Scanning and Transmission Electron Microscopic Studies on Sinusoidal Endothelium in the Embryonic, Neonatal and Adult Mouse Liver

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ABSTRACT. During intrauterine life, the liver serves as a hematopoietic organ. To clarify the relationship between the development of liver hematopoiesis and the surface structures of the sinusoidal endothelium, we examined ICR mouse livers from 11 days of gestation to 90 days after birth by scanning and transmission electron microscopy. The sinusoidal endothelium of neonatal and adult livers was generally fenestrated. On the basis of their size, endothelial pores could be classified into three types: small-sized pores (S pores) were 250 nm or less in diameter, medium-sized pores (M pores) were 0.5-2.5 μm in diameter, and large-sized pores (L pores) had a diameter larger than 4 μm. At 11 days of gestation, the sinusoidal endothelium of the liver was commonly non-fenestrated, although a few M pores could be seen at the endothelial cell periphery. At 13 and 15 days of gestation, large numbers of both M and L pores were observed on the sinusoidal endothelium, and reticulocytes and hematopoietic cells were passing through the L pores. In addition, large-scale defects of the sinusoidal lining were also observed at 15 days of gestation. In the neonatal liver, both L and M pores decreased in number, and, due to a marked increase in S pores, the sinusoidal surfaces of 7-day-old mice began to resemble those of adult mice. Therefore, the surface structures of the sinusoidal endothelium are considered to undergo remarkable changes in relation to the developmental stages of liver hematopoiesis.

Key words: liver hematopoiesis — sinusoidal endothelium — fenestration — mouse — scanning electron microscopy

Unlike the adult liver, the fetal liver is known to serve as a hematopoietic organ, and hematopoietic stem cells originating from the yolk sac and AGM region initiate liver hematopoiesis.[1,2] Liver hematopoiesis in mice declines in late fetal life, and the liver becomes an exocrine gland of the digestive system after birth. The dramatic changes in liver function between embryos and adults are considered to include transformation of not only the hepatic cells but also the sinusoidal endothelial cells. However, little information is available regarding alteration of the sinusoidal endothelium in the fetal and adult livers. The aim of this study was to clarify the relationship between the development

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of liver hematopoiesis and the surface structures of the sinusoidal endothelium. We observed the sinusoidal endothelium from the beginning of liver hematopoiesis through the neonatal period by scanning and transmission electron microscopy.

MATERIALS AND METHODS

A total of 20 ICR mice were used in this study: 11-day-, 13-day- and 15-day-fetal, 0-day-, 7-day- and 90-day-old mice. Adult female mice were mated overnight with males, and the next morning was taken as Day 0 of gestation. Mice were sacrificed under deep ether anesthesia, and livers were removed from embryos, neonates and adults to be studied by scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The sinusoidal ultrastructure was analyzed after both perfusion and immersion fixation.

Scanning electron microscopic observations

Under a stereomicroscope, the 13- and 15-day-old embryos were perfused with 0.15M NaCl containing 100U/ml heparin through the umbilical cord artery with a glass needle. This was followed by perfusion with 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) at 2 ml/min, and the pressure was maintained at 15 mmHg. Newborn, 7-day- and 90-day-old mice were also perfused via the aorta with the same solution. Livers were removed, cut into small blocks ($2\times2\times4$ mm), and immersed in the same fixative for 1h. The liver tissues were placed in dimethyl sulfoxide in 0.1M cacodylate buffer, pH 7.4, and then frozen and cut on a stainless plate cooled in liquid nitrogen. Since the livers of the 11-day-old embryos were too small for perfusion fixation and freeze cutting, the removed livers were cut with razor blades, and fixed in 2% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4, for 2.5h. The liver tissues of fetal, neonatal and adult mice were rinsed in 0.2M cacodylate buffer, and then postfixed in 1% osmium tetroxide in 0.2M cacodylate buffer for 2h. After placement in 1% tannic acid, liver tissues were re-immersed in 1% osmium tetroxide for 1h. After rinsing in cacodylate buffer, they were dehydrated in graded ethanols and transferred to t-butyl alcohol for freeze-drying. The dried tissues were coated with gold-palladium and observed in a HITACHI S-570 scanning electron microscope operating at 20 kV.

Transmission electron microscopy

The livers removed from the embryos, newborn and adult mice were immediately immersed in 2% glutaraldehyde in 0.1M cacodylate buffer, pH 7.4. After 15 min, they were cut into small blocks ($1\times1\times1$ mm), and immersed in the same fixative fluid for 2.5h. After washing in 0.2M cacodylate buffer, the tissue blocks were postfixed in 1% osmium tetroxide in 0.2M cacodylate buffer for 2h. After dehydration in graded ethanols, they were embedded in Epon 812. Ultrathin sections were impregnated with uranyl acetate and lead citrate and examined in a JEM-2000EX II electron microscope operating at 80 kV.

These experiments were approved by the Animal Research Committee of Kawasaki Medical School (No. 96047, 1996) and conducted according to the "Guide for the Care and Use of Laboratory Animals" of Kawasaki Medical School.

RESULTS

Sinusoidal Endothelial cells of the adult liver

The adult liver was composed of lobules consisting of liver cell cords and sinusoids which radially extended from central veins. The sinusoids were thin-walled vessels whose endothelial cells were characterized by fenestrations grouped in the form of so-called 'sieve plates'. In addition to these sieve plates, the endothelium also had a few large pores. The endothelial pores in the adult livers were classified, on the basis of their size, into two types: small-sized pores (S pores) 250 nm or less in diameter and medium-sized pores (M pores) 0.5-2.5 μm in diameter. The distribution pattern of the fenestration differed in the three zones of hepatic lobules. The sinusoidal endothelium of the central zone had S pores showing diffuse distribution, whereas that of the intermediate zone, not only had many sieve plates but a few M pores. The endothelium of the peripheral zone had only S pores diffusely distributed (Fig 1). The embryonic endothelium of the liver sinusoid, in addition to S and M pores, had large-sized pores (L pores) with a diameter of larger than 4 μm. The appearance of each type of pore showed remarkable changes in relation to the development of liver hematopoiesis.

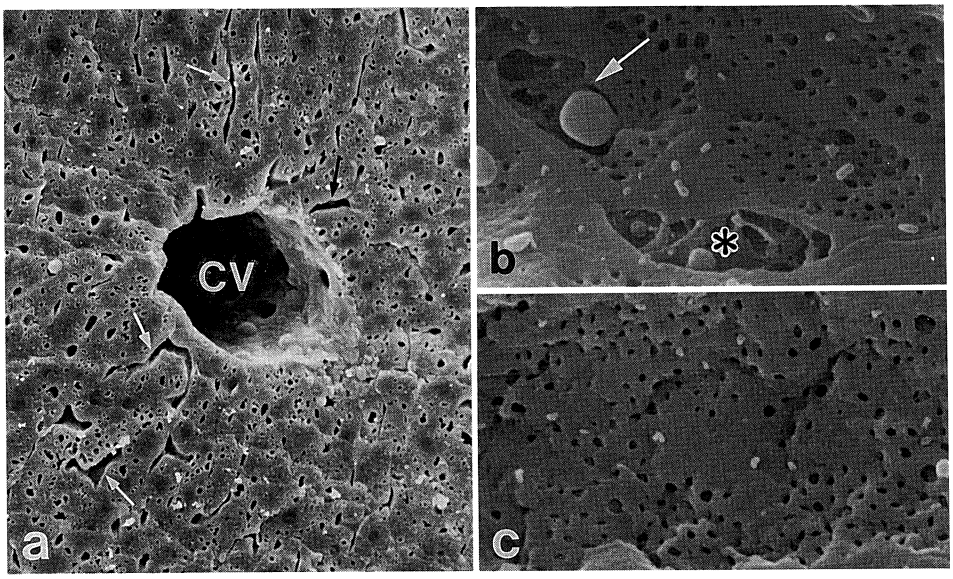

Fig 1. Adult liver: SEM images
a. Central vein (CV) and sinusoids (arrows). ×300
b. The endothelium of the intermediate zone not only has numerous S pores but also a few M pores (arrow). Asterisk indicates an interepithelial cell gap. ×14,000
c. Sinusoidal endothelium and randomly distributed S pores in the intermediate zone. ×14,500

Endothelium of the embryonic liver sinusoid

The liver of 11 days of gestation contained a few immature hematopoietic cells, which were located in hepatic cell cords and increased in number between 12 and 15 days of gestation. Thereafter, liver hematopoiesis gradually declined, and the neonatal liver contained very few hematopoietic cells within the

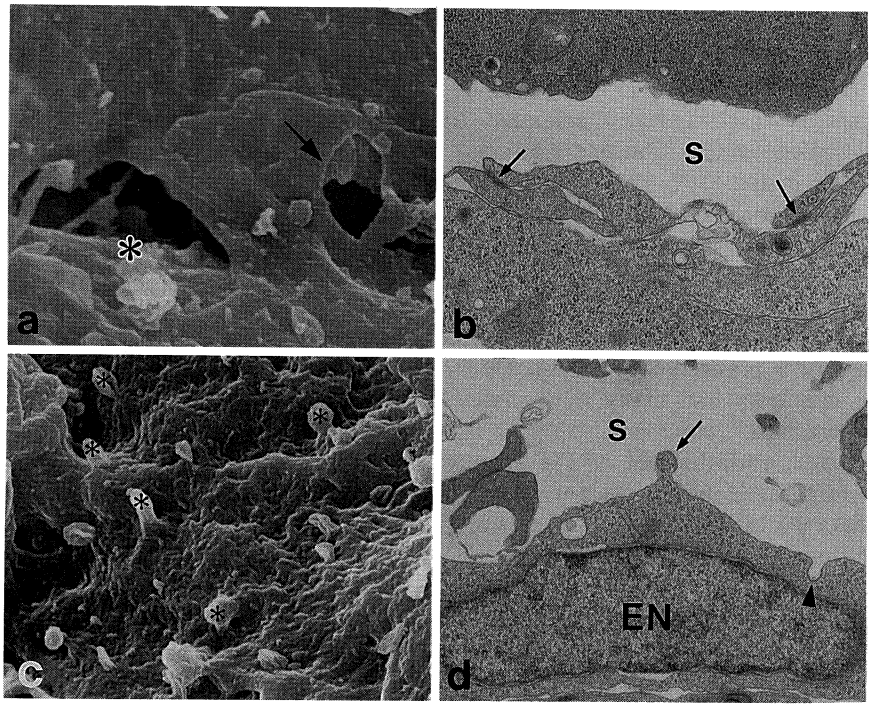

Fig 2. Sinusoidal endothelium of an 11-day embryonic liver
a. SEM image. An M pore (arrow) and an interepithelial cell gap (*) can be observed. ×9,000
b. TEM image of endothelial junctional complexes (arrows). S: sinusoidal lumen. ×17,600
c. SEM image of the endothelium. The endothelium has no fenestration, and many short droplet-like projections (*) can be observed. ×8,700
d. TEM image. A short cytoplasmic projection (arrow) and micropinocytosis (arrowhead) of an endothelial cell (EN). S: sinusoidal lumen. ×10,000

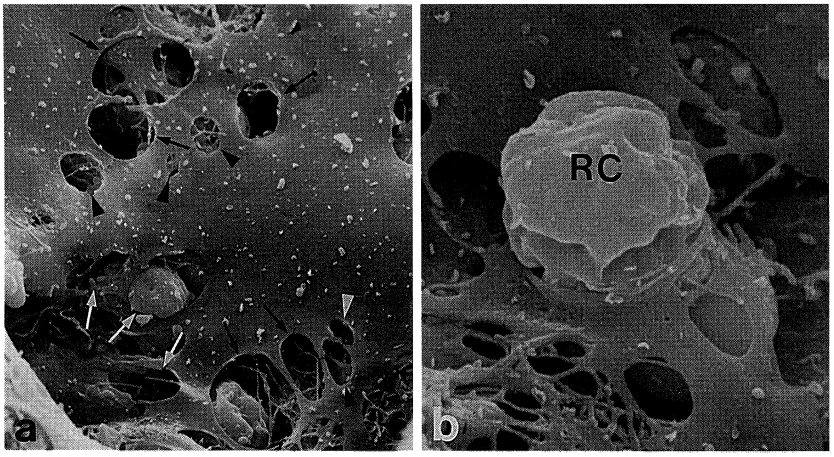

Fig 3. SEM images of sinusoids of a 13-day embryonic liver
a. M pores (arrowheads) and L pores (arrows) in the endothelium. ×2,000
b. A reticulocyte (RC) migrating through an L pore. ×5,600

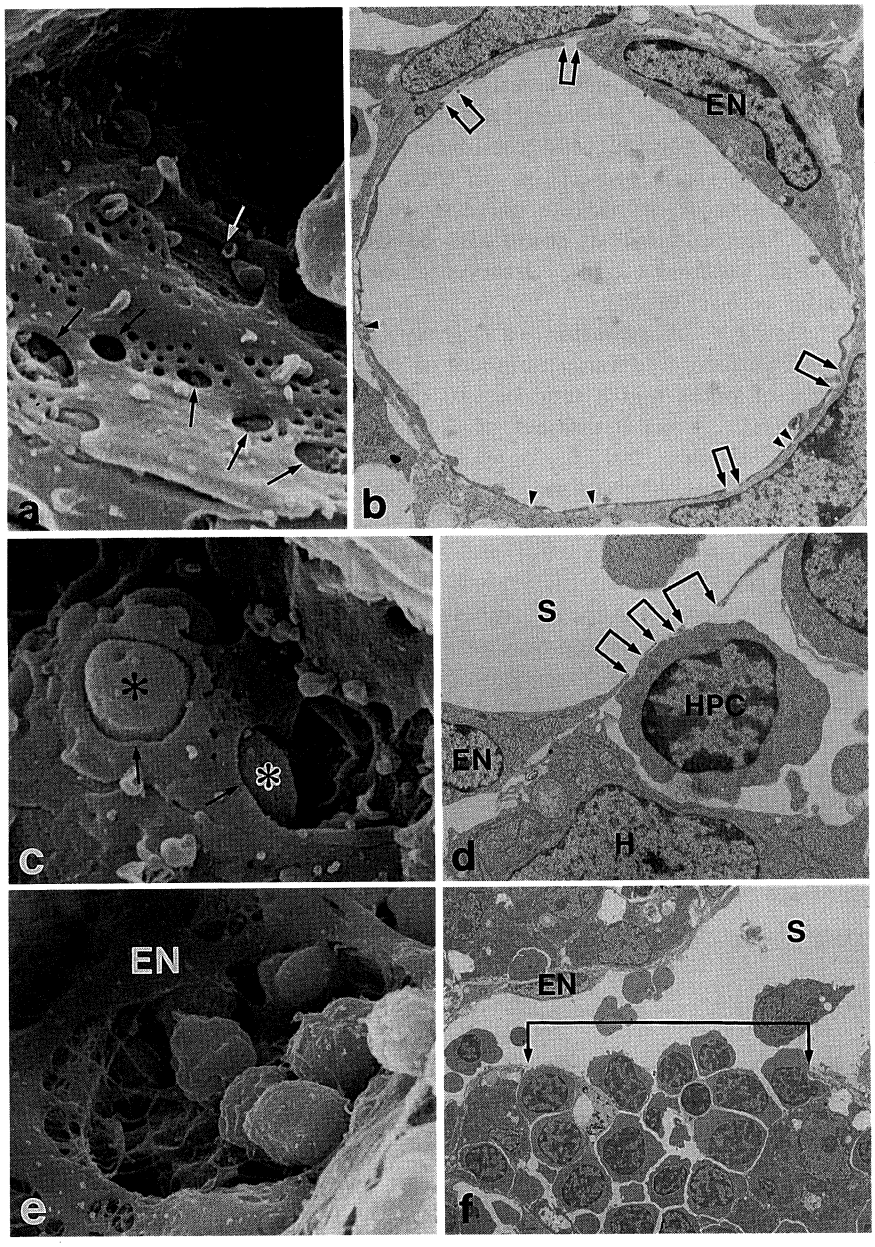

Fig 4. Sinusoids of a 15-day embryonic liver
a. SEM image of M pores (arrows) and S pores in the endothelium. ×12,000
b. TEM image of a sinusoid. The endothelial cell (EN) has M pores (arrows) and S pores (arrowheads). ×4,000
c. SEM image of M pores (arrows). Through the M pores, hematopoietic cells (*) in liver cell cords can be observed. ×15,000
d. TEM image of M pores (arrows). EN: endothelial cell, H: hepatocyte, HPC: hematopoietic cell, S: sinusoidal lumen. ×5,000
e. SEM image of a large intercellular gap in the sinusoidal wall. EN: endothelial cell. ×1,400
f. TEM image of a large intercellular gap (arrows) in the sinusoidal wall. EN: endothelial cell, S: sinusoidal lumen. ×2,400

hepatic cell cords.

At 11 days of gestation, the liver sinusoids had extremely expanded, and the epithelium appeared non-fenestrated. However, the endothelium had very few M pores, 1-2 μm in diameter, and narrow intercellular spaces could be seen at the border of adjacent endothelium (Fig 2a). The endothelial cells had numerous cytoplasmic projections on their luminal surface. These projections were droplet-like or nipple-like processes, 0.1-0.2 μm in width and 0.2-0.4 μm in length. Micropinocytosis could also be seen on the endothelial surface (Fig 2c, d), and desmosome-like structures were occasionally seen between endothelial cells (Fig 2b).

At 13 days of gestation, a variety of pores were observed on the endothelial surface, and large numbers of both M and L pores could be seen. Both types of pores had a tendency to occur in clusters of approximately six pores (Fig 3a). A small number of S pores also appeared around these pore clusters. Through the L pores, hepatocytes, mesenchymal cells and hematopoietic cells interspersed with hepatocytes could be observed. Some reticulocytes were migrating from hepatic cell cords to sinusoidal lumen through the L or M pores (Fig 3b). Compared with 11 days of gestation, cytoplasmic projections on the endothelial surface were decreased in number. At 15 days of gestation, the endothelium had S, M and L pores, and the S pores, in particular, had become more numerous and occurred in clusters (Fig 4a, b). Although the cytoplasmic projections became negligible in number at the border of adjacent endothelium, cytoplasmic flaps protruding to sinusoidal lumen were present. Hematopoiesis proceeded in both hepatic cell cord and sinusoidal lumina, and not only L pores but also intercellular gaps in the sinusoidal linings linked the sinusoidal lumina and the hemopoietic foci in the hepatic cell cords. The intercellular gaps were larger than 10 μm in length, and, through these gaps, numerous hematopoietic cells moved from the hepatic cell cords to sinusoidal lumina (Fig 4c, d, e, f).

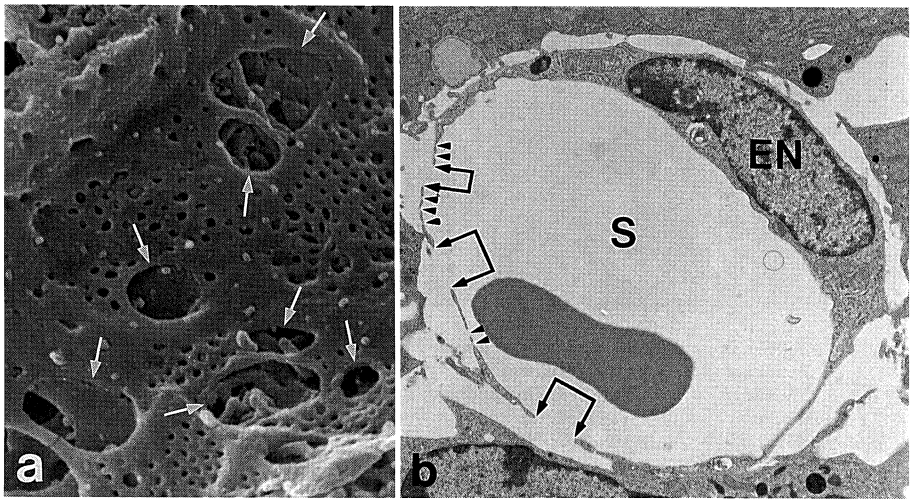

Fig 5. Sinusoidal endothelium of liver at 7 days after birth
a. SEM image. The endothelium has both M pores (arrows) and S pores. ×12,800
b. TEM image. Arrows indicate M pores and arrowheads indicate S pores. EN: endothelial cell, S: sinusoidal lumen. ×5,000

Endothelium of the neonatal liver sinusoid

Between 0 and 7 days after birth, liver hematopoiesis declined, and a small number of hematopoietic cells could be seen in the hepatic cell cords. The sinusoidal endothelium at Day 0 after birth had both numerous S pores and M pores, which tended to occur in clusters, but L pores could not be observed. Perisinusoidal spaces were expanded, and the microvilli of hepatocytes became obvious. The sinusoidal endothelium at 7 days after birth showed a decrease in M pores and an increase in S pores. The fenestration pattern at 7 days after birth became similar to that in adult livers (Fig 5a, b). The distribution patterns of S, M and L pores in liver sinusoidal endothelium are schematically shown in Figure 6.

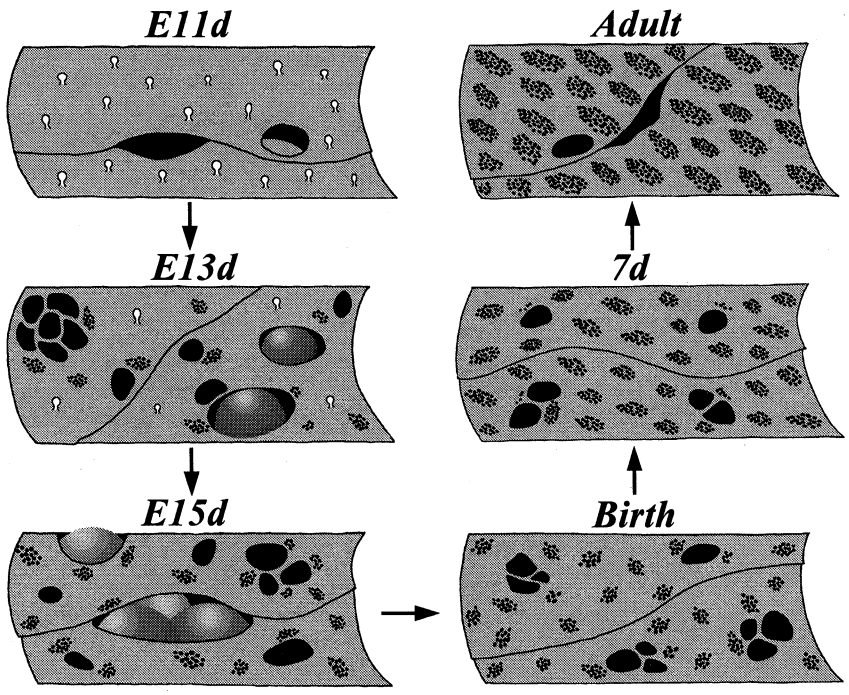

Fig 6. Schematic representation of liver sinusoidal endothelium during liver development

DISCUSSION

As mentioned above, the surface structures of the sinusoidal endothelium of embryos and neonates differed greatly from those of adult animals. The sinusoids of the adult liver are fenestrated capillaries[3] and, through the fenestrae, chylomicrons move from the sinusoidal lumen to liver cells.[4] The majority of the fenestrations are small-sized pores.[3,5,6] The sinusoidal endothelium markedly changed its surface morphology during intrauterine life. At the beginning of liver hematopoiesis, the endothelium of the primordial liver at 11 days of gestation was commonly non-fenestrated, although a few M pores could be seen at the endothelial cell periphery. Numerous M and L pores appeared on the sinusoidal endothelium at 13 and 15 days of gestation.

In the neonatal liver, both L and M pores decreased in number, and, due to a marked increase in S pores, the sinusoidal surfaces of young mice began to resemble those of adult mice.

Based on reports on the size distribution of chylomicrons in neonatal rat liver and latex administration indicating that chylomicrons or latex particles with a diameter larger than 250-330 nm were unable to pass through the endothelial lining, the S pores would serve as size filters on the passage of chylomicrons from the sinusoidal lumen to the space of Disse.[4,7] Therefore, the S pores could be important functional structures in the adult liver as a part of the digestive system. During intrauterine life, the liver does not serve as a digestive organ but as a hematopoietic organ. As shown in our results, the sinusoidal endothelium at 11 days of gestation had very few M pores and numerous cytoplasmic projections, and the hematopoietic stem cells were migrating from the sinusoidal lumen to hepatic cell cords and settling within the hepatic cell cords to start liver hematopoiesis. In this regard, the M pores may act as one of the main traffic routes when hematopoietic stem cells migrate from the sinusoidal lumen to the hepatic cell cords. Cytoplasmic projections on the endothelium surface were specific structures on the sinusoidal surface at 11 days of gestation, and showed a marked decrease in number after 13 days of gestation. Hence, the projections could be considered as structures for endothelium-hematopoietic stem cell contacts; i.e., the first step of stem cell migration into hepatic cell cords. At 13 and 15 days of gestation, when liver hematopoiesis culminated, numerous L pores could be observed on the sinusoidal endothelium, and the sinusoidal lumen contained numerous erythrocytes and erythroblasts which matured within the hepatic cell cords. The L pores are thought to be the main routes when erythroid cells pass through the endothelium from the hepatic cell cord to the sinusoidal lumen. Our results showed that some reticulocytes and hematopoietic cells were passing through L pores. Large-scale defects of the sinusoidal lining at 15 days of gestation were considered as changes to facilitate massive movement of hematopoietic cells into the sinusoidal lumen. In the neonatal liver, hematopoietic foci were decreased in size, and microvilli became obvious on the perisinusoidal surface of hepatocytes. M pores in the sinusoidal epithelium decreased, but S pores showed a marked increase in number. The surface structures of the sinusoidal endothelium of neonates are thought to reflect changes in the liver's function from an embryonic hematopoietic organ to an exocrine gland in the digestive system after birth. Among the three kinds of pores in liver sinusoids, only M pores could be recognized throughout gestational and adult life. In addition to S pores, liver sinusoids from various kinds of adult experimental animals have been found to have M pores.[5,8,9] On the basis of pore sizes, M pores could be considered to serve as one of the cell traffic routes not only for erythrocytes during gestational life but for Kupffer cells in the adult liver between the sinusoidal lumen and perisinusoidal space.

Concerning large-sized pores with a diameter larger than 1 μm, Wisse[3] and Vidal-Vanaclocha F *et al*[10] reported that large-sized pores were an artifact produced by the high pressure of perfusion fixation. YC Shin,[9] on the other hand, described large-sized pores not as artifacts but as normally present structures of the sinusoidal endothelium of adult livers under physiological conditions. Our SEM results confirmed that the sinusoidal endothelium of

embryonic livers perfused at physiological pressure had large-sized pores, and the pores could also be identified by TEM in embryonic livers fixed by immersion. In this regard, large-sized pores are considered to be present in fetal sinusoidal endothelium under physiological conditions for massive movement of erythrocytes from the hepatic cell cords to the sinusoidal lumen.

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