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Effects of Cholestatic Agents on the Structure and Function of Bile Canaliculi in Neonatal Rat Hepatocytes in Primary Culture

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KONO, Y., FUKUNAGA, M., SHIRAKI, K. and AKIYOSHI, H. *Effects of Cholestatic Agents on the Structure and Function of Bile Canaliculi in Neonatal Rat Hepatocytes in Primary Culture.* Tohoku J. Exp. Med., 1997, **181** (1), 9-18 —— The effects of cytochalasin B and colchicine on the structure and function of bile canaliculi were studied in neonatal rat hepatocytes in primary culture. Cellular contacts of neonatal hepatocytes were not as tight as those of adult hepatocytes. There was no remarkable difference in the ultrastructure of bile canaliculi between neonatal and adult hepatocytes. Neonatal hepatocytes treated with cytochalasin B were round in shape and aggregated in groups of several cells. Actin filaments stained by rhodamine-phalloidin were disrupted and condensed at the cell periphery or around dilated bile canaliculi. Markedly-dilated bile canaliculi with less microvilli were observed by transmission electron microscopy while the secretory function of horseradish peroxidase, which was used as a marker for uptake, transport and secretion into bile canaliculi, were maintained. The lumen of dilated bile canaliculi was found close to the undersurfaces of hepatocytes by scanning electron microscopy after turning over the cultured cells. By colchicine treatment, the filamentous structure of microtubules in neonatal hepatocytes disappeared. The ultrastructure of the bile canaliculi was not affected by the treatment, but transport and secretion of horseradish peroxidase into bile canaliculi were inhibited. The development of strict cellular polarity in neonatal hepatocytes may be suppressed in neonatal hepatocytes; however, cholestatic agents which rearrange the cytoskeleton caused the same morphological or functional changes of bile canaliculi as in adult hepatocytes. —— neonatal hepatocytes; cholestasis; bile canaliculus

The mechanism responsible for uptake, transport and secretion of bile in hepatocytes is important in understanding the pathogenesis of cholestasis. Previous studies have described that the integrity of the cytoskeletal structure of

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hepatocytes may be necessary in these processes (Feldmann 1989; Kawahara et al. 1989; Nathanson and Boyer 1991; Watanabe et al. 1991). The bile canaliculus (BC) is the smallest secretory channel in the liver and the development of a functioning BC is essential for the secretion of bile (Arias et al. 1993).BC domains are characterized by numerous microvilli in adult hepatocytes which exhibit striking cellular polarity (Evans 1980; Feracci et al. 1987). In the fetal liver, hepatocytes have not established complete cellular polarity (Ciofi Luzzatto 1981); columns of hepatocytes are not arranged in lines and grooves of BC are distorted.

We have reported previously using neonatal rat hepatocytes models in vitro that development of cellular polarity and BC in neonatal and adult hepatocytes are different (Kohno et al. 1993a). In neonatal hepatocytes, the formation of tight rigid cellular contacts was suppressed. The proliferating hepatocytes stimulated by human epidermal growth factor (hEGF) showed limited formation of functioning BC (Kohno et al. 1993b). Because of the immaturity of the hepatic excretory function of neonatal hepatocytes, susceptibility to cholestatic agents of neonatal hepatocytes might be different from that of adult hepatocytes, resulting in intrahepatic cholestasis in the neonatal period.

In this study, we investigated the effects of agents which rearrange the cytoskeleton on the structure and function of BC in neonatal rat hepatocytes in primary culture to elucidate the mechanism of cholestasis in the immature liver. Morphological changes caused by cytochalasin B and colchicine in neonatal hepatocytes were visualized by immunofluorescence and scanning electron microscopy (Kohno et al. 1993a, b). Secretory function into BC in hepatocytes was studied using transmission electron microscopy with horseradish peroxidase (HRP) as an electron-dense tracer (Sakisaka et al. 1988).

Materials and Methods

Culture of hepatocytes. Hepatocytes were isolated by perfusing the liver in situ with collagenase solution (type 1, Wako Pure Chemical Industries, Osaka) from neonatal rats (day 5) and adult male rats (7 weeks) as previously described (Kohno et al. 1991, 1993a, b). Cell viability as determined by trypan blue exclusion was over 90% in each experiment. The hepatocytes were resuspended in Williams' E medium (Kyokutou Pharmaceutical Co., Tokyo), containing 5% fetal bovine serum, insulin (1.0 nmol/liter, Sigma Chemical Co., St. Louis, MO, USA), and dexamethasone (1.0 nmol/liter, Sigma) and plated on collagen-coated dishes (Corning, Glass Works, Corning, NY, USA) (Kohno et al. 1991). They were incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. At 3 h after plating, the culture medium was replaced with serum-and hormone free-Williams' E medium.

Treatment with cholestatic agents. Cytochalasin B was dissolved in dimethyl sulfoxide (DMSO) and diluted to 5×10^{-7} mol/liter in a culture medium containing 0.1% DMSO (Kawahara et al. 1989). Colchicine (10^{-5} mol/liter) was prepar-

ed using the culture medium (Sakisaka et al. 1988). Neonatal hepatocytes were exposed to the agents for 2 hr and fixed at 6 hr or 24 hr in culture. Control hepatocytes were treated with 0.1% DMSO for 2 hr and fixed at 6 hr or 24 hr in culture.

Scanning electron microscopy. Ultrastructure of cellular contacts and BC were examined by scanning electron microscopy at 6 hr in culture. The method of preparation for scanning electron microscopy was the same as reported before (Kohno et al. 1993a, b).

Fluorescence microscopy. We visualized cytoskeletal structure of actin and microtubules by immunofluorescence. After 6 or 24 hr in culture, cells were first incubated with mouse IgG monoclonal antibody to α-tubulin (Amersham International, Amersham, England) at a dilation of 1 : 500 for 30 min at room temperature. The samples were incubated with fluorescein isothiocyanate (FITC)-conjugated rabbit anti mouse IgG (Zymed Laboratories Inc., San Francisco, CA, USA) for 30 min. Rhodamine-phalloidin (Molecular Probes Inc., Eugene, OR, USA) was applied to stain F-actin at a dilution of 1 : 20 for 30 min at room temperature. The labeled samples were examined with an Olympus fluorescence microscope (model BH-2).

Labeling with horseradish peroxidase and transmission electron microscopy. Hepatocytes were labeled with horseradish peroxidase (HRP, Sigma) to study transport and secretory function by transmission electron microscopy as previously reported (Sakisaka et al. 1988; Kohno et al. 1993b). The cells were incubated with 5 mg/ml HRP dissolved in culture medium for 3 min at 37°C after 6 hr or 24 hr in culture, then washed with HRP-free medium, and fixed after further incubation in HRP-free medium for 15 or 30 min. They were then fixed with 1% glutaraldehyde in 0.1 mol/liter phosphate buffer (pH 7.4) for 30 min and incubated with 0.2% 3,3′-diamino benzidine (Sigma) in tris buffer and 0.01% H_2O_2 for 30 min. After post-fixation, they were dehydrated and embedded in Epon 812. Thin sections made by en-face cutting were examined with a Hitachi-H500 transmission electron microscope.

Results

Scanning electron microscopy Neonatal control hepatocytes after 6 hr in culture varied in shape, but most of them were oblong (Fig. 1a). Cellular contacts were not straight or rigid as in adult hepatocytes. Some long fibers bridged intercellular spaces and connected adjacent hepatocytes. Short microvilli were observed on the entire surface. In contrast, adult control hepatocytes were flattened and polygonal (Fig. 1b). They were twice as large as neonatal hepatocytes and developed typical intercellular faces, which were straight segments of two parallel plasma membranes. There were numerous short microvilli on the cellular surface.

After treatment with cytochalasin B for 2 hr, neonatal hepatocytes cultured

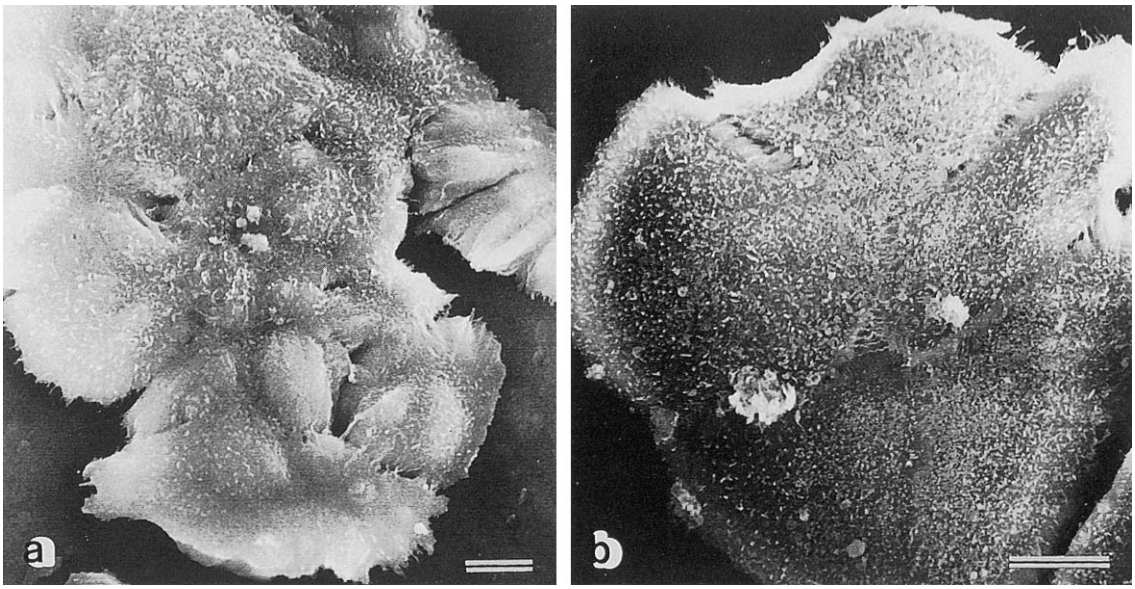

Fig. 1. Scanning electron micrographs of neonatal (a) and adult (b) control hepatocytes cultured for 6 hr. Hepatocytes were cultured in a basal medium without treatment. Cellular contacts in neonatal hepatocytes were not as tight as adult hepatocytes. (a) $\times$1,000, Bar, 10 μm; (b) $\times$1,500, Bar, 10 μm.

for 6 hr became round and smaller in shape (Fig. 2a). They aggregated in groups of several cells with some hepatocytes being overlaid on top of others. Many bleb formations on the cell surfaces were observed. No dilated lumen of BC was found on the apical surfaces of cultured hepatocytes. However, when cells were turned over with double-adhesive tape for viewing the undersurface of cultured hepatocytes, a markedly-dilated lumen close to the undersurface, which corresponds to the dilated BC observed by transmission electron microscopy, was found (Fig. 2b). Treatment with colchicine for 2 hr caused an irregular cellular shape and decrease of microvilli on the cell surface (Fig. 2c). Long fibrous structures connecting intercellular spaces were observed. The undersurface of the hepatocytes was flat and no dilated BC lumen was found.

Fluorescence microscopy We carried out fluorescent staining to investigate the relationship between the cytoskeletal elements and BC in neonatal hepatocytes after 24 hr in culture. In neonatal control hepatocytes, the filamentous structure of the microtubules was concentrated near the nucleus and spread out in a radiating pattern in the cytoplasm. Rhodamine-phalloidin staining showed that actin filaments were distributed near the cell membrane and were strongly stained between adjacent cells, possibly corresponding to BC (Fig. 3a). In adult hepatocytes, BC was more clearly surrounded by actin filaments and the number of BC was more than in neonatal control hepatocytes. The distribution of microtubules was the same as that in neonatal control hepatocytes. Incubation with cytochalasin B caused disruption and aggregation of actin filaments (Fig. 3b) which condensed into focal pools near the cell membrane and around markedly-dilated BC. Microtubules appeared unaffected by treatment with cytochalasin B.

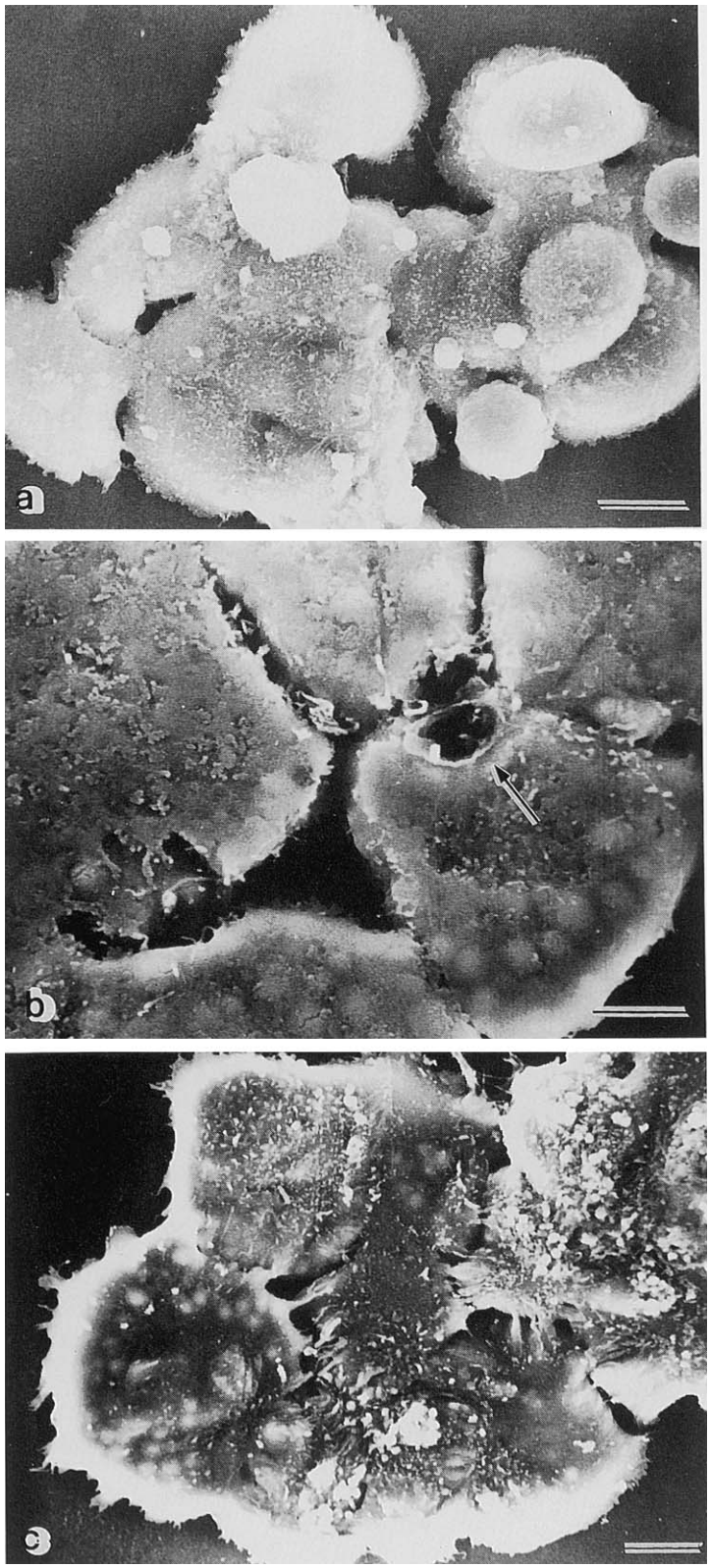

Fig. 2. Scanning electron micrographs of neonatal hepatocytes cultured in a basal medium for 3 hr and exposed to cytochalasin B (a) and (b), or colchicine (c) for 2 hr. (a) Apical surfaces faced the culture medium of neonatal hepatocytes treated with cytochalasin B. ×1,200, Bar, 10 μm.
(b) Undersurfaces attached to the culture dish of neonatal hepatocytes treated with cytochalasin B. A markedly-dilated BC lumen (arrow) was observed. ×3,000, Bar, 5 μm. (c) Apical surfaces of neonatal hepatocytes treated with colchicine. ×1,200, Bar,10 μm.

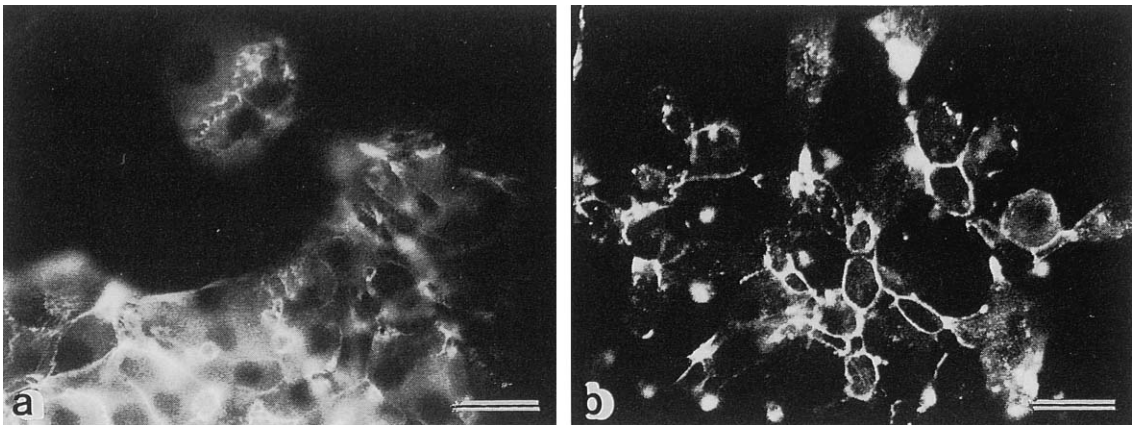

Fig. 3. Fluorescence micrographs of neonatal hepatocytes stained actin filaments with rhodamine-phalloidin. (a) Control neonatal hepatocytes cultured for 24 hr. ×400, Bar, 20 μm. (b) Cytochalasin B treated neonatal hepatocytes. Hepatocytes were cultured for 24 hr with incubation with cytochalasin B in the last 2 hr. ×400, Bar, 20 μm.

After treating with colchicine, the filamentous structure of the microtubules disappeared and the cytoplasm was stained diffusely, but not in a radiating pattern, with anti-α tubulin antibody. No difference in the distribution of actin filaments was found between the control cells and colchicine-treated cells.

Transmission electron microscopy and HRP secretion. Neonatal hepatocytes after 6 hr in culture showed a BC-like structure lined with short microvilli and sealed with faint electron dense material (Fig. 4a). There was no significant difference in the ultrastructure of BC observed by transmission electron microscopy between neonatal and adult rat hepatocytes. Preferential localization of cell organelles, such as Golgi complex, rough endoplasmic reticulum and lysosomes in the region of BC was observed. HRP uptake was found in endocytotic vesicles. After an incubation for 30 min, secretion of HRP into the canalicular lumen was observed (Fig. 4b).

Cytochalasin B treatment caused marked dilatation of BC with less microvilli (Fig. 5a). However, secretion of HRP into BC was normally observed. Colchicine did not affect the fine structure of BC. However, transport of HRP to BC was not found after a 30 min incubation. HRP-containing vesicles were presented throughout the cytoplasm, these rarely being seen in the pericanalicular region (Fig. 5b).

Discussion

As we have described in a previous report (Kohno et al. 1993a), the formation of tight rigid cellular contacts was suppressed in neonatal hepatocytes comparing with adult hepatocytes in primary culture. Organization of actin filaments and development of functioning BC were inhibited in actively proliferating neonatal hepatocytes by hEGF treatment (Kohno et al. 1993b). In contrast, by dexameth-

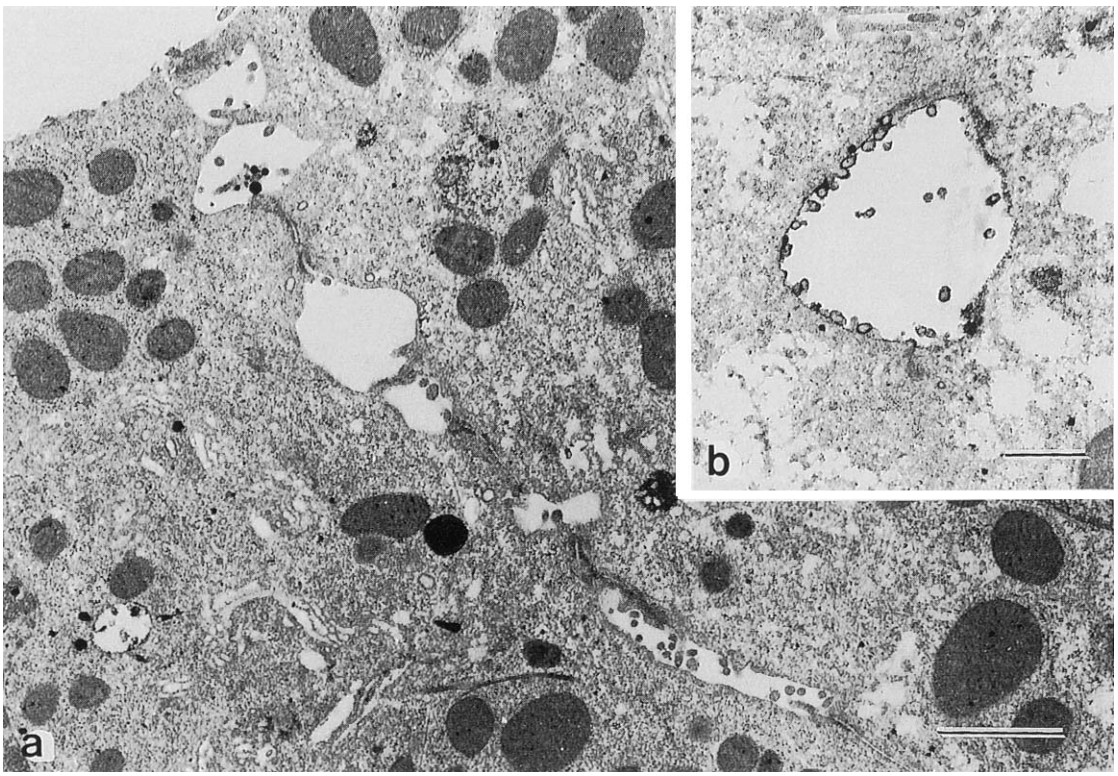

Fig. 4. Transmission electron micrographs of neonatal hepatocytes cultured for 6 hr. (a) Bile canaliculi (BC) were lined with microvilli and sealed with tight junctions. ×6,000, Bar, 5 μm. (b) Secretion of horseradish peroxidase (HRP) into the BC lumen in the control culture incubated for 30 min after a 3-min labeling was observed. ×10,000, Bar, 1 μm.

asone treatment, the development of tight cellular contacts and functioning BC was observed as adult hepatocytes (Kohno et al. 1993b). Thus, neonatal hepatocytes are considered to be not fully differentiated in the development of cellular polarity and secretory function of BC. There are many previous studies of the effect of cholestatic agents on the structure and function of BC in adult hepatocytes (Sakisaka et al. 1988; Feldmann 1989; Kawahara et al. 1989), but not in neonatal hepatocytes. In this study, using the technique of scanning electron microscopy for cultured hepatocytes that we have developed (Kohno et al. 1993a), a view of the entire cytoplasm and cellular contacts as well as the undersurface of neonatal hepatocytes treated with agents could be investigated.

Table 1 summarizes the effects of cytochalasin B and colchicine on the structure and function of BC in neonatal hepatocytes. Cytochalasin B inhibits the polymerization of G-actin to F-actin (Copper 1987). In the adult rat liver, it causes dissociation of actin filaments from the canalicular membrane and correlates with a decrease in bile flow (Oda and Phillips 1977). Dilatation of the canaliculus, loss of microvilli and loss of canalicular contractility after incubation with cytochalasin B are demonstrated in the model of adult couplet hepatocytes cultured in a short-term monolayer culture (Oda and Phillips 1977; Phillips et al.

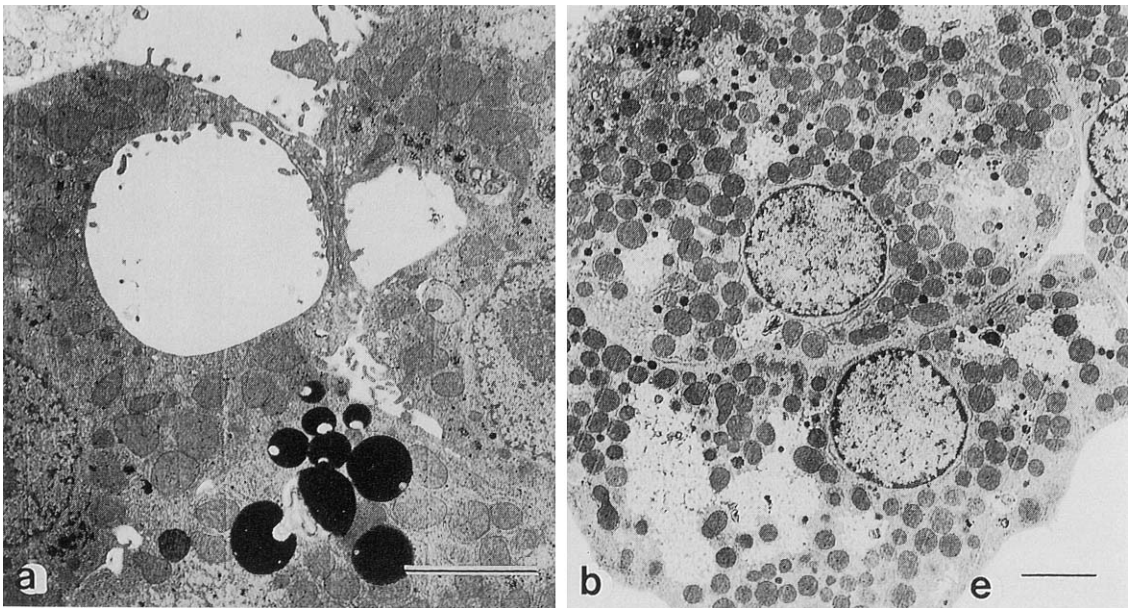

Fig. 5. Transmission electron micrographs of neonatal hepatocytes cultured for 6 hr and exposed to cytochalasin B (a) or colchicine (b) in the last 2 hr. (a) Roundly-dilated BC with less microvilli, which corresponds to a dilated lumen found close to the bottom of the cells by scanning electron microscopy, was observed. ×6,000, Bar, 1 μm, (b) No ultrastructural change of BC was observed after colchicine treatment. ×3,200, Bar, 5 μm.

TABLE 1. *Summary of morphological characteristics of neonatal and adult hepatocytes in primary culture with or without treatment of cholestatic agents*

	Treatment	Cellular shape	Cellular contacts	Actin distribution	BC formation	HRP secretion
Neonatal	Control[a]	Oblong	Loose	Around BC, cell peripheral	+	+
	Cytochalasin B	Round, smaller	Slightly loose	Disruption, aggregate	+ Markedly-dilated	+
	Colchicine	Oblong	No change	Around BC, cell peripheral	+ No change	−[c]
Adult	Control[1]	Polygonal	Very tight	Around BC	++[b]	+

[a]Control hepatocytes were not treated with any agents.

[b]The number of BC in adult hepatocytes observed by fluorescent microscopy was more than in the neonatal control hepatocytes.

[c]Horseradish peroxidase (HRP) secretion into BC lumen was not observed.

1983). The same morphological changes as adult hepatocytes by transmission electron microscopy were observed in neonatal hepatocytes. On the apical surfaces that faced the culture medium, no orifice of BC was observed. However, we could find the dilated lumen of BC on the undersurface of hepatocytes by using our technique of scanning electron microscopy. We could speculate that the BC structure of the neonatal hepatocytes in primary culture would be formed close to the bottom of the adjacent cells and the direction of the canaliculi would not be

parallel with the direction of monolayer of hepatocytes. These results coincide with the reports by Gautam et al. (1987) and Sakisaka et al. (1988) showing that the modulation of cell membranes appeared on the undersurface of hepatocytes in early culture stages. Even while BC was remarkably dilated by cytochalasin B treatment, HRP secretion to BC remained intact in neonatal hepatocytes. Investigation on the contractility of BC using time-lapse photomicrography (Oshio et al. 1985) has shown that cytochalasin B inhibits and alters canalicular contraction. Actin filaments seemed to play an important role not in the secretory process but in the contraction of BC, that is the pumping action of bile flow in vivo (Tsukada et al. 1995).

Colchicine, which is an inhibitor of microtubules (Mimura et al. 1995), is known to impair the excretion of lipids or lipoprotein into the bile (Gregory et al. 1978). However, the effects of colchicine on the secretory function of adult hepatocytes are still controversial. In couplets of hepatocytes cultured for 3 hr (Sakisaka et al. 1988), colchicine inhibited the movement of HRP-containing vesicles to the BC. In differentiated hepatocytes cultured for 48 hr with dexamethasone (Kawahara et al. 1989), colchicine did not affect uptake or transport of HRP, but did completely inhibit secretion of HRP into BC, suggesting that colchicine decreases the contraction of BC after a lag by impairing the organization of microtubules. In the neonatal hepatocytes in this study, colchicine did not have an effect on the ultrastructure of BC but suppressed HRP transport through cytoplasm into BC lumen. Microtubules appeared to play a role both in the transport and secretion of HRP to BC at least in neonatal hepatocytes after 6 hr in primary culture.

In summary, the structure and function of BC in neonatal hepatocytes was equally affected by cholestatic agents to adult hepatocytes. Since proliferative activity of immature hepatocytes in the neonatal period may lead to differences in cellular polarity and development of functioning BC, intrahepatic cholestasis could be easily caused by hormones and other agents in the neonatal liver on the basis of immaturity of hepatic excretory function.

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