT has been disappointing, except in a few cases. HT may become an alternative for liver transplantation in the future; however, many efforts should made before establishing an effective method for HT and liver replacement therapy.

Key words Hepatic stem cells · Hepatocyte transplantation · Liver repopulation

Introduction

Hepatocyte transplantation is an attractive therapeutic alternative to liver transplantation. A number of experiments have shown the feasibility of total liver parenchymal cell replacement by transplanted hepatocytes.[1–5] However, researchers are still looking for more efficient methods of hepatocyte transplantation to employ for clinical treatment.

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The most recent important finding is the discovery of which liver stem cells can be derived from bone marrow cells.[6,7] Part of the phenomenon can be explained by cell fusion;[8,9] however, genetic disorders can be corrected by either cell engraftment or cell fusion.

Despite preclinical studies that suggest the potential benefits of hepatocyte transplantation, a clinical protocol for such transplantation has not yet been established. A number of issues must be resolved for total liver replacement by hepatocyte transplantation to succeed. The source cells should be few in number and proliferate vigorously without transformation. A cell marker should be identified to detect and evaluate transplanted cells. Techniques for manipulation and transplant procedures need to be safer and more effective than they are now. We reviewed the literature to investigate experimental and clinical experiences of hepatocyte transplantation.

Potential cell source

Normal hepatocytes can be an ideal cell source for hepatocyte transplantation. However, very few healthy donor cells are available. Therefore, researchers have tried to increase cell numbers by culturing hepatocytes. The obstacle to this strategy is that mature normal hepatocytes hardly proliferate in classical culture conditions.[10] Therefore, various strategies have been employed to increase the cell numbers. These include attempting to develop immortalized cells by gene transduction, and searching for hepatic stem cells or an alternative stem cell source that will transdifferentiate into hepatocytes (Table 1).

Immortalized cells

A number of genes have been successfully transferred into hepatocytes to proliferate eternally in vitro. The

Table 1. Potential cell sources

	Reference
Immortalized hepatocytes	
SV 40 large T-antigen	Isom et al.[11] 1981
Thermolabile mutant SV40 large T, antigen	Fox et al.[12] 1995
Hepatitis C virus core protein	Ray et al.[13] 2000
hTERT	Wege et al.[14] 2003
$p19^{ARF}$	Mikula et al.[16] 2004
C/EBP alpha	Soriano et al.[17] 1998
Truncated Met	Amicone et al.[18] 1997
Hepatic stem cells	
Oval cells	Yasui et al.[22] 1997
Small hepatocytes	Gordon et al.[23] 2002
Other stem cells	
Hematopoietic cells	Petersen et al.[6] 1999
Pancreatic stem cells	Dabeva et al.[25] 1997
Salivary gland progenitor cells	Okumura et al.[26] 2003
Umbilical cord blood cells	Kakinuma et al.[27] 2003
Cell fusion	Wang et al.[8] 2003
	Vassilopoulos et al.[9] 2003
Myelomonocytic cells (cell fusion)	Willenbring et al.[28] 2004
Embryonic stem cells	Yamamoto et al.[29] 2003
Low asialoglycoprotein receptor cells	Ise et al.[30] 2004

SV, simian virus; hTERT, human telomerase reverse transcriptase; C/EBP, CCAAT/enhancer binding protein

initial experiment, using simian virus (SV) 40 large T-antigen transferred into hepatocytes, in a rat model resulted in immortality of the cells, but with tumor development.[11] This strategy appears to have been ignored for a decade because of its tumorigenesis; however, hepatocytes conditionally immortalized using thermolabile mutant SV40 large T-antigen were successfully transplanted into rats without tumor development.[12] Hepatitis C virus core protein promotes an immortalized phenotype in primary human hepatocytes, although possible tumorigenesis has not yet been investigated.[13] Transformation, using the human telomerase reverse transcriptase (*hTERT*) gene, has been shown to immortalize human fetal hepatocytes without disrupting their differentiation potential,[14] although the mature hepatocyte itself cannot be immortalized. Inactivation of $p16^{INK4a}$ expression in accord with *hTERT* transformation may immortalize mature hepatocytes, as it does other cells.[15] Other genes, such as $p19^{ARF}$, *C/EBP* alpha, and truncated *Met*, have been used to successfully immortalize murine hepatocytes,[16–18] but have not yet been tried for human hepatocytes.

Hepatic stem cells

The existence of hepatic stem cells has been debated for a long time, because the stem cell-like properties of mature hepatocytes are sufficient for them to regenerate without a stem cell population. However, once the mature hepatocyte is unable to proliferate, due to hepatic damage, as in the D-Galactosamine (D-GalN)-treated rat liver, Cu^{2+}-deficient rat pancreas, or retrorsine-treated rat, hepatic stem cells emerge to restore liver regeneration.[2,19,20] This peculiar condition is rarely seen in the clinical setting, except during the process of hepatocarcinogenesis.

Two types of liver progenitor cells have been discovered. Oval cells are considered to be progenitor cells of both hepatocytes and cholangiocytes.[2] On the other hand, small hepatocytes (SHs) are considered to be progenitor cells of hepatocytes.[21] Both cell types have been used as cell sources for transplantation and engraftment to the host liver, with various functions being shown.[22,23] The difference between these cell types is that the basic phenotype of the oval cell is for cholangiocytes and that of SH is for hepatocytes. In the clinical setting, SHs are preferable to oval cells for various reasons, as outlined below.

Hepatocytes have a wide variety of functions and are regulated in various ways to achieve each function. Hepatocyte function is tightly regulated at the transcription level, and liver-enriched transcription factors regulate the expression of many genes associated with protein production and detoxification in a hierarchical manner.[24] Basically, SHs express all the liver-enriched transcription factors that need to be expressed in the mature hepatocyte. On the other hand, oval cells do not express all of these factors. Therefore, it is a reasonable hypothesis that SHs would achieve full hepatocyte func-

Table 2. Markers to detect exogenous transplanted hepatocytes in the recipient liver

	Reference
Markers	
DPPIV	Thompson et al.[31] 1991
HBsAg	Gupta et al.[32] 1994
Y-chromosome	Krishna Vanaja et al.[33] 1998
111-indium	Gupta et al.[35] 1994
	Bohnen et al.[36] 2000
99m-technetium	Gupta et al.[37] 1981

HBsAg, hepatitis B virus antigen; DPPIV, dipeptidyl peptidase IV

tion more rapidly than oval cells. Another reason to avoid the clinical use of oval cells for cell transplantation is their malignant potential.

Other stem cells

The natural plasticity of stem cells means that they could generate hepatocyes in vivo. Hematopoietic cells, pancreatic stem cells, salivary gland stem cells, and umbilical blood cells generate functional hepatocytes in vivo after cell transplantation.[6,25–27] Cell fusion could be the reason why stem cells with different origins can transdifferentiate into other cell types, and cell fusion is sufficient for treating some gene disorders.[28] Therefore, most stem cells in any organ could be feasible cell-source candidates for cell transplantation to manage liver disease in the clinical setting. However, an efficient method for the transdifferentiation of stem cells with other origins to hepatocytes should be developed before such cells can be used clinically. Another potential cell source could be embryonic stem cells.[29] However, the efficacy and safety of this approach should be carefully determined before clinical use can begin. Recently, a selective hepatocyte population with low asialoglycoprotein receptor-expressing cells was suggested to have the ability to repopulate the host liver after cell transplantation.[30] This approach could be used for clinical treatment by selecting a small cell population that could effectively repopulate the host liver.

Markers to detect exogenous transplanted hepatocytes in the recipient liver

A marker for donor cells is important to evaluate the cells' fate after transplantation (Table 2). In animal models, various genetic markers are available when using such genetically deficient animals as the dipeptidyl peptidase IV (DPPIV)-deficient rat[31] and the hepatitis B virus surface antigen (HBsAg) transgenic mouse model.[32] This approach cannot be used for clinical treatment in humans. The Y chromosome can be used in the case of sex-mismatch cell transplantation if a specimen is available for molecular evaluation.[33,34] However, as this approach needs tissue specimens, an invasive approach to obtain them is unavoidable. Cell labeling, with either 111-indium[35,36] or 99m-technetium,[37] may be an ideal strategy to detect donor cells after cell transplantation, without the need for an invasive approach. Indeed, 111-indium has been used for a clinical study to detect the transplanted cells in a 5-year-old child.[36] However, it can be used only in the early peroid after cell transplantation, because the label may be transferred to other cells after exposure for longer periods. Without a method to detect the transplanted cells, the exact mechanism by which hepatocyte transplantation ameliorates liver function will be difficult to determine.

Method for enhancement of liver repopulation

The purpose of hepatocyte transplantation is to support hepatocyte function that has been disrupted by either a congenital gene abnormality or acquired cell damage. The liver is an ideal place for the transplanted hepatocyte to work properly. However, very limited space is available under normal conditions without any manipulation. Therefore, manipulation is necessary to make a space for hepatocyte engraftment (Table 3).

Animal models created by gene manipulation

The engraftment of hepatocytes after transplantation has been considered to be limited, because the proliferation of mature hepatocytes is believed to be limited and does not last forever. Hepatocyte transplantation of the urokinase plasminogen activator (*uPA*) gene into the liver, under control of the albumin promoter gene, in a transgenic mouse (uPA-alb) model showed massive engraftment of transplanted hepatocytes, which replaced nearly all parenchymal cells.[38] Subsequently, similar extensive liver repopulation was seen in the fumaryl acetoacetate hydrolase (FAH)-knockout mouse model, which is a model of human hereditary tyrosinemia type 1.[39] These models showed that hepatocyte transplantation could be feasible for the replacement of liver tissue, as an alternative to liver transplantation. In addition, the results indicated the existence of highly proliferative mature hepatocytes.

The strategy for efficient hepatocyte transplantation is to make a space for the engraftment and increase the proliferative activity of the donor cells. In the initial genetically manipulated models, such as the uPA-alb and FAH models, the manipulation resulted in critical damage to the recipient liver and produced a space for

Table 3. Methods for enhancement of liver repopulation

	Reference
Animal models	
Alb-uPA transgene	Sangren et al.[38] 1991
FAH mutant	Overturf et al.[39] 1996
Bcl-2 transgene	Mignon et al.[40] 1998
Bcl-x(L) transgenic donor cell	Mitchell et al.[41] 2002
p27kipl-deficient donor cell	Karnezis et al.[42] 2001
Hepatotoxic agents	
Retrorsine and hepatectomy	Laconi et al.[43] 1998
CCl_4 and hepatectomy	Gupta et al.[44] 1999
DDC	Camargo et al.[45] 2004
Radiation	
With hepatectomy	Guha et al.[46] 1999
With ischemic reperfusion	Malhi et al.[47] 2002
With FasL	Takahashi et al.[48] 2003
Others	
HGF	Kato et al.[49] 1996
Thyroid hormone	Oren et al.[52] 1999

uPA, urokinase-type plasminogen activator; FAH, fumarylacetate hydrolase; CCl_4, carbon tetrachloride; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; FasL, Fas ligand; HGF, hepatocyte growth factor

donor cells to proliferate in the liver. The resistance of hepatocytes to apoptosis was generated by the gene transfer of either *Bcl-2*[40] or *Bcl-xL*,[41] and the donor cells successfully repopulated the host liver after transplantation. In this model, Fas-mediated apoptosis killed mature hepatocytes in the recipient liver and the transgenic hepatocytes resisted Fas ligand-induced apoptosis and proliferated to repopulate the host liver.

Genetically manipulated donor cells with knockout of the $p27^{Kipl}$ gene enhanced liver repopulation in the FAH model.[42] The deficiency of the $p27^{Kipl}$ gene enhanced cell proliferation, because loss of $p27^{Kipl}$ activates cyclin-dependent kinase (Cdk2)-kinase activity, which promotes cell-cycle progression. These studies using engineered cells show that the basic strategy for liver repopulation is to damage the recipient liver and enhance the proliferation of donor cells.

Hepatotoxic agents

Pyrrolidine alkaloids, such as retrorsine, are hepatotoxic and block the hepatocyte cell cycle in the late S and/or G2 phase. Although the halflife of pyrrolidine alkaloids is very short, their inhibitory effect on hepatocyte proliferation lasts for several weeks. Therefore, a retrorsine model has been successfully used with transplanted hepatocytes to repopulate the liver after cell transplantation combined with partial hepatectomy.[43] In this model, donor cells are the only cells that can proliferate after stimulation for liver regeneration, as proliferation of the host liver is inhibited by the retrorsine treatment. Hepatic toxins have also been used instead of genetically manipulated models to ablate liver cells and make a space for the engraftment of hepatocytes after cell transplantation. Carbon tetrachloride (CCL_4), which depletes perivenous hepatocytes (zone 3 hepatocytes), has been used to enhance liver repopulation.[44] The concept of requiring space for liver repopulation has been proven. Another hepatic toxin, 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC),[45] has been employed in a model of hematopoietic stem cell transplantation.[28] In this model, myelomonocytic cells were sufficient to produce functional hepatic repopulation.

Radiation

In the clinical strategy of liver repopulation, it is preferable to induce only mild hepatic damage. After radiation-induced liver damage, it has been shown that massive liver repopulation is accomplished by hepatocyte transplantation combined with partial hepatectomy.[46] Radiation can inhibit host hepatocyte proliferation in the recipient of hepatocyte transplantation. However, the surgical procedure is necessary to stimulate liver regeneration, and this is an obstacle to clinical application. Non-surgical alternatives have been investigated, and successful liver repopulation by cell transplantation has been demonstrated in an ischemia-reperfusion model after irradiation.[47] *FasL* gene transfer to the host liver also resulted in significant liver repopulation when combined with irradiation after cell transplantation.[48] These results indicate that irradiation of the liver prior to hepatocyte transplantation is feasible for liver repopulation therapy.

Enhancement of donor-cell proliferation

Hepatocyte growth factor (HGF) and a thyroid hormone have been used for the enhancement of donor-cell proliferation after hepatocyte transplantation. HGF administration after hepatocyte transplantation improves donor-cell proliferation.[49] However, it does not enhance liver repopulation in the CCl_4-treated hepatocyte transplantation model.[44] Serum HGF levels are markedly high after partial hepatectomy.[50,51] Therefore, it is a matter of debate whether additional HGF can enhance cell proliferation in liver regeneration when the HGF level is already high. On the other hand, a thyroid hormone (triiodothyronone) has been shown to stimulate liver repopulation in a retrorsine model without partial hepatectomy.[52] Other hepatic mitogens should be tested to see whether they stimulate liver repopulation in models for clinical treatment.

Table 4. Clinical reports of hepatocyte transplantation

Applications	Number of patients	Outcome	Reference
Chronic liver failure			
Chronic liver disease	10	Transplanted cells detectable for 1 to 6 months	Mito et al.[53] 1992
Chronic end-stage liver disease	5	Three recovered	Strom et al.[54] 1997
Acute liver failure			
Fulminant hepatic failure	7	Three recovered	Habibullah et al.[55] 1994
Acutse liver failure	19	Two recovered; six liver transplantations; 11 died	Strom et al.[1] 1999
TPN sepsis and HCV	2	Both died	
Acute liver failure	5	All died within 52 days	Bilir et al.[56] 2000
Hereditary metabolic liver disease			
Familial hypercholesterolemia	1	Stable for 18 months	Grossman et al.[57] 1994
Familial hypercholesterolemia	5	Three improved	Grossman et al.[58] 1995
A1-antitrypsin deficiency	2	Liver transplantation	Strom et al.[59] 1997
Crigler-Najjar syndrome type I	1	Liver transplantation after 4 years	Fox et al.[60] 1998
OTC deficiency	1	One died after 42 days	Strom et al.[59] 1997
	1	One had liver transplantation after 6 months	Horslen et al.[61] 2003
	1	One had liver transplantation after 7 months	Mitry et al.[62] 2004
Glycogen storage disease type Ia	1	9 Months, improved	Muraca et al.[63] 2002
Infantile Refsum's disease	1	18 Months, improved	Sokal et al.[64] 2003
Factor VII deficiency	1	Not described	Horslen et al.[65] 2004
Bile salt export protein deficiency	1	Not described	Horslen et al.[65] 2004

OTC, ornithine transcarbamoylase; TPN, total parenteral nutrition

Clinical reports of hepatocyte transplantation

Despite the results of preclinical studies promising clinical benefits for cell therapy, the clinical experience of hepatocyte transplantation has been disappointing, except in a few cases (Table 4). The first clinical hepatocyte transplantation was reported by Mito et al.,[53] in 1992, who transplanted autologogous hepatocytes in ten patients with chronic liver disease. Although the transplanted hepatocytes survived in the host body, no clinical benefit of the hepatocyte transplantation was seen. Strom et al.[54] reported hepatocyte transplantation in five patients with chronic liver disease. Three of the five patients survived; however, orthotopic liver transplantation eventually had to be conducted for all three patients.

Hepatocyte transplantation has been tested for acute liver failure. Habibullah et al.[55] transplanted human fetal hepatocytes in 7 patients with fulminant hepatic failure and reported that 3 patients recovered. Strom et al.[1] reviewed 30 cases of hepatocyte transplantation in the United States. Although 11 of 19 patients died, 2 of the patients recovered as a result of hepatocyte transplantation alone. An other clinical experience was reported by Bilir et al.[56] in 2000. Three of their patients survived for more than 48 h, although they would not have been expected to survive without treatment including liver transplantation. However, all their patients died within 52 days of the hepatocyte transplantation.

The greatest clinical benefit of hepatocyte transplantation could be for patients with hereditary metabolic liver diseases.[57–65] Most of such patients reported to have received hepatocyte transplantation received liver transplantation eventually; however, some of them showed improved clinical status for more than 6 months with the transplantation. Therefore, at present, hepatocyte transplantation is not an alternative for liver transplantation, but it could be a bridge to liver transplantation. Hepatocyte transplantation may become an alternative for liver transplantation in the future; however, this will not be easy. This dream may come true as a result of the many efforts currently being made to develop an effective method for hepatocyte transplantation and liver replacement therapy.

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ORIGINAL ARTICLE

Liver Repopulation and Long-Term Function of Rat Small Hepatocyte Transplantation as an Alternative Cell Source for Hepatocyte Transplantation

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Hepatocyte transplantation (HT) is an attractive therapeutic modality for liver disease as an alternative for liver organ transplantation. Primary fresh hepatocytes (FHs) are the exclusive cell source that has been used for clinical HT. However, the use of FHs is limited due to a shortage of donor cells. Small hepatocytes (SHs) are hepatic progenitor cells and can be isolated not only from rodents but also from humans. SHs can proliferate in vitro and express liver functions, although conventional hepatocytes lose them within a short period after culture. SH functions in vivo have never been studied. We therefore investigated HT using SHs to evaluate cell engraftment and function compared to HT using FHs. The donor cell number in the SH group was smaller than that in the FH group at HT. The cell engraftment in the SH group was smaller in the liver and larger in the spleen than in the FH group. The cell engraftment in the liver increased after HT; however, that in the spleen decreased after HT in both groups. HT using SHs supported the serum albumin level in the NAR experiment as well as that using FH, and albumin mRNA was detectable in the recipients' tissues at 12 weeks after HT. In conclusion, HT using SHs showed hepatic repopulation similar to that using FHs. This suggests that both SHs and FHs can repopulate the liver as if they were hepatic stem cells. In addition, HT using SHs supported liver functions such as albumin correction at the same level as that using FHs. These observations strongly support the idea that SHs could be an alternative to primary FHs as a novel cell source for future HT. *Liver Transpl 12:78–87, 2006.* © 2005 AASLD.

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Liver transplantation (LT) has become a golden treatment for liver diseases, including inherited metabolic errors, acute liver failure, and chronic liver failure.[1,2] However, the shortage of donor organs is an obstacle for applying LT to every patient. Therefore, hepatocyte transplantation (HT) has been considered as an alternative for LT and has long been investigated.[3,4]

Primary fresh hepatocytes (FHs) are the main cell source not only for experimental investigations but for clinical HT.[5] Other cellular sources such as xenogenic hepatoyctes, fetal hepatocytes, hepatic progenitor cells, and immortalized hepatocytes have been tested in experimental investigations.[3] All these cells

Abbreviations: HT, hepatocyte transplantation; FH, fresh hepatocyte; SH, small hepatocyte; LT, liver transplantation; DPPIV, dipeptidyl peptidase IV; SD, Sprague-Dawley; NAR, Nagase analbuminemic rats; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; Asc2P, ascorbic acid 2-phosphate; EGF, epidermal growth factor; DMSO, dimethyl sulfoxide; BrdU, 5-bromo-2'-deoxyuridine; PCR, polymerase chain reaction; SD, standard deviation; SDS, sodium dodecyl sulfate.

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can feasibly support liver functions; however, FHs are the exclusive cell source for clinical therapy because of safety issues. The use of FHs is also limited due to a shortage of donor cells. Therefore, we have been attempting to obtain sufficient numbers of donor cells for HT that can express and support liver functions just like FH.

Small hepatocytes (SHs) are hepatic progenitor cells that possess high proliferative activity and can generate mature differentiated hepatocytes in vitro.[6] The mature hepatocytes generated from SHs can express liver functions similar to those seen in vivo, including protein production and bilirubin metabolism. Unlike conventional hepatoyctes, which fail to express liver functions within a few days in vitro, SHs can proliferate and express liver functions in extended long-term culture.[7] SHs can be isolated not only from rodents but also from humans. The SH character in humans is very similar to that in rodents.[8] In addition, SHs tolerate cryopreservation well compared to FHs and can be stored for an extended period.[9] This suggests that enormous numbers of SHs can be obtained for clinical treatment. However, the functions of SHs in vivo have never been studied to determine if they can express and support liver functions as seen in vitro.

Liver repopulation by transplanted hepatocytes has been shown in a number of experiments. Hepatic toxins such as retrorsine,[10] carbon tetrachloride,[11] and 3,5-diethoxycarbonyl-1,4-dihydrocollidine,[12] and radiation[13-15] have been used for providing a space for cell repopulation and fatal damage to the host hepatocytes to block their proliferation. In addition, hepatectomy has been combined with these manipulations in order to stimulate the transplanted cells to proliferate and repopulate in the recipients. However, these strategies are difficult to apply to clinical treatment due to their toxicity and surgical invasiveness, except for hepatic irradiation, in which liver damage can be controlled by the energy. Treatment of metabolic disorders may be one of the best applications for HT. Total liver repopulation is the ideal therapy in any case; however, it may not be necessary to support or correct liver functions. Clinical therapy should be safe and minimally invasive for the patient. Therefore, we selected hepatic irradiation alone, even though it might not stimulate total liver repopulation, as it supported part of the liver function.

Several markers have been investigated to identify the donor cells in the recipient organ after HT, including congenital genetic abnormalities, genetically manipulated markers, and sex mismatch markers.[5] The combination of sex mismatch cells and other markers is a simple method to identify donor cells with double markers in the recipient organ, which allows us to detect cells by protein, and mRNA and DNA levels. We used female dipeptidyl peptidase IV$^-$ (DPPIV$^-$) Fisher 344 rats, which are spontaneously mutated animals without other abnormalities compared to male DPPIV$^+$ rats.[16] Although this model can be suitable to investigate cell physiology and morphology after HT, it is far from the clinical situation because of no immunogenicity. Therefore, we investigated further, using analbuminemic rats, to test if the donor cells could exhibit hepatocyte functions in an immunosuppressive environment in a situation similar to the clinical setting.

The purpose of this study was to see if cultured SHs could be used for liver repopulation or supporting liver function compared to primary FHs as an alternative cell source for HT with or without immunosuppression.

MATERIALS AND METHODS

Animals

Male dipeptidyl peptidase IV$^+$ (DPPIV$^+$) Fisher 344 rats were purchased from Japan SLC (Shizuoka, Japan). Japanese-strain female DPPIV$^-$ Fisher 344 rats, which possess a spontaneously mutated gene, were purchased from Charles River Japan (Kanagawa, Japan). Male Sprague-Dawley (SD) rats were purchased from Japan SLC. Female Nagase analbuminemic rats (NAR) were purchased from Japan SLC. All animals were 8 to 12 weeks old. All animals used in the experiments received humane care and the experimental protocol was approved by the Committee of Laboratory Animals according to Sapporo Medical University guidelines.

Primary Hepatocyte Preparation for Donor Cells

Primary donor cells were isolated from either male DPPIV+ F344 rats or male SD rats by the two-step collagenase liver perfusion method.[17] The cells were washed with Dulbecco's modified Eagle's medium (DMEM; GIBCO Laboratories, Grand Island, NY) supplemented with 20 mM HEPES, 30 mg/L L-proline, 10% fetal bovine serum (Hy-Clone, Logan, UT). The cell viability was determined by the trypan blue exclusion test to be more than 90%. These cells were used for experiments. The cells were resuspended at 4×10^7 viable cells/ml of saline for transplantation.

Small Hepatocyte Isolation and Culture

The details of isolation and culture of liver cells were previously described.[6] Finally, the cells were suspended in L-15 medium (GIBCO Laboratories) with 20 mmol/L HEPES (Dojindo, Kumamoto, Japan), 1.1 g/L galactose (Katayama Chemical Co., Osaka, Japan), 30 mg/L L-proline, 0.5 mg/L insulin, 10^{-7} mol/L dexamethasone (Sigma Chemical Co., St. Louis, MO), and antibiotics, and the number of viable cells was counted. Two $\times$ 10^6 viable cells were inoculated on 100 mm culture dishes (Corning Glass Works, Corning, NY) coated with rat tail collagen (50 μg of dried tendon/0.1% acetic acid) and placed in a 100% air incubator at 37°C. Two to three hours after plating, the medium was changed to DMEM with 20 mmol/L HEPES, 25 mmol/L $NaHCO_3$, 30 mg/L L-proline, 0.5 mg/L insulin, 10^{-7} mol/L dexamethasone, 10% fetal bovine serum (FBS; Hy-Clone), 10 mmol/L nicotinamide (Katayama Chemical Co.), 1 mmol/L ascorbic acid 2-phosphate (Asc2P; Wako Pure Chem., Tokyo, Japan), 10 ng/ml epidermal growth factor (EGF; Collaborative Research Inc., Lex-