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Original Research

F-Actin Plaque Formation as a Transitional Membrane Microstructure Which Plays a Crucial Role in Cell-Cell Reconnections of Rat Hepatic Cells after Isolation

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Abstract
Cell-cell reconnection after isolation or during healing process after injury is a cell membrane function. It is assumed that each tissue has its own architecture and accordingly, cells in different tissues could have different adaptability to resume their tissue-specific architectures. How membranes adapt themselves to make cell-cell reconnections has not been elucidated. This study investigated how rat hepatic cell membranes adapt themselves after isolation to establish cell-cell reconnections, which is essential to liver tissue healing process. Hepatic cells were obtained from rat liver by collagenase perfusion. The cells were maintained in suspension using a gyratory-mediated cell spheroid culture method. F-actin, claudin-2 and connexin-32 on cell membranes were stained and observed by confocal microscopy. Cells and spheroids were also observed by Scanning Electron Microscopy. It was found that hepatic cells underwent adaptive change after enzymatic isolation by diminishing membrane F-actin residues and forming F-actin plaques. Once an F-actin plaque is formed (within 1-4 h), it is ready to make initial contact with other cells through plaque-plaque overlapping in a manner similar to 'Velcro-like' binding. A cell could form one or more F-actin plaques and establish plaque-plaque contacts with one or more other cells. When the cells had established plaque-plaque contacts, their contacts would further develop to make full cell-cell bindings. Claudin-2, a tight-junction molecule, and connexin-32, a gap-junction molecule, gradually diminished and lost their original distribution patterns within 4 h after isolation. They did not show involvement in establishing initial cell-cell connections. It is concluded that F-actin plaque formation is a characteristic change that precedes cell-cell reconnection. F-actin plaques are transitional microstructures that serve as a special device to facilitate cell-cell contacts in the early stage after cell isolation. F-actin can be used as a biomarker to study membrane adaptability and the healing process of hepatic cells.

INTRODUCTION

Cell membrane plays an essential role in establishing cell-cell connections and tissue architecture. The cells in most tissues or organs undergo replacement by new cells due to injury and death of old cells [1]. In both cases existing cells need to establish cell-cell connections with new cells and other existing neighbour cells. The liver is a multicellular organ and contains four major cell types including hepatocytes, Kupffer cells, stellate cells and sinusoidal endothelial cells [2]. They form the special architecture of liver tissue. In the normal liver, all these cells undergo division to replace aged or dead cells and can resume liver-specific architecture such as tight-junctions and canaliculi. The cellular mechanism of how cell membranes initiate cell-cell connections and resume

tissue/organ architectures has not been well elucidated.

We have shown that isolated primary hepatic cells can re-aggregate together to form three-dimensional spheroids when cells were cultured in gyratory-shaking conditions [3-4]. When primary cells in liver tissues are isolated by collagenase, this means that all cell-cell connections have been disconnected. This method thus provides a model to study changes of membrane microstructures and the critical interactions between hepatic cells in establishing cell-cell reconnections after cell isolation, which is relevant to healing process after liver tissue injury.

Tight-junctions and gap-junctions are two major junctions between hepatocytes in the liver [5-7], which are involved in cell-cell connections and communications. Connexin 32 (Cx32) is involved in gap-junction formation and Claudin-2 is involved in forming tight-junctions. In addition, actin is an essential cell component existing in virtually all cell types. It constitutes 10% of the total cytoplasmic protein and exists either as a globular monomer (G-actin) or a filament form (F-actin, a polymer) [8]. F-Actin is involved in forming the cytoskeleton, remodelling to facilitate membrane fusion, cell movement or migration, cell-cell binding and signal transduction [9-14]. In the current study, we have investigated the roles of F-actin, connexin32 and claudin-2 in establishing cell-cell reconnections of hepatotic cells after isolation.

MATERIALS AND METHODS

Isolation and maintenance of hepatic cells

Liver cells were obtained from male adult Wistar rats (Charles River) at body weight 250 g, by a collagenase perfusion method as described previously [4]. Briefly, the obtained cell suspension was a mixture of hepatic cells including hepatocytes, stellate cells, Kupffer cells and sinusoidal endothelial cells. The cell suspension was diluted with culture medium containing Hepatocyte Medium (Sigma-Aldrich, UK) supplemented with 5 % foetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin sulphate to give 5×10^5 viable cells/ml. After dilution, the cell suspension was plated into 6-well plates, 3 ml/well. The plates were incubated on a gyratory shaker (New Brunswick) at 37 °C in a 5% CO_2 incubator. The shaker rotating speed was adjusted to 83 rpm to prevent cells from sticking on the bottom of the wells and promote cells colliding randomly and freely.

F-actin staining and observation

Cell suspensions were sampled at 0 (immediately after enzymatic isolation), 0.5, 1, 2, 3, 4, and 24 h, up to 6 days after isolation. A well of cells was collected at each time point and put in an Eppendorf tube. Each of the collected suspensions was centrifuged at 150 *g* for 30 s. Supernatant was removed and cell pellets were washed with phosphate buffered saline (PBS, GIBCO) twice. After washing, the cell pellets were fixed in 4% glutaraldehyde in PBS for 1 h. After fixation, the cells were washed twice with PBS and extracted with ice-cold acetone for 3 min. Cells were then rinsed twice with PBS. After washing, 200 μl PBS was added to each tube followed by 10 μl Alexa Fluor 488 phalloidin (Invitrogen, Cat. A12379). The tubes were wrapped with foil and incubated at room temperature for 20 min. After staining, the cells were washed with PBS twice and observed using a spinning disk confocal microscope (Ultraview, Perkin Elmer), at ex/em = 488/518.

Connexin32 and claudin-2 co-stained with F-actin

Cells were collected as mentioned above and fixed with 4% glutaraldehyde in PBS for 60 min followed by three washes with PBS. After fixation, cells were pre-extracted with 1 ml on ice-cold acetone for 3 min and washed three times with blocking buffer (containing 10 mM glycine, 0.05% sodium azide, 0.5% BSA in PBS). Cell pellets were incubated in 200 μl of either mouse anti-connexin 32 (Invitrogen, Cat. No. 13820) or mouse anti-claudin-2 (Invitrogen, Cat. No. 325600) solution diluted at 1: 50 in blocking buffer at room temperature for 40 min. After incubation, the pellets were washed three times with blocking buffer and incubated with 20 μl goat anti-mouse IgG (H+L) AlexaFluor 568 conjugate (Invitrogen, Cat. A11031) diluted at 1: 50 at room temperature in dark conditions for 20 min, which was followed by 10 μl Alexa Fluor 488 phalloidin and further incubation for 20 min. The stained cells were washed three times with PBS and imaged using a spinning disk confocal microscope in multi-tracking mode. Z step increments were set to 0.3 um for Z-stack acquisition.

Sample preparation for Scanning Electron Microscope (SEM)

Cells were collected at the time points mentioned above and fixed in 4% glutaraldehyde in PBS for 1 h at room temperature. The cells were washed three times with PBS. After washing, cells were dehydrated with 20, 30, 50, 70, 80, and 100% ethanol and then ethanol: hexamethyldisilazane (HMDS, Sigma) at 2:1, 1:1 and 1:2 respectively, 5 min for each concentration. This was followed by three rinses in HMDS. After dehydration, the cells were sputter coated with gold and observed using a Philips XL30 Environmental Scanning Electron Microscope.

RT-PCR for actin gene expression

Cells were harvested at the time points of 1, 2, 3, 4 and 24 h after isolation, 1 well of cells was collected at each time point. Cellular RNA was isolated using 0.5 ml TRIzol LS reagent (Invitrogen, Paisley, UK) for each well of cells according to the manufacturer's instructions. A total of 1 μg RNA was digested with 1 unit RQ1 RNase-free DNase (Promega, Southampton, UK), and then reverse-transcribed using random hexamers and the Superscript II pre-amplification system (Invitrogen). Amplifications were performed by running real-time PCR (PTC-200, MJ Research) in 50 μl reaction mixtures containing PCR buffer (10 mM Tris–HCl, 1.5 mM MgCl2, and 50 mM KCl, pH 8.3), 0.2 mM of each deoxynucleotide triphosphate, 2 units of Taq DNA polymerase (Invitrogen); 0.2 μM of each rat β-actin primer: forward, ACC ACC ATG TAC CCA GGC AT and reverse, CCG GAC TCA TCG TAC TCC TG; 0.2 μM of each rat glyceraldehydes-3-phosphate dehydrogenase (GAPDH) primer: forward, TCC CTC AAC ATT GTC AGC AA and reverse, AGC TCC ACA ACG GAT ACA TT. GAPDH was used as an internal reference. After initial denaturation (94 °C for 5 min), 25 cycles were performed with denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and synthesis at 72 °C for 2 min, followed by 7 min of a final extension step. PCR products were separated by electrophoresis on 2% agarose gels, stained with ethidium bromide, and visualized under UV light by DNR Bio-Imaging Systems (MiniBis, Stretton Scientific Ltd).

RESULTS

F-Actin plaque formation and residue clearance

Residues of membrane surface microstructures on the isolated hepatic cells at the earliest time point (0 h) after isolation can reflect the situation of cell-cell connections in the liver tissue. The residues of F-actin stripes on the cell membrane surfaces were clearly observed (Fig.1A). They exhibit either a network- or ring-like structures. The F-actin stripes showed clear double 'lines' suggesting that they might be the residues of tight-junctions forming bile canaliculi between hepatocytes in the liver.

The membrane surface of hepatic cells, including residues of F-actin stripes, underwent change with time after isolation. Some cells showed early surface changes 30 min after isolation (Fig.1B) and the F-actin stripes were seen to widen locally. The cells marked by arrows (including the inserted cell picture in Fig.1B) show that the F-actin stripes remained but their surfaces showed what appeared to be irregular bulges or a bumpy surface with small bulges.

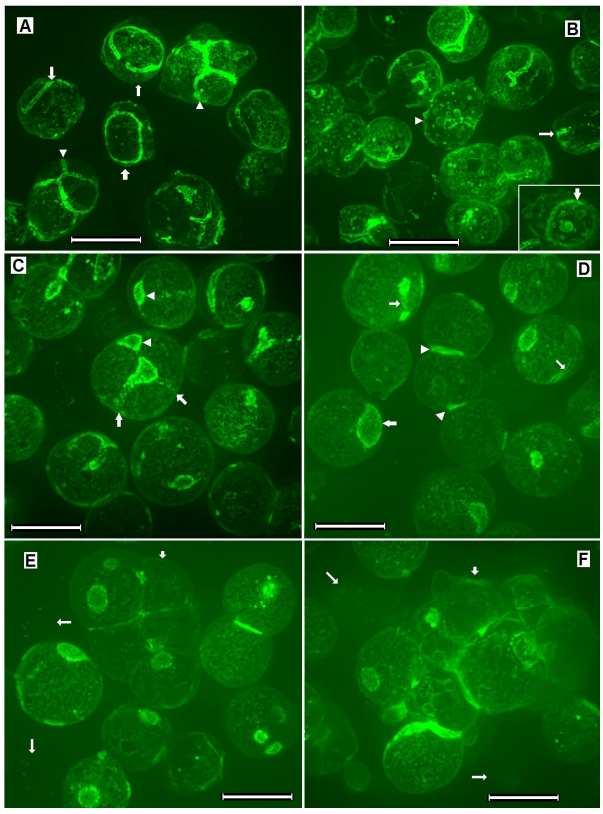

Figure 1. F-actin plaque formation and cell-cell reconnection of hepatic cells after isolation. A: hepatic cells 0 h post isolation. F-actin stripes were strongly stained in various forms from a simple stripe (the thinner arrow), ring-shapes (the thicker arrows) to networks (arrowheads); B: 30 min post isolation. F-actin stripes started changing (the thinner arrow) and bulges appeared on some cell surfaces (arrowhead). The cell in the insertion show both large bulges and F-actin stripe network (the thicker arrow); C: 1 h. F-actin plaques are forming (arrowheads) and F-actin stripe residues are diminishing (arrows); D: 2 h. A typical F-actin plaque (the thicker arrow) and plaque-plaque contacts (arrowheads). Trails of F-actin stripes on some cells can still be seen (the thinner arrows); E: 3 h. A small aggregate with 5 cells (the thicker arrow). The thinner arrows indicate the cells with very low F-actin stain and without F-actin plaque formation; F: 4 h. A larger aggregate with more cells (the thicker arrow). The cells with very low F-actin stain (the thinner arrows) remained singles. The white bars represent 20 μm.

One hour after isolation, some F-actin stripes on cell membranes expanded to form plaques at certain sites along stripes (Fig.1C), while the other parts of the F-actin stripes showed reduced stain (Fig.1C). Comparing the cells indicated by arrows in Fig.1A and C it can be seen that both the disappearance of F-actin stripes and plaque formation were going on simultaneously.

By 2 h after isolation, the F-actin stripes on most cells had disappeared from the cell surfaces or only had some trails left (Fig.1D). Meanwhile, more regular and circular F-actin plaques were formed (Fig.1D). F-actin plaques exhibited oval or round shapes and often have

a densely F-actin-stained edge. Once a plaque had been formed, the cells were ready to connect with other cells through plaque-plaque contact in a manner similar to 'Velcro-like' binding (Fig.1D). Thus, F-actin plaque formation is a crucial step for hepatocytes to make initial connects with each other and the F-actin plaque is a new microstructure and serves as a special device to initiate cell-cell reconnection.

Three hours after isolation, small aggregates had formed (Fig.1E). These small aggregate could be further expanded by binding more single cells or small aggregates through plaque-plaque contacts (Fig.1E). Once a cell-cell connection had been established, F-actin remodelling and binding between cells would develop to make full cell-cell contacts. As a result, strong F-actin stains were seen between cells in aggregates/spheroids (Fig.1E).

Four hours after isolation, small aggregates were further enlarged by binding more cells or small aggregates through plaque-plaque contacts (Fig.1F). The irregular shape of an aggregate at this stage relates to the distribution of F-actin plaques on the cell surfaces.

The kinetic process of the F-actin plaque formation and cell-cell connection described above is a typical one. Some cells may form plaques faster than others. The cells with very low or no F-actin stain on the cell membranes could not form F-actin plaques and would remain singles (Fig.1E and F). In the whole process of establishing cell-cell connection after isolation, F-actin plaques were key transitional microstructures to serve as a 'Velcro-like' device in initiating cell-cell reconnections.

The sizes of F-actin plaques were found to range from 4 to 8 μm in diameter. Some F-actin plaques (Fig.2A) show typical ring-shaped F-actin stain. One cell can form one or more than one plaques (Fig.2A). F-actin plaque-plaque Velcro-like overlapping (Fig.2B) can form firm and rapid initial binding once two cells meet each other through plaque-plaque contact. In this way, smaller aggregates can enlarge continuously to form larger aggregates (Fig.2C). F-actin stain between cells is clearly seen and cells are tightly bound together in aggregates or spheroids. After 6 days, mature liver spheroids were formed (Fig.2D). It can be seen that F-actin stain between cells is strong and cell-cell connections are tight. The size and shape of liver spheroids after day 6 tended to be stabilized and they looked spherical and more regular than aggregates at earlier time points as viewed by light microscopy and ESEM (Fig.2E and F).

Formation of other membrane micro-structures

Small F-actin plaques and spots: In addition to larger plaques as mentioned above, some other small F-actin plaques and spots (1-4 μm in diameter) were observed using confocal microscopy (Fig.3A). This type of plaque showed a small solid patch on the surface of cell membrane using SEM (Fig.3B). They were clearly seen 1 h and onwards after isolation, but not at earlier stages.

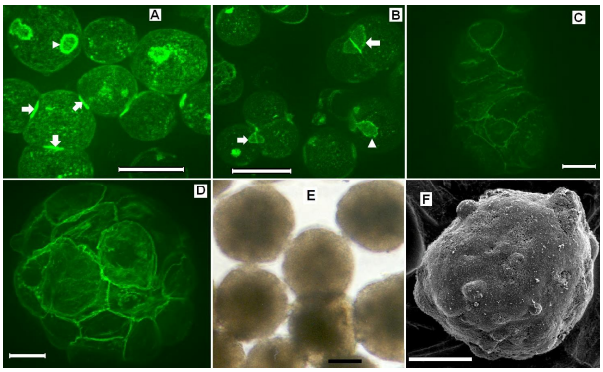

Figure 2. Plaque-plaque contacts and liver spheroid formation. A-D are confocal images. A. 3 h post isolation. A typical ring-shaped plaque (arrowhead) formed and plaque-plaque contacts (arrows) established; B. 4 h after isolation. Plaque-plaque overlapping (arrows) can be seen occasionally. In some cases, two plaques are close to each other (arrowhead); C. Image of an aggregate 24 h post isolation; D. a 6-day liver spheroid; E. light micrograph of 6-day liver spheroids. F. Scanning electron micrograph of a 6-day liver spheroid. The white bars in A-D represent 20 μm. The black bar in E represents 100 μm. The white bar in F represents 50 μm.

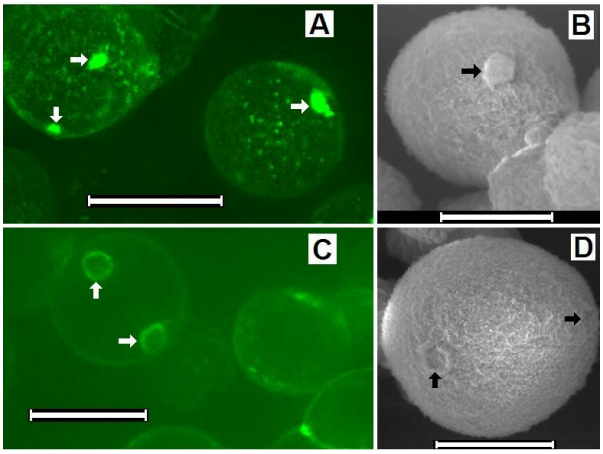

Figure 3. Some other F-actin microstructures formed on the surface of hepatic cells after isolation. A. small F-actin plaques (white arrows). B: a small plaque (black arrow) imaged using SEM; C: a pair of small depressions (white arrows) on the same cell imaged using confocal microscopy. D: a pair of small depressions (black arrows) on the same cell imaged using SEM. The white bars in the confocal pictures represent 20 μm and those in the SEM pictures represent 10 μm.

Paired polar depressions: Under confocal microscopy, paired depressions on some hepatocytes were observed. They locate at opposite 'poles' to each other and show

ring-like F-actin stain (Fig.3C). They are, therefore, termed paired 'polar' depressions here. This type of depressed microstructure appeared circular and ranged from 3-5 μm in diameter and was seen 2 h after isolation, but not at earlier stage. Under SEM, a pair of depressions can also be observed clearly (Fig.3D). According to their surface characteristics, these cells appear to be hepatocytes (see Fig.4).

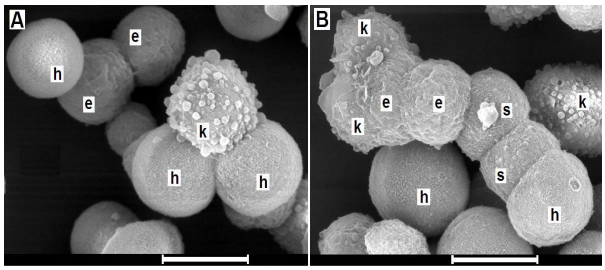

Figure 4. Cell-cell connections between different hepatic cell types. SEM images A and B show cells at 4 h post isolation. h: hepatocyte; k: Kupffer cell; e: sinusoidal endothelial cell; s: stellate cell. All these cell types can aggregate together in any combination. The white bars represent 20 μm.

Cell-Cell Connections between Different Cell Types

Fig.4 shows how different cell types reconnect together after isolation. From the SEM images taken 4 h after isolation, at least four different hepatic cell types are recognizable as shown in Figure 4. According to the membrane surface characteristics the individual cells may be defined as the following cell types: hepatocyte (h): their surface looks smoother than other cell types and often with paired polar depressions (Fig. 4A and B); Kupffer cells (k): This type of cell shows many large bulges on the membrane surface (Fig.4A and B) which resemble the appearance of macrophages. A Kupffer cell can form a large F-actin plaque, which allows the Kupffer cell to attach on other cells at one side and form firm cell-cell connection with other cell types (Fig.4A and B). Thus, Kupffer cells cannot move freely once they connect with other hepatic cells. Sinusoidal endothelial cells (e) have a bumpy cell surface (Fig.4B) but have no bulges. The cells labelled with 's' are likely to be stellate cells. Their membrane surfaces have small bead-like bulges (Fig.4B). They are a quiescent cell type which can potentially proliferate and transform into Kupffer cells and myofibroblasts.

All four different cell types established cell-cell connections through F-actin plaque-plaque binding in any combination as shown in Figure 4: *e.g.* h-k, h-e, h-s, k-e, e-s, in addition to cell-cell-connections between the cells of the same type.

Actin Gene Expression

F-actin plaque formation is a common change for all hepatic cell types after cell isolation. Its monomers (G-actin) gene expression after cell isolation is shown in Fig.5. It shows that actin gene expression remained at a stable level within the first 24 h after isolation. This suggests that F-actin re-modelling happened during membrane adaptive change rather than an increase in actin monomer synthesis after isolation.

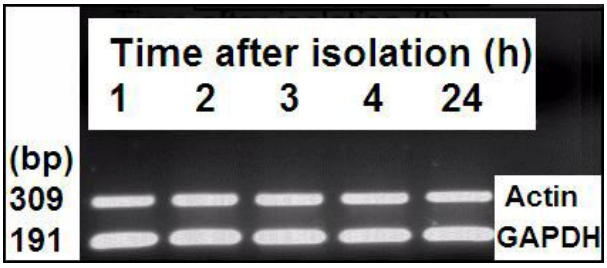

Figure 5. Actin gene expression over 24 h after cell isolation.

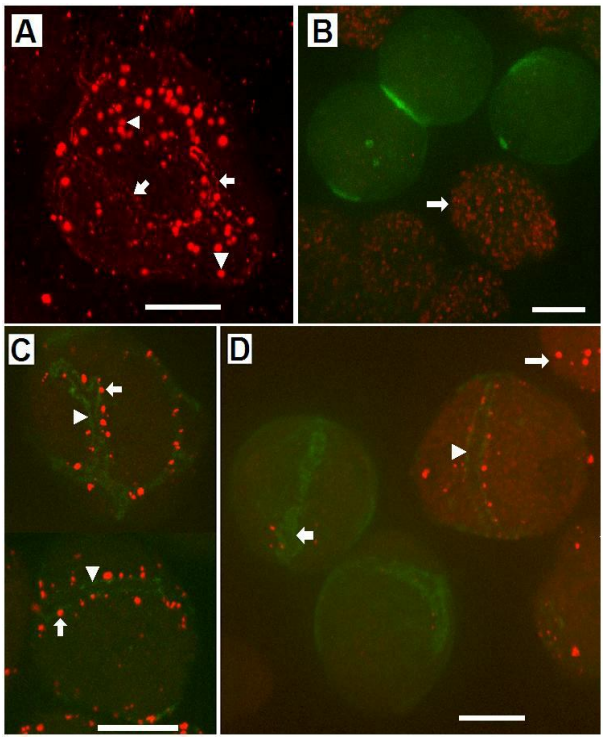

Figure 6. Distribution and kinetic changes of claudin-2 and Cx32 on hepatocytes after isolation. A. Claudin-2 stained, 0 h post isolation. Claudin-2 existing in two forms: network-like form (arrows) and spot form (arrowheads) randomly distributing on membrane; B. F-Actin and claudin-2 co-stained cells, 4 h post isolation. The cells with claudin-2 red spots (arrow) were hepatocytes. The spots becoming fainter and claudin-2 network-like stripes disappeared. The cells stained green may be new hepatocytes or non-parenchymal cells. C. newly isolated hepatocytes (0 h). Cx32 spots (arrows) mainly distribute along outsides of edges of the F-actin stripes (arrowheads). D. 1 h post isolation. Cx32 spots along F-actin stripes (arrowhead) remained on some cells and when F-actin stripes started to change (the thicker arrow) or disappeared, the Cx32 spots showed irregular distribution (the thinner arrow). The white bars represent 10 μm.

Distribution of Claudin-2 and Cx32 and Their Changes after Isolation

Fig. 6 shows the distribution and changes of Claudin-2 and Connexin 32 (Cx32) after isolation. It can be seen from Fig. 6A that at 0 h (just after isolation) claudin-2 is distributed on the cell surface in two forms: randomly distributed spots (Fig.6A) and continuous 'network-like' stripes. Their distribution at 0 h apparently reflects their distribution in normal liver tissue. The Claudin-2 spots were 0.5 - 1 μm in diameter at 0 h and showed reduced stain (Fig.6B). They became very faint by 24 h. The network-like stripes of claudin-2 show stronger staining at the edges of the network stripe residues (Fig.6A). This form of claudin-2 disappeared within 2-3 h after cell isolation.

Fig.6C and D show dual staining of Cx32 (red spots) and F-actin (green) on cells just after isolation and 1 h later respectively. Cx32 exists on cell membrane in spot form. It can be seen from Fig.6C and D that Cx32 spots distribute along both sides of F-actin stripes, which are residues of bile canaliculi. The distance between these spots varies from 0.5 to 2 μm. When F-actin residues started to change (Fig.6D) or disappeared, Cx32 spots remained but showed irregular arrangement within 4 h after isolation (Fig.6D) and diminished within 24 h (picture not shown), which implies the whole membrane is undergoing adaptive changes.

It is clear that claudin-2 and Cx32 diminish slowly after isolation and are not involved in establishing initial cell reconnections after cell isolation.

DISCUSSION

The many varied functions of cell membranes in maintaining cell shape, signal transduction, substance exchange, cell movement and cell division are well known. To our best knowledge, however, how individual hepatic cells adapt themselves to make cell-cell reconnections with other cells after isolation has not been well elucidated. The current study clearly demonstrates, for the first time, that the membranes of all the hepatic cell types are able to undergo adaptive changes before establishing cell-cell connections after isolation. The adaptive changes are characterised by redistributing or remodelling F-actin to form F-actin plaques and clearing up original F-actin residues simultaneously. The F-actin plaques serve as 'Velcro-like' devices to make firm initial cell-cell contacts. In the whole process of cell-cell reconnection, the F-actin plaque was a key transitional membrane 'device' to facilitate cell-cell contact. It may be also an important way to resume tissue architecture such as forming bile canaliculi between new hepatocytes and existing neighbour cells in the normal process of hepatocyte replacement. The whole process can be termed membrane adaptability of hepatic cells for establishing cell-cell reconnections. Thus the adaptive change is a special function of hepatic cell membranes after original cell connections disconnected.

Actin remodelling includes two processes: the G-actin (monomer) can assemble to form F-actin (polymer) and F-actin can be disassembled into G-actin. The G-actin/F-actin ratio is a factor in regulating F-actin assembly [15,16]. Small GTPases of the Rho-family are involved in regulating F-actin polymerisation. GTPase-activating protein can catalyse the transition between GTP-bound (active) and GDP-bound (inactive) states of GPTases. This type of regulation does not require an increase in G-actin synthesis to facilitate rapid F-actin remodelling [17]. In the current study, we observed that G-actin gene expression remained at a stable level while F-actin on the cell membranes underwent dramatic adaptive change. This suggests that actin remodelling plays an essential role in F-actin plaque formation rather than an increase in G-actin synthesis. As shown in the current study, F-actin plays a distinctive role in the early stage of establishing cell-cell connections by forming F-actin plaques. Thus, F-actin can serve as a biomarker in studying membrane adaptability of hepatic cells.

The mechanism to trigger membrane adaptive changes is not fully understood. Nitric oxide (NO) may play a role in initiating this response. We previously reported that cell isolation significantly induced NO synthesis and increased arginine uptake by hepatic cells [18]. NO is an important modulator and is known to play an important role in the wound repair process [19]. Perhaps F-actin itself plays a part in initiating membrane adaptive changes when F-actin attachment is interrupted [9,10].

It was reported that claudin-2 is involved in liver tight-junction formation [7,20,21] and its distribution showed a lobular gradient increasing from periportal to pericentral hepatocytes in rat liver [6,7,20,21]. However, how claudin-2 distributes on the hepatocyte membrane surface had not been clearly elucidated before. We have demonstrated in the current study that claudin-2 has two forms of distribution on hepatocytes. One of them is the 'network-like' form, which is distributed along tight-junctions [7,20], and the other is the spot form randomly distributing on the cell surface (Fig.6A), which has not been reported previously. The current finding suggests that only the network-like form of claudin-2 is involved in tight-junction formation, which is relevant to bile canaliculi formation between hepatocytes.

Cx32 is known to be involved in gap junction formation on hepatocyte membranes [5, 22]. It has been

observed by freeze-fracture replicas that gap junctions distribute peripherally or within the tight-junction network [22]. The current study has clearly shown that Cx32 spots are distributed on outside edges along both sides of tight-junctions on hepatocytes (Fig.6C and D) and underwent change after isolation. It did not play a role at the initial stage of cell-cell connections.

As shown in the current study, F-actin plaques formed on all hepatic cell types and different cell types were able to establish reconnections with each other by F-actin plaque-plaque contacts, whereas claudin-2 and Cx32 mainly exist on hepatocytes [5,22]. Both Claudin-2 and Cx32 did not show involvement in initiating cell-cell reconnections at early stages after cell isolation. In monolayer culture of hepatocytes, claudin-2 and Cx32 disappeared gradually and reappeared on cells 10 days after isolation [22]. Thus it is suggested that F-actin is an essential molecule for establishing initial cell-cell reconnections of hepatic cells at an early stage after isolation. In addition, the current study has clearly demonstrated that F-actin is also one of essential molecules in maintaining tight-junctions (Fig.1A) and cell-cell adhesion in the liver.

It is concluded that hepatic cell membranes undergo adaptive change after enzymatic isolation. The change include forming F-actin plaques and the reduction of original residues of F-actin and other molecules such as claudin-2 and connexion 32. In the whole process, F-Actin plays an essential role in the F-actin plaque formation and cell-cell reconnection at early stage after isolation and can be used as a biomarker to study adaptive change of hepatic cells after cell-cell disconnections. We have, for the first time, demonstrated that the F-actin plaque formed on hepatic cell membrane after cell isolation is a transitional microstructure and serves as a special device to initiate cell-cell connection. It implies that cell membranes of some cell types need to undergo adaptive change to be ready for establishing cell-cell connection after cell isolation or tissue injury and the formation of transitional microstructures is a way to make cell-cell contact and resume tissue-specific architecture.

REFERENCES

1. Timchenko NA. Aging and liver regeneration. Cell 2009; 20:171-176.
2. Raven PH, Johnson R. Biology, fifth edition, WCB/McGraw-Hill, London, pp 105-126 and 1169-1192, 1999.
3. Ma M, Xu J, Purcell WM. Biochemical and functional changes of rat liver spheroids during spheroid formation and maintenance in culture: I. Morphological maturation and kinetic changes of energy metabolism, albumin synthesis and activities of some enzymes. J Cell Biochem 2003; 90:1166-1175.
4. Xu J, Ma M, Purcell W. Characterisation of some cytotoxic endpoints using rat liver and Hepg2 spheroids as *in vitro* models and their application in hepatotoxicity studies: I. glucose metabolism and enzyme release as cytotoxic markers. Toxicol Appl Pharmacol 2003; 189:100-111.
5. Kojima T, Sawada N, Duffy HS, Spray DC. Gap and tight junctions in liver: composition, regulation and function. In: Arias I M, Boyer J L, Chisari F V (eds), The Liver: Biology and Pathology, fourth edition, Williams and Wilkins, Pennsylvania, pp 29-46, 2001.
6. Kojima T, Yamamoto T, Murata H, Kokai Y, Sawada N. Regulation of the blood-billiary barrier: interaction between gap and tight junctions in hepatocytes. Med Electron Microsc 2003; 36:157-164.
7. Imamura M, Kojima T, Lan M, Son S, Murata M, Osanai M, Chiba H, Hirata K, Sawada N. Oncostatin M induces upregulation of claudin-2 in rodent hepatocytes coinciding with changes in morphology and function of tight junctions. Exp Cell Res 2007; 313:1951-1962.
8. Lambrechts A, Toys MV, Ampe C. The actin cytoskeleton in normal and pathological cell motility. Internat J Biochem Cell Biol 2004; 36:1890-1909.
9. Ridley AJ. Membrane ruffling and signal transduction. Bioessays 1994; 16 (Suppl):321-327.
10. Zigmond SH. Signal transduction and actin filament organization. Curr Opin Cell Biol 1996; 8:66-73.
11. Furukawa R, Jinks T M, Tisharten T, Mazzawi M, Morris DR. Elongation factor-1 beta an actin-binding protein. Biochim Biophys Acta 2001; 1527:130-140.
12. Tzanakakis ES, Hansen LK, Hu WS. The role of actin filaments and microtubles in hepatocyte spheroid self-assembly. Cell Motility Cytoskelet 2001; 48:175-189.
13. Rombouts K, Knittel T, Machesky L, Braet F, Wielant A, Hellemans K, De Bleser P, Gelman I, Ramadori G, Geerts A. Actin filament formation, reorganization and migration are impaired in hepatic stellate cells under influence of trichostatin A, ahistine deacetylase inhibitor. J Hepatol 2002; 37:788-796.
14. Eitzen G. Actin remodelling to facilitate membrane fusion. Biochim Biophys Acta 2003; 1641: 175-181.
15. Jaffe AB, Hall A. Rho GTPases: Biochemistry and biology. Ann Rev Cell Dev Biol 2005; 21: 247-269.
16. Ladwein M, Rottner K. On the Rho'd: The regulation of membrane protrusions by Rho-GTPases. FEBS Lett 2008; 582:2066-2074.
17. Welch MD, Mallavarapu A, Rosenblatt J, Mitchison TJ. Actin dynamics in vivo. Curr Opin Cell Biol 1997; 9:54-61.
18. Xu J, Ma M, Purcell WM. Biochemical and functional changes of rat liver spheroids during spheroid formation and maintenance in culture: II. Nitric oxide synthesis and related changes. J Cell Biochem 2003; 90:1176-1185.

19. Schwentker A, Vodovotz Y, Weller R, Billiar TR. Nitric oxide and wound repair: role of cytokines? Nitric Oxide 2002; 7:1-10.

20. Tsukita S, Furuse M. Pores in the wall: Claudins constitute tight junctions' strands containing aqueous pores. J Cell Biol 2000; 149:13-16.

21. Rahner C, Mitic LL, Anderson JM. Heterogeneity in expression and subcellular localization of claudins 2, 3, 4, and 5 in the rat liver, pancreas, and gut. Gastroenterol 2001; 120:411-422.

22. Kojima T, Yamamoto M, Tobioka H, Mizuguchi T, Mitaka T, Mochizuki Y. Changes in cellular distribution of connexin32 and 26 during formation of gap junctions in primary cultures of adult rat hepatocytes. Exp Cell Res 1996; 223:314-326.