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Immunocytochemistry of perinatal rat livers with a special reference to the roles of mesenchymal cells in hepatic differentiation

T. Hayashida[1], N. Nagata[1], Y. Doi[2], T. Ozaka[1] and H. Itoh[1]

Departments of [1]Surgery and [2]Anatomy, University of Occupational and Environmental Health, School of Medicine, Kitakyushu, Japan

Summary. To investigate the roles of extracellular matrix produced by hepatic mesenchymal cells in the organization of hepatic cell cords, perinatal rat livers were examined with immunocytochemistry of fibronectin (FN) and laminin (LM). Some hepatocytes in a free state at prenatal day 15 actively produced FN and LM in the rough endoplasmic reticulum but lost this synthetic activity when such cells were incorporated into hepatic cell cords. On the other hand, hepatic mesenchymal cells, especially those associated with the perisinusoidal space, retained this synthetic activity throughout the stages examined. In the differentiating hepatic cell cords, positive immunoreactions for FN and LM were preferentially seen on the cell surface facing both sinusoidal space and differentiating bile canaliculus concomitant with the expression of the tight junction protein, ZO-1, from prenatal day 17. Since such hepatocytes have lost or reduced their synthetic activities of both glycoproteins in the rER, the immunoreactions appear to be mainly due to hepatic mesenchymal cells which seem to play a role in the formation of the hepatic cell cords and the bile canaliculi.

Key words: Developing rat liver, Fibronectin, Immunocytochemistry, Laminin, Tight junction protein, ZO-1

Introduction

The roles of hepatic mesenchymal cells (HMCs) in the differentiation of hepatocytes (HCs) have been investigated from the viewpoint of epithelial-mesenchymal interactions in in-vivo and in-vitro studies (Sherer, 1975; Fukuda, 1979; Fukuda-Taira, 1981; Yamamoto et al., 1989; Teresa Donato et al., 1990). Also, recent immunocytochemical studies have focused on the roles of extracellular matrix (ECM) in such reciprocal parenchymal-nonparenchymal interactions, with emphasis on the roles of fibronectin (FN) and laminin (LM) in the induction of hepatic tissue organization (Wartiovaara et al., 1979; Hahn et al., 1980; Hirata et al., 1983; Wan et al., 1984; Landry et al., 1985; Bissel et al., 1986, 1987; Bockman and Gulati, 1989; Reif et al., 1990; Baloch et al., 1992). However, immunoelectron microscopic surveys of these problems in differentiating hepatic tissues have been limited (Baloch et al., 1992).

Liver parenchymal morphogenesis is a composite of two processes, the fragmentation of hepatic endoderm and the organization of HCs into cell cords with bile canaliculi between adjacent HCs. Baloch et al. (1992) described that LM provides a scaffold of the developing liver, but once the differentiation is complete, the perisinusoidal LM expression is suppressed. Further immunoelectron microscopy is indicated to elucidate the spatiotemporal alterations in the production of ECM components in HMCs and/or HCs and the roles of ECM produced by HMCs in hepatic differentiation. In addition, whether the formation of tight junctions between apposed HC's modifies synthetic activities of such cells, as previously suggested by Tavoloni et al. (1985), remains uncertain.

On these grounds, the present work was designed to investigate immunocytochemically the localization of FN, LM and the tight junction protein, ZO-1, in perinatal rat livers. The main purpose of this study was to elucidate the roles of FN and/or LM, released from HMCs during hepatic tissue organization, especially in the differentiation of hepatic cell cords using ultrastructural investigations.

Materials and methods

Animals

Wistar rats of prenatal days 15, 17, 19 and 21, and

Offprint requests to: Dr. Sunao Fujimoto, Department of Anatomy, University of Occupational and Environmental Health, School of Medicine, Kitakyushu 807, Japan

postnatal days 1 and 5 were used.

Conventional electron microscopy

Livers were fixed in a mixture of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer for 2 hr at 4 ºC, postfixed in 1% osmium tetroxide in the same buffer, dehydrated in graded concentrations of acetone, and embedded in epoxy resin. Ultrathin sections were made on an ultramicrotome, stained with uranyl acetate and lead citrate, and then examined in a JEM 1200 EX electron microscope.

Light microscopic immunocytochemistry

Livers were fixed in 10% formalin fixative for 6 h at room temperature, dehydrated in graded concentrations of ethanol, embedded in paraffin, and cut into approximately 2 µm-thick sections. After deparaffinization, sections were digested with 0.4% pepsin in 0.01 N HCl in 0.1M phosphate buffered-saline (PBS) for 2 h at 37 ºC and treated with 0.3% H_2O_2 in absolute methyl alcohol for 20 min to reduce endogenous peroxidase activity. After non-specific binding was blocked with 10% normal goat serum for 5 min, sections were reacted to rabbit anti-rat FN (LSL. INC.) and rabbit anti-rat LM (CMN. INC.), respectively, at a dilution of 1:100 in 0.1M PBS for 1 hr at room temperature. The sections were rinsed in PBS, and reacted to both biotinylated goat anti-rabbit IgG and peroxidase conjugated streptavidin for 40 min each (BSA method), and were then developed in a mixture of 0.05% diaminobenzidine (DAB) and 0.01% H_2O_2.

Immunoelectron microscopy

Livers were fixed in a periodate-lysine-paraformaldehyde solution (McLean and Nakane, 1974) in 0.1M PBS containing 10% sucrose for 12 hr at 4 ºC. Approximately 20 µm-thick sections were made on a microslicer (DSK. Co.), treated with 0.3% H_2O_2 in absolute methyl alcohol, and reacted to the above mentioned antibodies and to rabbit anti-rat ZO-1 antibody (CMN. INC.) at a dilution of 1:100 in 0.1M PBS for 1 hr at room temperature. After rinsing in 0.1M PBS, immunostainings were carried out using the BSA method as described above. Sections were postfixed in 0.1% osmium tetroxide in 0.1M PBS for 5 min, dehydrated in graded concentrations of acetone, embedded in epoxy resin, cut into ultrathin sections, and examined in the electron microscope without counterstaining.

Controls

The specificity of the immunoreactions was confirmed by substituting the normal rabbit sera for the primary antisera.

Results

1) Conventional electron microscopy

At prenatal day 15, the preexisting vessels derived from the vitelline veins were associated with an abundance of HCs, hematopoietic cells (HPCs), and HMCs possessing thin cytoplasmic projections (Fig. 1). Adjacent HCs occasionally possessing thin cytoplasmic

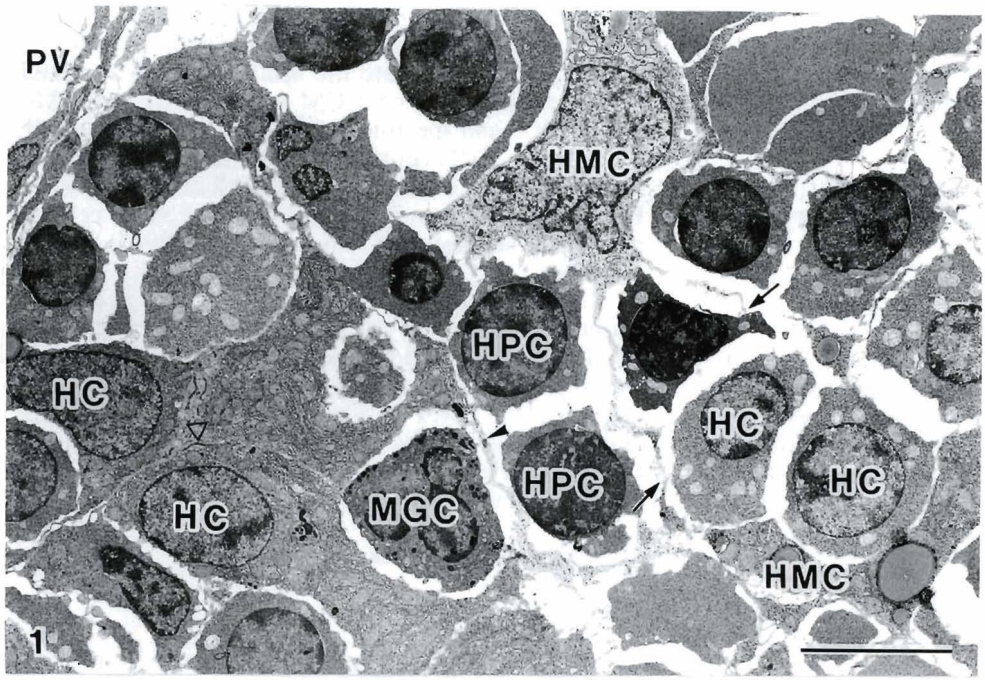

Fig. 1. Developing liver at prenatal day 15. HCs, HMCs and HPCs are associated with the preexisting vessel (PV). HMCs extend thin cytoplasmic projections among HPCs forming the microenvironment (arrows). Adjacent HCs are conjugated to each other by simple attachment devices (open arrowhead) and the thin cytoplasmic projection (arrowhead) is juxtaposed to a megakaryocyte (MGC). Bar: 5 µm.

projections were conjugated to each other by simple attachment devices (Fig. 1).

At prenatal day 16, profiles of growing capillaries, of which endothelial cells were very similar in ultrastructure to HMCs, were frequently seen (Fig. 2). Some HMCs containing lipid droplets were occasionally associated with the endothelium of such growing capillaries which were devoid of the basal lamina (Fig. 2). At prenatal day 17, the growing capillaries consisted of the continuous attenuated endothelium and were associated with HMC, of which cytoplasmic projections were in contact with the endothelium (Fig. 3).

The continuous endothelium developed pores of various sizes without diaphragm by prenatal day 21. In our electron micrographs, the endothelium was seen to be involved in the transmural diapedesis of HPCs indicating the differentiation into sinusoidal capillaries by the formation of temporary migration pores (Fig. 4). The differentiation into sinusoidal capillaries possessing numerous pores with or without diaphragm was

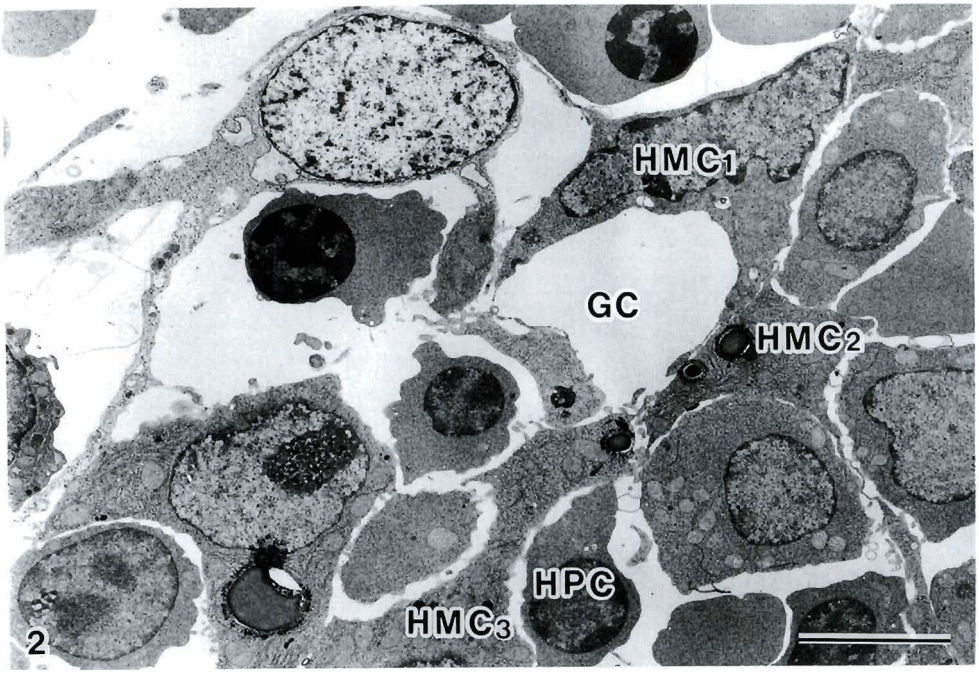

Fig. 2. An HMC (HMC_1) forms a growing capillary lumina (GC), and HMCs (HMC_2 and HMC_3) containing lipid droplets exist near the growing capillary. At prenatal day 16. Bar: 5 μm.

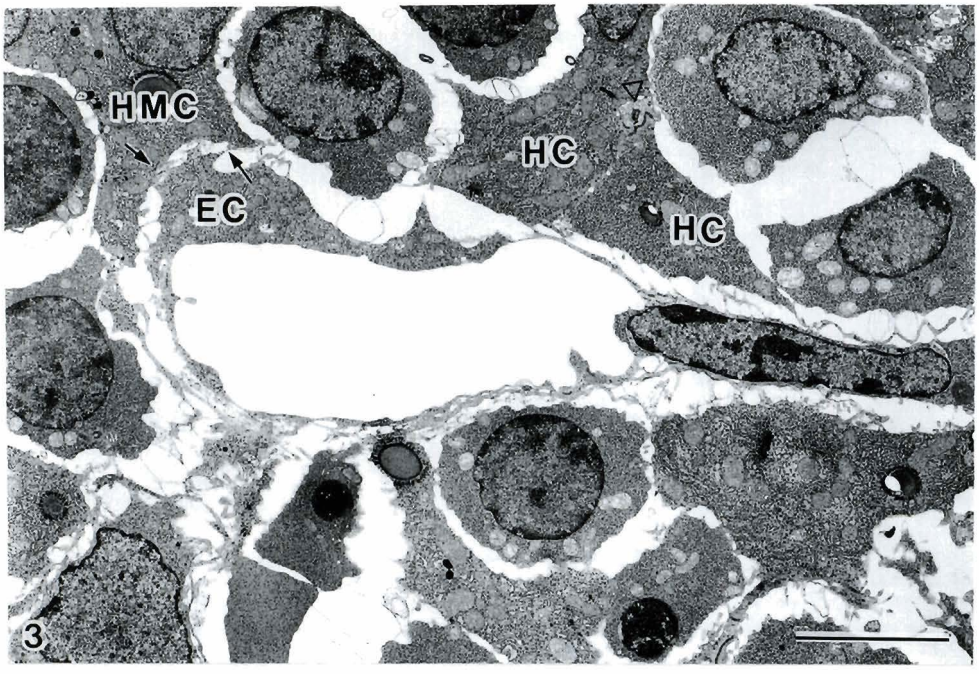

Fig. 3. A young endothelial cell (EC) of the growing capillary is similar in ultrastructure to the surrounding HMC which is in contact with the endothelial cell by the cytoplasmic projections (arrows). HCs are in contact with each other forming the differentiating bile canaliculi (open arrowhead). At prenatal day 17. Bar: 5 μm

pronounced after birth. In such sinusoidal capillaries, the basal side of young endothelial cells occasionally possessed deep infoldings lined with discontinuous basal lamina (Fig. 5). HMCs containing lipid droplets were often located below the endothelium.

2) Light microscope immunocytochemistry of FN and LM

At prenatal day 15, some HMCs and HCs showed immunoreactivity of both FN (Fig. 6) and LM (Fig. 6b). A positive reaction for both glycoproteins was also apparent in megakaryocytes, as already reported in the case of FN by Bockman and Gulati (1989). The perisinusoidal space of newly-formed capillaries was also immunoreactive for both glycoproteins. However.

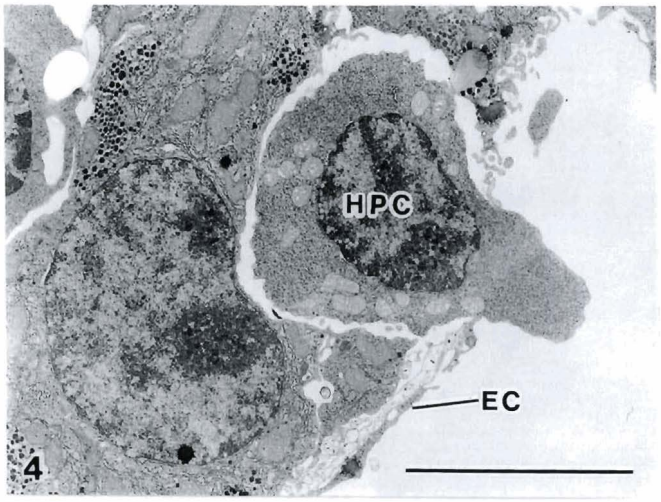

Fig. 4. Diapedesis of an HPC through the endothelium (EC) of the sinusoidal capillary is shown. At prenatal day 21. Bar: 5 μm.

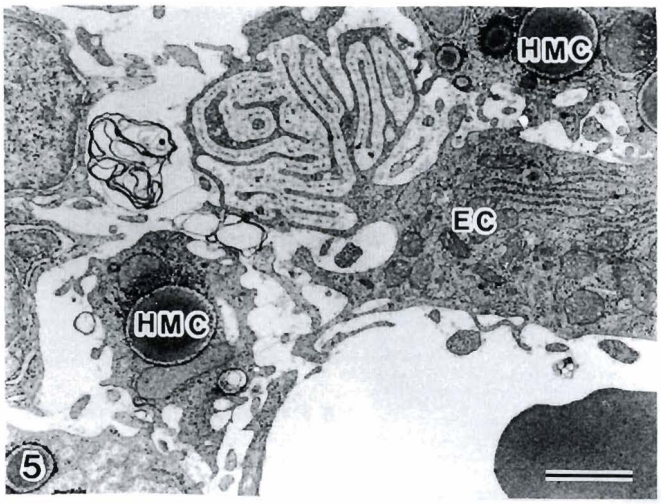

Fig. 5. Basal infoldings lined with the discontinuous basal lamina of an endothelial cell (EC) of the sinusoidal capillary are shown. HMCs containing lipid droplets are associated with the capillary. At postnatal day 5. Bar: 1 μm.

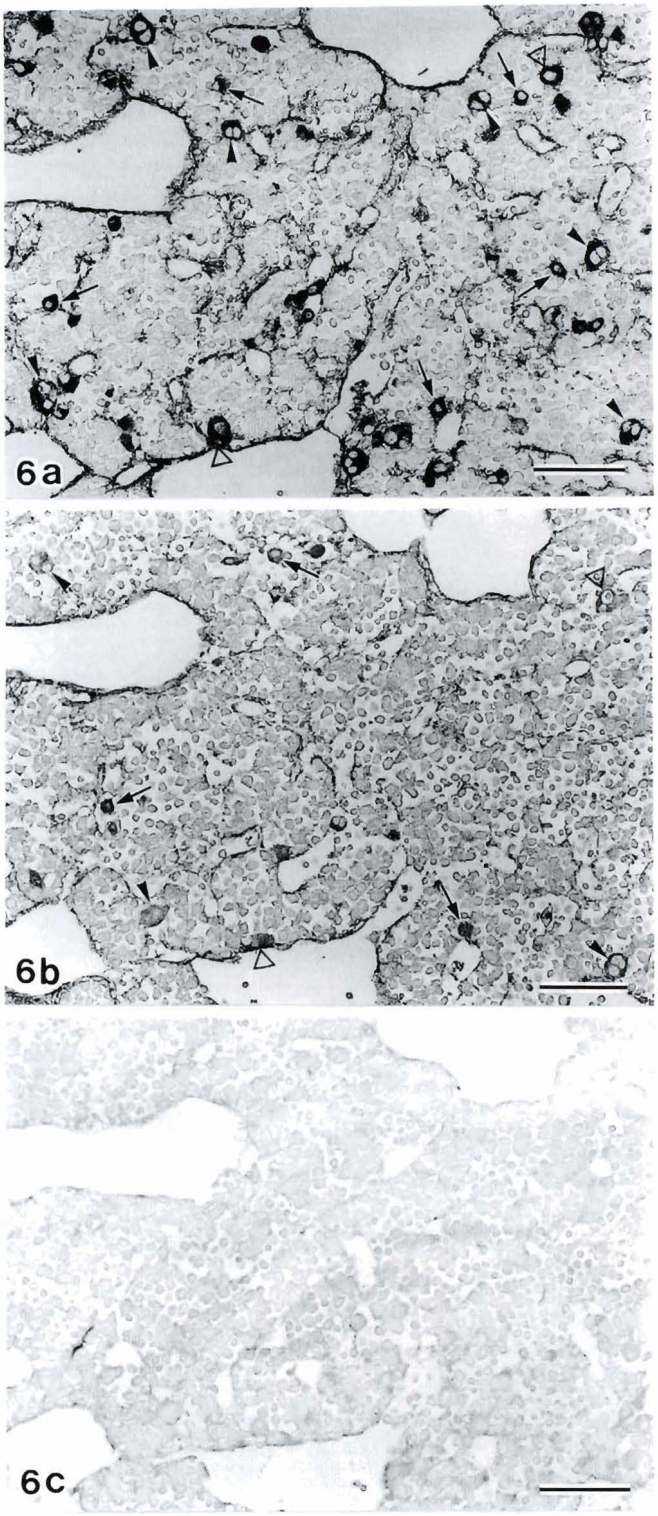

Fig. 6. Immunoreactions of FN (a), LM (b) and control (c) at prenatal day 15 in each adjacent section. HCs (arrows), HMCs (open arrowheads) and megakaryocytes (arrowheads) occasionally show immunoreactions of both FN and LM. The perisinusoidal space is also immunoreactive for both glycoproteins. Bar: 20 μm.

the FN and LM immunoreactivities in HCs began to decrease after prenatal day 17 as the conjugation between adjacent HCs was progressing (Fig. 7) and completely disappeared at postnatal day 1 (Fig. 8). On the other hand, the perisinusoidal space and HMCs, especially those associated with the perisinusoidal space, retained immunoreactivities of both glycoproteins throughout the stages examined (Figs. 7-9).

3) Immunoelectron microscopy of ZO-1, FN and LM

Although adjacent HCs were occasionally conjugated to each other by simple attachment devices from prenatal day 15 (Fig. 1), the formation of hepatic cell cords in which individual HCs were conjugated to each other by tight junctions progressed in accordance with the development of bile canaliculi after prenatal day 17. Apparent immunoreactions of ZO-1 already appeared in these areas of the two apposed HCs at prenatal day 17 (Fig. 10).

HCs in a free state at prenatal day 15 showed FN and LM immunoreactivities in the rough endoplasmic reticulum (rER), but not on the cell surface (Figs. 11a,b). However, once HCs were successively integrated into hepatic cell cords after prenatal day 17, the immunoreactions inside the HCs disappeared, but those on the cell surface both facing the sinusoidal capillaries (Figs. 12a,b) and the differentiating bile canaliculi between each apposed HC (Figs. 13a,b) became evident. On the other hand, HMCs, especially associated with the perisinusoidal space, retained FN and LM immunoreactions in the rER throughout the stages examined (Figs. 14a,b).

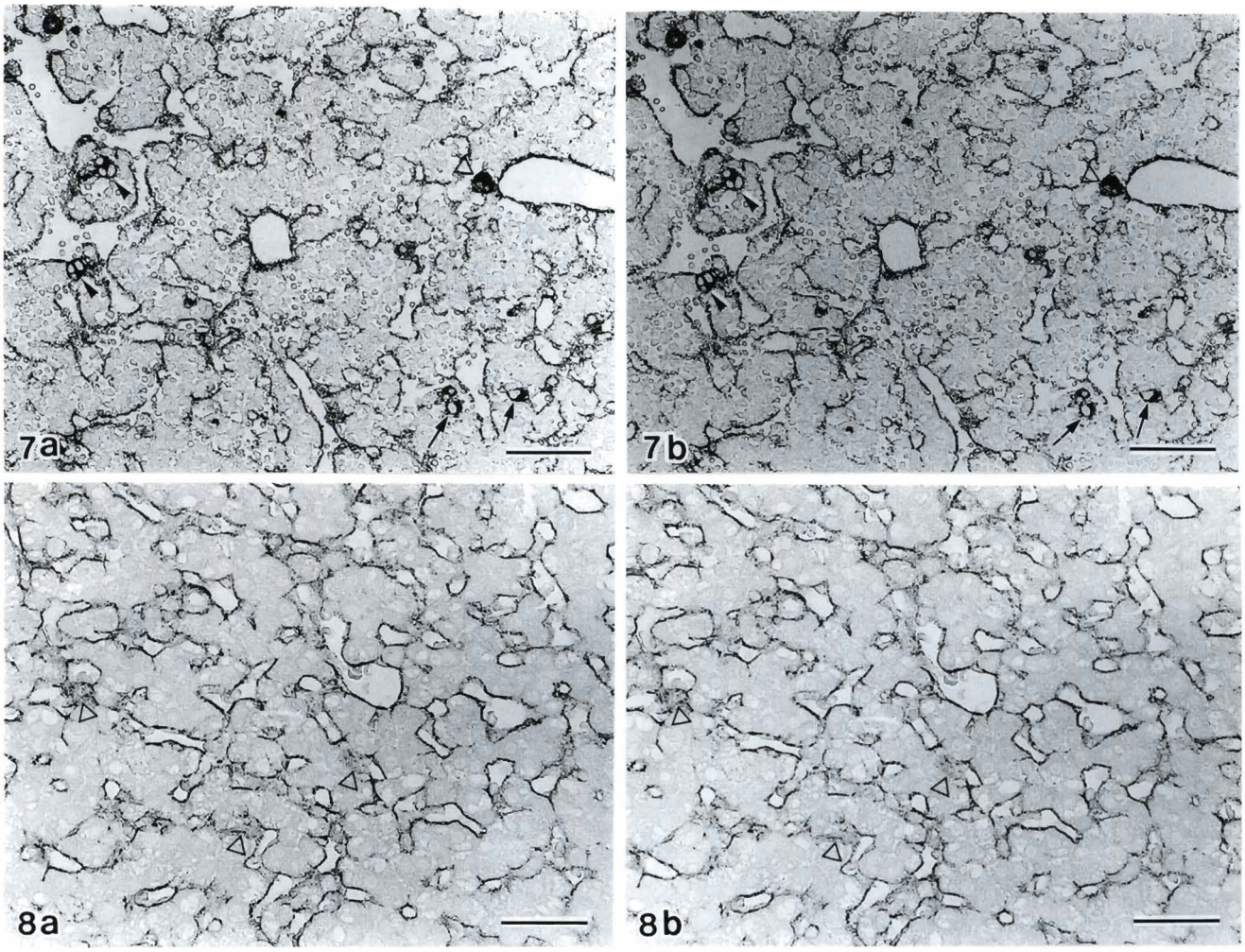

Fig. 7. Immunoreactions of FN (a) and LM (b) at prenatal day 17 in each adjacent section. Immunoreactive HCs for both glycoproteins (arrows) appear to decrease in number when compared to those at prenatal day 15, as shown in Fig. 6. Megakaryocytes (arrowheads), HMCs (open arrowheads) and perisinusoidal space show the immunoreactivities. Bar: 20 μm.

Fig. 8. Immunoreactions of FN (a) and LM (b) at postnatal day 1 almost completely disappear in HCs, but HMCs associated with the perisinusoidal space retain the immunoreactions (open arrowheads). Bar: 20 μm.

Young endothelial cells of the growing capillaries occasionally showed immunoreactions of FN and LM in the rER and at the cell surface (Figs. 15a,b), but well-differentiated endothelial cells lost these immunoreactivities.

Discussion

The effects of nonparenchymal cells on the morphological and functional differentiation of HCs have already been reported (Sherer, 1975; Fukuda, 1979; Fukuda-Taira, 1981; Yamamoto et al., 1989; Teresa Donato et al., 1990; Sirica, 1995). In our samples, HCMs seem to be actively involved in the transformation to endothelial cells of the growing capillaries in a manner suggestive of vasculogenesis: young endothelial cells are similar in ultrastructure to the adjacent HMCs of which cytoplasmic projections are often in contact with the endothelium. The present study suggests the classic data (Le Douarin, 1964) that HMCs are transformed into endothelial cells during the formation of the hepatic microvasculature.

By immunoelectron microscopy, it can be seen that HMCs, especially those associated with the growing capillaries, are actively involved in the production of FN and LM in their rER throughout the stages examined. Bissel et al. (1987), Grant et al. (1989), Martínez-Hernández et al. (1991) and Mori et al. (1992) reported the involvement of both FN and LM in vasculogenesis in the in-vivo and in-vitro specimens. The immature endothelial cells of the growing capillaries also retain this synthetic ability in the rER, as shown in Figs. 15a,b. We consider at present that FN and LM, synthesized and released by HMCs and by young endothelial cells which

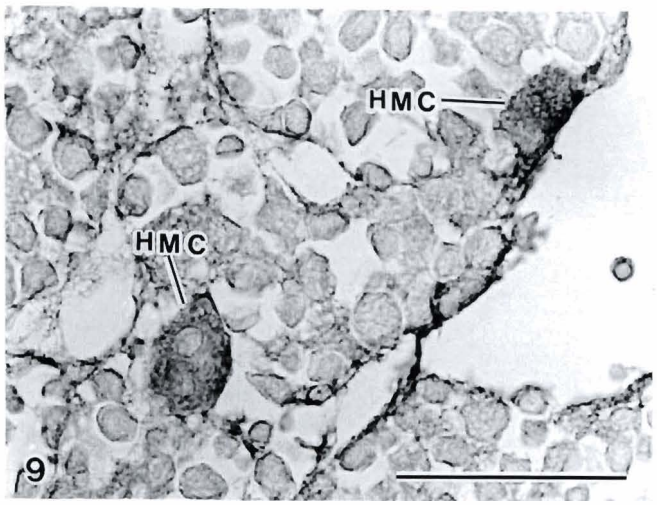

Fig. 9. Highly magnified HMCs associated with the perisinusoidal space showing immunoreactions of FN at postnatal day 1. Bar: 20 µm.

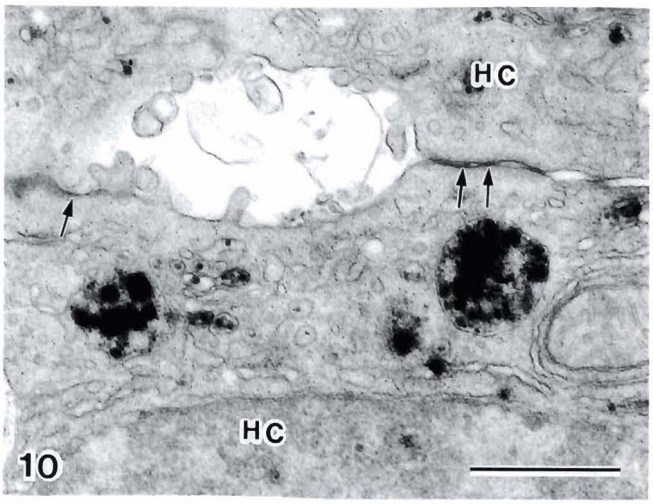

Fig. 10. Immunoreactions of ZO-1 (arrows) are seen along tight junction areas of the apposed HCs forming the bile canaliculus at prenatal day 17. No counterstain. Bar: 1 µm.

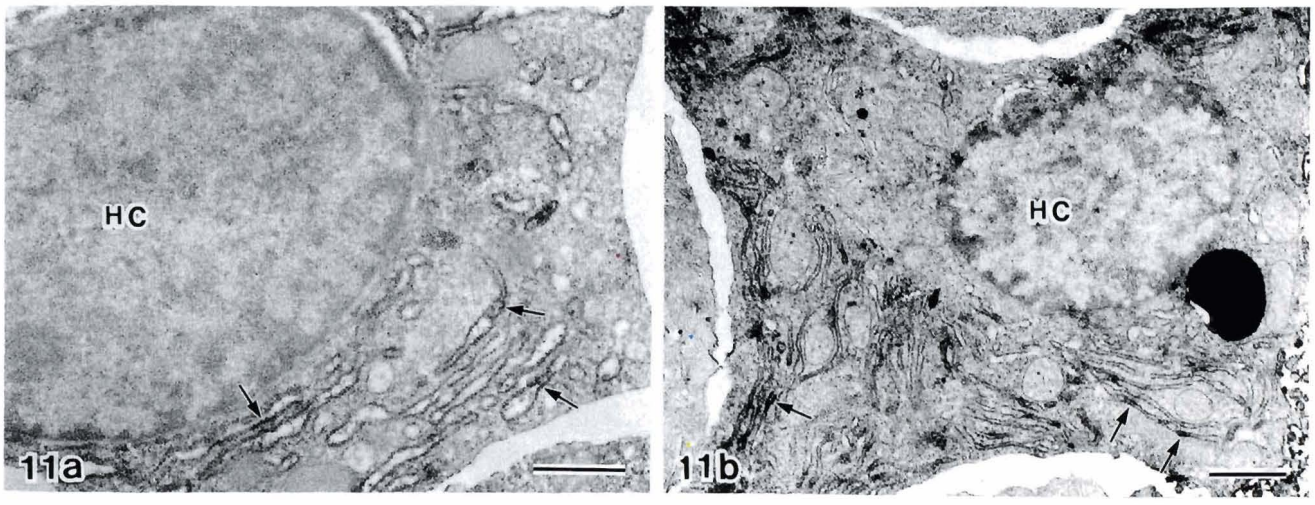

Fig. 11. Immunoreactions of FN **(a)** and LM **(b)** in HCs at prenatal day 15. They are preferentially seen in the rER (arrows). No counterstain. Bar: 1 µm.

may be transformed from HMCs, participate in the development of hepatic microvasculature. However, the effects of growth factors such as epidermal growth factor and insulin-like growth factor, and hormones such as corticosteroid and thyroxin on the hepatic neovascularization cannot be ruled out, since the roles of such substances on the neovascularization have been analyzed by Díaz-Flores et al. (1994). Taking the above into consideration, the reason why the production of FN and LM becomes gradually reduced in endothelial cells as they differentiate is under investigation. In addition, whether HCs are responsible for the induction and maintenance of the sinusoidal phenotype should be further investigated, since Módis and Martínez-Hernández (1991) suggested that HCs modulate the hepatic microvascular phenotype.

The ability of HCs in developing livers to synthesize FN and LM has already been described by light microscopic immunocytochemistry (Sakakibara et al., 1978; Wartiovaara et al., 1979; Rescan et al., 1989). Our immunoelectron microscopy reveals that HCs in a free state are occasionally involved in the production of both FN and LM in the rER but those integrated into hepatic cell cords are not. Araki et al. (1992) revealed that HCs incorporated into hepatic cell cords have lost α-fetoprotein synthetic activity in contrast to the enhancement of albumin synthesis in the perinatal rat liver. The present data add weight to the interpretation that the establishment of hepatic cell cords modulates various synthetic activities of individual HCs.

Our immunoelectron microscopic results indicate that the immunoreactions of both FN and LM in differentiating hepatic cell cords are preferentially seen on the cell surface facing both the sinusoidal space and

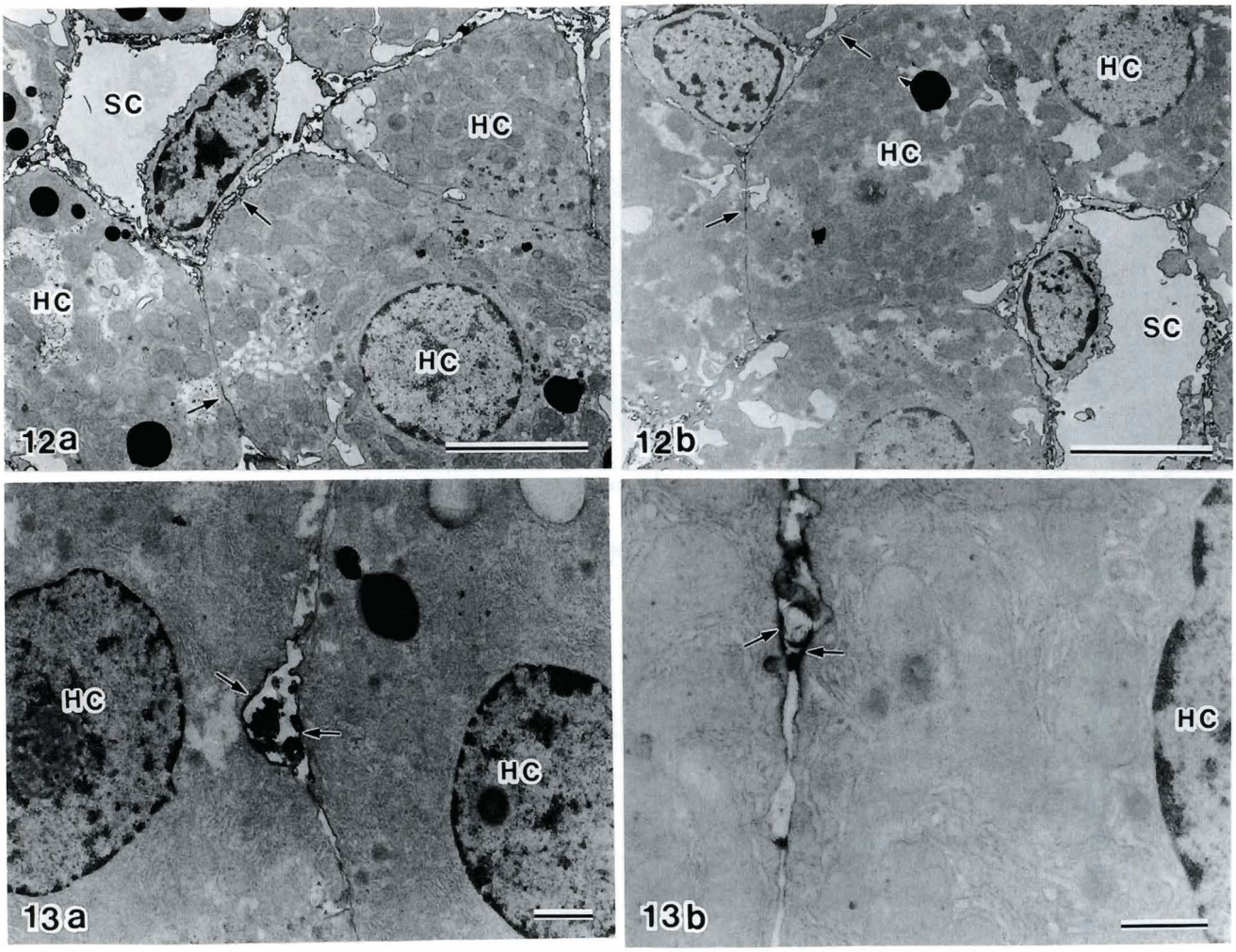

Fig. 12. Immunoreactions of FN **(a)** and LM **(b)** in HCs at postnatal day 1. Immunoreactions of both glycoproteins inside the HCs disappear, but can be seen at the cell surface facing the sinusoidal capillaries and cell to cell contact areas of the differentiating bile canaliculi (arrows). The young endothelial cell surface of the sinusoidal capillaries (SCs) is also immunoreactive for both glycoproteins. No counterstain. Bar: 5 μm.

Fig. 13. Immunoreactions of FN **(a)** and LM **(b)** of each apposed HC at prenatal day 19. They are preferentially seen at areas of the differentiating bile canaliculus (arrows). No counterstain. Bar: 1 μm.

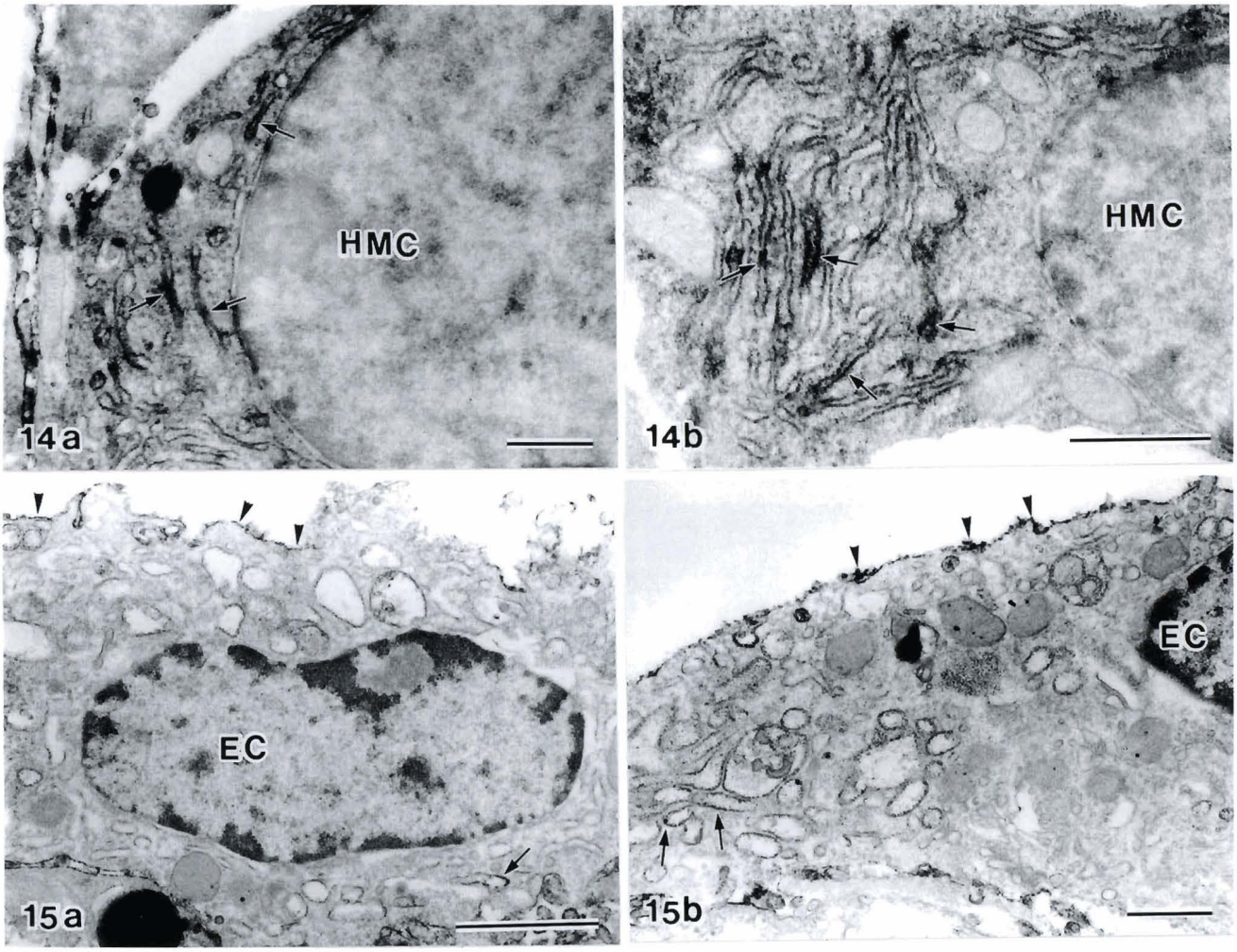

Fig. 14. HMCs associated with the growing sinusoidal capillaries show immunoreactions of FN (**a**) and LM (**b**) in the rER (arrows) at postnatal day 1. No counterstain. Bar: 1 μm.

Fig. 15. Young endothelial cells (EC) of the growing sinusoidal capillaries show immunoreactions of FN (**a**) and LM (**b**) in the rER (arrows) and at the cell surface (arrowheads) at postnatal day 1. No counterstain. Bar: 1 μm.

the differentiating bile canaliculi concomitant with the expression of ZO-1. Since HCs that are in contact with each other reduce or completely lose their immunoreactivities of both FN and LM in the rER, it seems reasonable to consider that FN and LM that are synthesized and released from the HMCs existing in the perivascular space play a role in the induction of hepatic cell cords associated with the formation of intercellular bile canaliculi. Megakaryocytes which are immunoreactive for FN and LM may also have similar roles in the early developing liver.

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