DEVELOPMENT OF THE BILE CANALICULAR SYSTEM IN RAT LIVER: WITH SPECIAL REFERENCE TO BILE CANALICULAR ACTIN FILAMENTS AND Mg^{2+} , Ca^{2+} -ATPase ACTIVITY

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Developmental changes of the bile canaliculi (BC) in rat (fetal 13th–21st day, postnatal and adult) livers were cytochemically examined by fluorescence, light and electron microscopy. Bile canalicular actin filaments (F-actin), consisting of the circumferential pericanalicular F-actin and microvilli (MV) core F-actin, were observed by 7-nitrobenz-2-oxa-1,3-diazole phalloidin (NBD-ph) fluorescence microscopy and electron microscopy. Mg^{2+} , Ca^{2+} -ATPase activity, which is presumed to be involved in bile acid secretion across the bile canalicular membrane, was detected by a modified Wachstein and Meisel method at both light and electron microscopic levels. Bile canalicular F-actin sparsely appeared from the 13th day of gestation. Since then, the amount of bile canalicular F-actin gradually increased accompanying the bile canalicular formation by 2 weeks after the birth when the development was almost completed. Mg^{2+} , Ca^{2+} -ATPase activity appeared on the fetal 19th day in less than 10% of the BC. The number of Mg^{2+} , Ca^{2+} -ATPase-positive BC rapidly increased around the time of birth (fetal 21st day–postnatal 1st day). The rate of ATPase positive BC reached 40–70% during this period. On the 5th day after birth, most BC showed Mg^{2+} , Ca^{2+} -ATPase activity. These findings indicated that the expression of bile canalicular F-actin and ATPase can be regarded as a structural marker and a functional marker for bile secretion, respectively. Structural development of BC began earlier but at a rather slower rate than functional maturation.

Bile canalicular F-actin has been thought to play a significant role in bile transport of the bile canalicular system (22). Bile canalicular F-actin changes its volume in various liver cholestasis. In experimental animals, phalloidin and cytochalasin B treatments make bile canalicular F-actin either overpolymerized or depolymerized, respectively, to cause intra-canalicular MV disappearance and intrahepatic jaundice (4, 10, 19, 23). Abnormality of bile canalicular F-actin was frequently observed in patients and animals with obstructive jaundice, drug induced liver injury and other hepatic disorders (9, 31). Recently, the contraction motility of BC was observed both *in vitro* and *in vivo* (33–35). These observations suggested that pericanalicular F-actin worked as the physical machinery of BC movements to make a flow of bile juice (33–35). Moreover, it was also reported that F-actin was associated with the formation of bile canalicular MV (6, 28). Thus, the bile canalicular F-actin may play an important role in the intrahepatic

bile flow and the structural formation of BC, although the developmental changes associated with pericanalicular F-actin and MV-core F-actin have not been investigated yet. Therefore, it is of interest to examine the change in bile canalicular F-actin during the development of the liver.

On the other hand, the bile canalicular membrane is an important place where bile acids are secreted from hepatocytes into the bile canalicular lumen. Recent biochemical studies indicated that the membrane transport of the bile acids was mediated by a carrier protein (25, 26). Since the carrier transport system required ATPase-hydrolysis (21), it is possible that Mg^{2+} , Ca^{2+} -ATPase activity on the BC membrane may be involved in bile acid transport across the BC membrane. Actually, it is known that ethynylestradiol treatment, which induces cholestasis, weakens ATPase activity of the hepatocyte membrane (24). In contrast, epomediol which reverses cholestasis activates ATPases in hepatocytes (17).

Thus, it seems ATPase activity in hepatocytes is closely associated with bile secretory function. From these aspects, bile canalicular F-actin and Mg^{2+} , Ca^{2+} -ATPase may be used as distinct type indicators of BC maturation with regards to their structure, contractility and secretory function during the developmental period.

The aims of the present study are to resolve the following questions. We would first like to determine the relationship between the appearance of bile canalicular F-actin and the morphological development of BC system. Next, when and how Mg^{2+} , Ca^{2+} -ATPase activity on bile canaliculi appear are of interest. Finally, we want to determine whether Mg^{2+} , Ca^{2+} -ATPase can be used as an indicator of bile acid secretion from hepatocytes. In this study, NBD-ph-fluorescence staining methods (2) and enzyme cytochemical techniques were applied to demonstrate the developmental changes of bile canalicular F-actin and Mg^{2+} , Ca^{2+} -ATPase in rat liver. Furthermore, the double staining technique of F-actin and Mg^{2+} , Ca^{2+} -ATPase, which we recently reported on (12), was also employed.

MATERIALS AND METHODS

Fetal 13th–21st day (on every other day), postnatal 1st, 3rd, 5th, 7th, 14th day and adult (more than 3 months after birth) Wistar rats were used in this study. Male and female rats were mated to obtain fetal and perinatal rats, and the day of gestation was determined as fetal 0 day when the vaginal plug was observed at 8–10 am. Laparotomy of infant, adult and pregnant rats was carried out under pentobarbital anesthesia. Delivered fetuses and newborn rats were decapitated before laparotomy. Liver samples of each developmental stage were obtained from 4–6 rats. The livers were immediately removed from rats and cut into small pieces to be processed by the following methods.

Observation of F-actin

a) Fluorescence microscopy

The removed liver was immediately cut into small pieces and treated with a solution consisting of 0.03% saponin, 8% sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 5 μ M leupeptin in PHEM buffer (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.8) for 15 min at room temperature. After rinsing in the same buffer without saponin, they were fixed with 4% paraformaldehyde (PFA) in PHEM buffer containing 8% sucrose for 2 hr at 4°C. The fixed liver tissues were cut into 7 μ m-thick frozen sections with a cryostat. These frozen sections were

placed on a gelatin-coated glass slide, and air-dried. Then, a drop of NBD-ph solution (Molecular Probes Inc., 5.0 units/ml in PHEM buffer) was applied to the tissue on the glass slides and the incubation of F-actin staining was carried out in a dark box for 30 min. Then, the specimens were washed by PBS and sealed with PBS-glycerin. The fluorescence micrographs were taken with an Olympus BH-2 using Kodak Tri-X pan films.

b) Electron microscopy

An improved technique for observing both cytoskeletal elements and membrane organelles in hepatocytes was previously described (1). Briefly, liver tissues were treated with 0.03% saponin in PHEM buffer, and the pieces of liver tissues were fixed with 2% PFA, 0.5% glutaraldehyde (GA) and 0.4% tannic acid in PHEM buffer at pH 6.8, containing 8% sucrose for 2 hr at 4°C. Then, the tissues were post-fixed in 1% osmic acid for 1 hr at 4°C. After block staining with uranyl acetate, the specimens were dehydrated and embedded in Spurr's resin. Ultrathin sections, counter-stained with uranyl acetate and lead citrate, were examined with a Hitachi H-800 transmission electron microscope (TEM) at 100–150 kV.

Cytochemistry for Mg^{2+} , Ca^{2+} -ATPase

The liver was cut into small pieces in a fixative, 2% PFA, 0.5% GA, 30 mM Pipes buffer at pH 7.4, containing 8% sucrose. After the immersion fixation for 2 hr at 4°C in the same fixative, the fixed tissues were cut into 7 μ m and 50 μ m-thick sections with a cryostat for light and electron microscopic cytochemistry, respectively. Enzyme cytochemical incubation for Mg^{2+} , Ca^{2+} -ATPase was carried out using a modified incubation medium of Wachstein and Meisel (32) consisting of 2.0 mM ATP-2Na, 80 mM Tris-maleic acid, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 3.6 mM lead nitrate, 5% sucrose at pH 7.4. The concentrations of Mg^{2+} and Ca^{2+} in the incubation medium were based on the biochemical data on bile acid transporters (ATPase) in rat bile canalicular membranes (21). As a cytochemical control, either substrate (ATP-2Na) or Ca^{2+} , Mg^{2+} was omitted from the incubation medium. The tissues were incubated in the medium for 30 min at 37°C. For light microscopic observation, the incubated sections were treated with 2% ammonium sulfide to form lead sulfate. For electron microscopy, these specimen were postfixed in a mixture of 1% osmic acid and 0.8% potassium ferrocyanide for 30 min at 4°C. They were then dehydrated, embedded, counter stained with uranyl acetate and examined with a Hitachi H-800 TEM.

The proportion of ATPase-positive BC in whole BC was estimated by counting more than 50 BC on the electron micrographs for each stage.

Double staining of NBD-ph and Mg^{2+} , Ca^{2+} -ATPase

The details were documented in our previous paper (12). Briefly, liver tissues were fixed in 4% PFA and 8% sucrose in 30 mM Pipes for 2 hr at 4°C. Then, detergent-extraction with 0.1% saponin in 10% DMSO and 30 mM Pipes was performed for several hours. Next, 7 μ m-thick frozen sections were placed on a gelatin-coated glass slide. For histochemical incubation of Mg^{2+} , Ca^{2+} -ATPase, these glass slides with tissues were immersed in the same medium as mentioned above for 20 min. Then, NBD-ph staining and fluorescence microscopy were carried out by the same procedure described above. Coloring of Mg^{2+} , Ca^{2+} -ATPase activity and light microscopic examination were performed on the same preparation after completing fluorescence microscopy of NBD-ph.

RESULTS

NBD-ph fluorescence

In adult rat liver (Fig. 1), BC were most preferentially revealed by the strong fluorescence of NBD-ph owing to the abundant pericanalicular F-actin. BC were revealed as strong fluorescent spots in cross sec-

tion, and as some bold irregular lines in longitudinal section at the light microscopic level. They anastomosed with each other to form BC networks. The sinusoidal and lateral membranes were also moderately positive for NBD-ph, since they had membrane-associated F-actin. The cytosol of hepatocytes also showed slight fluorescence, but there was not reflection in the nucleus.

Figs. 2 showed the morphological changes in appearance of the pericanalicular F-actin during the developmental period. In fetal liver, bile canalicular F-actin were recognized from the 13th day of gestation, although the distribution of bile canalicular F-actin spots was sparse and the bile canalicular F-actin lines representing the BC continuity were short (Fig. 2A). Short canals of BC seen in the early liver gradually increased in number and length, day by day, as the area that the hepatocytes were in contact became larger and larger. From 15th to 17th day of gestation, it seemed that development of the BC system somewhat slowed down since hematopoietic stem cells (HSC) in the liver were most abundant during those days. The early BC network was formed by branching and anastomosing around the 19th day of gestation (Figs. 2B, C). Irregularly dilated parts of the BC were frequently seen during the perinatal period especially from the fetal 21st day to the

FIG. 1. Fluorescence micrograph of adult rat liver stained with NBD-ph.

Bile canalicular F-actin are revealed as strong spots or bold lines of NBD-ph fluorescence, featuring the profile of BC (large arrows). BC are often recognized between two opposite hepatocytes. In some sections, they appeared in a hexagonal pattern (small arrows). The lateral and sinusoidal membranes of the hepatocytes are moderately positive for NBD-ph, due to the membrane-associated F-actin (arrowheads). $\times 400$

postnatal 3rd day (Fig. 2D). This irregular dilation tended to disappear by the postnatal 7th day, as hepatocyte arrangement became more regular and BC branch channeling became more complicated (Fig. 2E). The BC network appeared almost the same as those of adults on the postnatal 14th day.

Electron microscopic observation of BC and bile canalicular F-actin

Pericanalicular F-actin appeared as a fuzzy web structure around the BC. F-actin were also seen in the MV of the BC. The BC fluorescence revealed by NBD-ph seemed to correspond to the F-actin in both pericanalicular web and MV (Figs. 3).

By electron microscopic observation, three types of BC were distinguished by the degree of their structural maturation, i.e., the formation of MV through the development of the BC (Figs. 3A-E). The first type is an immature type of BC having very few or no MV. The BC of this type were recognized only by their tight junction. Immature type BC might not be visible by NBD-ph fluorescence, because those are poor in F-actin. The second type of BC observed is a mature type of BC which have a lot of MV. Mature type BC were rich in bile canalicular F-actin which consisted of a considerably thick web of F-actin around the bile canalicular lumen, a part of them raised from the adherent junction. However, the most abundant part of F-actin seemed to be the MV-core where F-actin rose from the pericanalicular F-actin web extending into the MV. The volume of F-actin seemed to be largely dependent on the number of MV. The third type of BC observed by electron microscopy is an in-

termediate type in which the BC have a moderate number of MV with irregular lengths. Pericanalicular F-actin of intermediate type BC are somewhat less than those of mature type. On the 13th day of gestation, more than half of the recognized BC were of the immature type, although the mature type BC were less than 10%. The proportion of intermediate and mature type of BC in the whole BC was increasing. It reached about 90% at the time of birth. A small number of immature BC, which were presumed to be newly differentiated, were still recognized even on the postnatal 3rd-7th day. However, immature BC were hardly seen on the 14th day after birth.

Light microscopic observation of Mg^{2+} , Ca^{2+} -ATPase activity

The appearance of Mg^{2+} , Ca^{2+} -ATPase activity was largely located on the BC membrane in rat hepatocytes (Figs. 4). During the fetal period, the ATPase activity began to appear on some BC from the 19th day of gestation, but no reaction was observed before the 17th day (data not shown). During the perinatal period, on the 21st day of gestation and the 1st day after birth, the ATPase-positive BC markedly increased in number. Double staining with NBD-ph and ATPase demonstrated that about half of the BC revealed by NBD-ph showed ATPase activity just before birth. In adult liver, all BC showed the strong ATPase activity.

Electron microscopic observation of Mg^{2+} , Ca^{2+} -ATPase activity

The BC showing Mg^{2+} , Ca^{2+} -ATPase activity were mostly of the mature type. Although some intermediate type BC showed ATPase activity, im-

FIGS. 2A-F. Fluorescence micrographs of fetal (2A-2C) and postnatal rat livers (2D-2F) stained with NBD-ph. $\times 400$

A. On the 13th day of gestation, the distribution of BC revealed by bile canalicular F-actin (arrows) is sparse. B. On the 17th day of gestation, BC are increasing in number and in length, although branching is rarely seen and is short (small arrow). C. On the 19th day of gestation, an early BC network is established. D. On the postnatal first day, BC are somewhat dilated, especially at the junction of BC (arrow). E. On the postnatal 5th day, hepatocytes arrangement becomes more regular, the BC network is becoming more complicated, resembling more those networks of adults. F. On the 14th day after birth, the BC channeling has been well-established.

FIGS. 3A-E. Electron micrographs of the bile canalicular region of hepatocytes treated with a saponin extraction.

A. Low magnification of the adult rat liver. The membrane structure indicating BC (arrows) and other organelles are well preserved even after the detergent extraction. F-actin and other cytoskeletal elements are clearly observed. $\times 8,300$ B. Immature BC from a liver on the 13th day of gestation. MV are few in number, and pericanalicular F-actin are poor. $\times 20,000$ C. A mature BC, which has abundant MV and bile canalicular F-actin (small arrows), is rarely seen on the 13th day of gestation. $\times 20,000$ D. Intermediate type BC from a liver on 17th day of gestation. $\times 20,000$ E. Mature BC from an adult liver. $\times 20,000$ N: nucleus. G: Golgi apparatus. MT: mitochondria. sER: smooth endoplasmic reticulum. rER: rough endoplasmic reticulum.

FIGS. 4A-F. Double staining of F-actin and Mg^{2+} , Ca^{2+} -ATPase in the same field. (A, C, E) Fluorescence of NBD-ph, (B, D, F) Mg^{2+} , Ca^{2+} -ATPase. $\times 400$

A, B. On the 19th day of gestation. Some BC recognized by NBD-ph (large arrows in A) show weak or trace ATPase activity (small arrows in B), although most BC show no activity. Some HSC are also positive (arrow heads). C, D. On the 21st day of gestation. About half the number of BC show moderate activity of ATPase (large arrows) and others show little activity (small arrows). E, F. Adult rat liver. All BC show intense ATPase activity.

mature BC had no ATPase activity. On the 19th and 20th day of gestation, about 10% of the mature and intermediate type of BC had ATPase activity. This proportion rapidly increased to 40–60% on the fetal 21st day (Fig. 5A), and 50–70% on the 1st day after birth. They reached about 90% from the 3rd to 7th day after birth (Fig. 5B). In the adult, more than 95% of the

BC (almost all BC were of the mature type) showed the ATPase activity (Fig. 5C).

There was no clear difference in the ATPase of the BC before suckling and after suckling on the 1st day of gestation.

These results are summarized in Table 1.

FIGS. 5A–C. Electron micrographs of livers cytochemically reacted for Mg^{2+}, Ca^{2+} -ATPase activity.

A. On the 21st day of gestation. Some mature BC show ATPase activity (large arrows), while other mature BC have no reaction products (small arrows). $\times 5,300$ B. On the 3rd day after birth. Most BC show ATPase activity. Some BC are dilated (arrows). $\times 5,300$ C. Adult rat liver. All recognizable BC show strong ATPase activity. $\times 7,600$

TABLE 1. *Development of bile canalicular system*

days	fetus					infant					
	13	15	17	19	21	birth-1	3	5	7	14	adult
mature BC/total BC ¹⁾	±	+	+	2+	2+	2+	2+	3+	3+	4+	4+
BC network formation ²⁾	—	—	±	+	+	+	2+	2+	2+	3+	3+
irregular dilation of BC ³⁾	—	—	+	+	2+	2+	2+	+	—	—	—
ATPase activity ⁴⁾	—	—	—	±	+	+	2+	2+	2+	3+	3+

1) Proportion of BC with well developed MV in whole BC was estimated from electron micrographs (EM).
± 10%, + 20–40%, 2+ 50–60%, 3+ 70–80%, 4+ >90%

2) The degree of BC network formation was expressed as follows, based on the fluorescence microscopy (FL) with NBD-ph.
— no branching short BC, ± rarely branching short BC, + early network formation, 2+ incomplete hexagonal network, 3+ complete network formation

3) Frequency of BC dilation was estimated both from EM and FL.
— no dilation, + occasionally, 2+ frequently

4) Proportion of ATPase-positive BC in whole BC.
— 0%, + <10%, + 40–70%, 2+ 80–90%, 3+ >95%

DISCUSSION

Bile canalicular F-actin

F-actin widely exist in various cell types and play an important role as a member of the cytoskeleton to support the cell membrane and to mediate cell movement, etc. (20). Hepatocytes have an abundant amount of F-actin, especially in the bile canalicular region (1, 22). The formation of bile canalicular F-actin was mainly studied using cultured hepatocytes (10, 18). To our best knowledge, the developmental changes of bile canalicular F-actin in fetal and postnatal rat liver have never been reported, so far.

The prominent organ of the rat liver appears from the 11th–12th day of gestation (8, 36). The early form of BC was detected from the 12th–13th day of gestation by electron microscopic observation (36). However, no information is available about the formation of bile canalicular F-actin at these early stages. Our observations by NBD-ph and electron microscopy revealed that the mature type BC which were rich in pericanalicular F-actin and MV were already present at the 13th day of gestation, although, the number of mature BC was very little. This suggested that morphological maturation of each BC began early and bile canalicular F-actin had great importance in the fetal BC formation as well as in the postnatal formation.

Until the fetal 17th day, BC, even of the mature type, were short in length. It seemed that MV might develop in newly formed BC before the formation of BC connection. This finding suggested that the maturation of each BC was at an earlier time and at a

faster event than the whole BC network formation.

As for the irregular dilation of the BC which was most frequently seen during the perinatal period, the fetal 21st day to the postnatal 3rd day, a possible explanation may be that the incomplete channeling of the BC network among the loosely contacted hepatocytes cause accumulation of some bile juice or pre-bile products in the BC (3). In addition, immature BC with less pericanalicular F-actin might prohibit the normal bile flow. A little time lag was present between the beginning of BC dilation and ATPase expression. It may be owing to the sensitivity of cytochemical detection of ATPase or ATPase-independent bile secretion. The BC dilation during the neonatal cholestasis disappeared on the postnatal 7th day when the BC network construction was almost completed. From these aspects, it can be expected that morphological development of BC began earlier but at a rather slower rate than maturation of bile secretory function. Actually, this expectation was confirmed by our observation on Mg^{2+} , Ca^{2+} -ATPase activity expression in the developmental liver.

Mg^{2+} , Ca^{2+} -ATPase

The style of Mg^{2+} , Ca^{2+} -ATPase appearance in fetal rat was different from that of alkaline phosphatase (ALP) which is also used as a histochemical marker of BC. De Wolf-Peeters *et al.*, (3) reported that ALP activity on BC was seen from the 14th day of gestation. It was not conceivable that the early expression of ALP activity was associated with bile secretory function. On the other hand, Mg^{2+} , Ca^{2+} -ATPase activity appeared from the 19th day and increased during the

perinatal period. About 40–70% of BC during the perinatal period showed ATPase activity. This fairly wide variation of the proportion may be due to the rapid changing from ATPase-negative BC to positive ones.

According to earlier biochemical data on bile acid metabolism during development (14), rat hepatocytes begin bile acid secretion at the 19th day of gestation, although bile acid synthesis in hepatocytes starts at the 17th day of gestation. The bile acid synthesis and secretion fully mature after birth.

As Mg^{2+} , Ca^{2+} -ATPase activity examined in our study showed a correlation to this bile acid secretion from developing liver, the ATPase may be involved in bile acid secretion from the BC of hepatocytes. The rapid increase of the ATPase in the last stage of the fetal period correlates well with the timing when bile acid secretion becomes necessary for the first milk taken within the several hours after birth.

Vectorial transport of bile acids from sinusoidal blood across the hepatocytes into the BC is a critical physiological process in bile formation (11). The uptake of unconjugated bile acids from the sinusoidal membrane is carried out by Na^+ , K^+ -ATPase (localized in the sinusoidal membrane) which mediates carrier protein transport system (16, 30).

During hepatocellular transcytosis, unconjugated bile acids are mainly conjugated at the smooth endoplasmic reticulum to reach around the bile canaliculi. These conjugated bile acids are excreted into the bile canicular lumen across the canicular membrane, but the mechanism of this secretory process has not yet been understood in detail. Recent biochemical studies indicated that the canicular membrane contained an ATP-dependent bile acid carrier protein (21, 29). ATP-hydrolysis may play a role in giving an electrogenic membrane potential to mediate this ATP-dependent carrier transport (21). This carrier protein is thought to be a 100–110 kDa membrane glycoprotein (7).

Mg^{2+} , Ca^{2+} -ATPase, a representative form of ecto-enzyme in the BC membrane which hydrolyses extracellular ATP, is presumed to mediate some membrane transport system, but its function has not been elucidated (13).

To our interest, recent biochemical studies gave rise to a possibility that ecto-ATPase in the BC membrane was the same protein as HA-4 which was considered as a bile acid transporter (15, 25, 27). HA-4 is also a 100–110 kDa glycoprotein which is predominantly localized in the BC membrane. Similar to our observations of ATPase activity, HA-4 was also detected on the 19th day of gestation, showing a strong

signal on the fetal 21th day (5). Therefore, it is strongly suggested that Mg^{2+} , Ca^{2+} -ATPase activity cytochemically detected in this study, at least partially, corresponds to HA-4, i.e. a bile acid transport carrier protein. It may be concluded that cytochemical detection of Mg^{2+} , Ca^{2+} -ATPase activity can be used as a useful and convenient indicator for bile secretory function in hepatocytes.

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