Morphological Studies on the Forming Processes and Patterns of the Platelet Demarcation Membrane System in the Megakaryocytic Series of Embryonic Rat Livers

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Summary. Using injections of horseradish peroxidase (HRP) and the osmium-tannic acid method, megakaryocytic cells in the livers of rat embryos at 12-16 days of gestation were examined for the purpose of classification of the stages of formation of the platelet demarcation membrane. Megakaryoblasts were classified into the following three types according to the formation patterns of the demarcation membrane. 1) The P-type megakaryoblasts showed plate-like membrane invaginations in large localized areas at early stages. The invaginating membrane developed toward the periphery of the nucleus. 2) The L-type megakaryoblasts showed localized labyrinthine membrane invaginations but no definite direction in its development. 3) The T-type megakaryoblasts had tubular invaginations at multiple sites on the plasma membrane. The P- and L-type cells were observed at 12 and 13 days of gestation. The T-type cells were found after the 14th day. In all the types of megakaryoblasts the membrane invagination occurred in the areas making contact with hepatocytes. It was agreed that the cells of the megakaryocytic series in which the demarcation membrane developed contrary to the basic pattern were ordinary promegakaryocytes. The megakaryocytes forming networks of the demarcation membrane dividing into platelet areas were small in cell size. Examination of the patterns of formation of the demarcation membrane proved useful for classifying the megakaryocytic series at each stage of maturation.

Since the first descriptions of the platelet demarcation membrane, which segregates the cytoplasm into numerous platelets (YAMADA, 1957), morphological interest in megakaryocytes has centered upon the origin and formation processes of this membrane system. BEHNKE (1968, 1969) reported that the demarcation membrane is derived from tubular invaginations of the plasma membrane. Using specific staining of the plasma membrane or injection of electron dense materials, he demonstrated that the cavernous invaginations remained open to the cell surface at all times. However, immature megakaryocytic cells in the materials used by BEHNKE (1968, 1969) were so scarce as to render it difficult to collect various images on early demarcation membrane formation or to visualize different modes of membrane invagination.

Erythrocytic or megakaryocytic hematopoiesis in human fetal liver parenchyme was reported by ZAMBONI (1965) and by EMURA et al. (1983, 1984). Some types of mega-
karyoblasts were studied in embryonic livers of mouse by Sugisaki (1981). They observed many megakaryoblasts in the initial stages of hematopoiesis.

In this study, the relationship between hematopoietic cells and the liver parenchyme is investigated in rat embryonic livers. Furthermore, the cells of the megakaryocytic series are morphologically classified and the processes of demarcation membrane formation examined at every stage of its maturation.

MATERIALS AND METHODS

Embryonic livers of Sprague-Dawley rats at 12–16 days of gestation were used in this study.

Tannic acid method (Mizuhira and Futarsaku, 1972)
The embryonic livers were removed, immediately immersed in 2% glutaraldehyde and 2% tannic acid in 0.1 M cacodylate buffer at pH 6.8 for 12 hrs. Tissues were then postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer at pH 7.4 for 2 hrs, dehydrated in a graded series of ethanol, and embedded in Epon 812.

Control livers were fixative in the same fixatives, excluding tannic acid, and treated with the same method.

Injection of horseradish peroxidase
Embryos were removed from the uterine horn under ether anesthesia. Horseradish peroxidase (HRP) (8 mg/ml, 4 mg/ml, 2 mg/ml or 1 mg/ml in Tyrode solution, at 37.5°C was injected (0.15 ml/min, for 10–12 min) into the umbilical vein, and then washed with Tyrode solution. The fetal livers were removed and cut into small pieces to be fixed for 20 min in a mixture of 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4. The tissues were then washed in 0.5 M cacodylate buffer with 7% sucrose for 4 hrs and immersed in Karnovsky’s medium using diaminobenzidine (DAB) (Graham and Karnovsky, 1966) for demonstration of peroxidase activity. After postfixation in 1% osmium tetroxide in 0.1 M cacodylate buffer, the tissues were embedded in Epon 812.

Controls were obtained by immersing the liver tissues of non-injected embryos in a full Karnovsky’s medium, or by incubating the tissues of embryos injected with HRP in a medium excluding hydrogen peroxide.

Electron microscopy
Sections were cut with a Porter-Blum MT2-type ultramicrotome, post-stained with uranyl acetate and examined under a JEA 100B electron microscope.

RESULTS

Stereoscopic microscopic observations
Embryonic livers at 12–13 days of gestation showed pinkish coloration. In 12–13 day embryos they were pinkish red; in 15 and 16 day embryos, they were red, amounting to one third the size of the embryos.
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Light microscopic observations
Livers of 12–16 day embryos were composed of cell masses containing parenchymatous cells or hepatocytes, hematopoietic cells and hepatic sinuses. The hematopoietic cells were gathered among the hepatocytes. Megakaryocytic cells were observed mingled with hemocytoblasts of other series after 12 days of gestation. After 13 days, the number of hematopoietic cells and hepatocytes increased rapidly. At 15 and 16 days, many hematopoietic cells were observed in the perisinusoidal spaces and within sinuses.

Electron microscopic observations

Erythrocytic and granulocytic series
Proerythroblasts and basophilic erythroblasts were observed in the liver parenchyme at 12 days. Polychromatophilic erythroblasts and orthochromat erythroblasts were small in number at 12 days, but multiplied after 13 days of gestation. At 14 days, various erythroblasts at different stages of maturation could be seen intermingling in the hematopoietic cell masses, and numerous orthochromat erythroblasts showing the nuclear extrusion phenomenon were revealed in the hepatic sinuses. Erythroblasts did not show any physiologically significant attachment to the surrounding cells. A small quantity of immature neutrophilic granulocytic series was observed in the parenchyme after 14 days. Basophilic and eosinophilic granulocytes were not found in the parenchyme.

Megakaryocytic series
Cells of megakaryocytic series were dispersed in the periphery of hematopoietic cell masses, and were not crowded. They were in close contact with erythroblasts and frequently enclosed by hepatocytes. However, desmosome-like attachments were not observed among them at any of the maturation stages.

1) Megakaryoblasts
The most immature megakaryoblasts were round or oval in shape, and were similar in cell size (10–18 μ) to the proerythroblasts (Fig. 1). They possessed a round or slightly bilobed nucleus showing a smooth nuclear profile with nucleoli. The cytoplasm contained abundant polysomes, a small quantity of rough endoplasmic reticulum, mitochondria and specific granules which showed low electron density and were in contact with the Golgi apparatus; there were none of such lysosome-like dense granules existent in the proerythroblasts. At this stage, membranous structures indicating early formation of the demarcation membrane were observed in the cytoplasm. These structures were vesicular or tubular in shape. In animals injected with HRP, the cavities of these membranous structures showed a positive DAB reaction. Furthermore, staining with the osmium-tannic acid method showed a continuity of the plasma membrane and the demarcation membrane. Megakaryoblasts were classified into the following three types according to the invaginating forms of the plasma membrane.

a) Megakaryoblasts showing plate-like membrane invaginations, or folded belt-shaped membrane invaginations (P-type megakaryoblast) (Fig. 2A, 2B, 7): Some long, paired-line profiles in the cytoplasm were seen to stretch from the plasma membrane to anastomose to each other within the cytoplasm. Under the electron microscope, the long, extended lines clearly had the appearance of transverse sections of membranous structures. The length of these lines grew in proportion to the increase in specific
granules and cytoplasmic matrix, and to the progressive lobulation of the nucleus. They tended to surround the nucleus as double paired-line profiles in the intermediate cytoplasmic zone (Fig. 2B).

b) Megakaryoblasts showing labyrinthine membrane invaginations (L-type megakaryoblast) (Fig. 3, 7): Some of the megakaryoblasts showed several long, paired-line profiles with irregular whirling patterns. It is clear that these line profiles represent transverse sections of whirling membrane. The membranes continuous from the plasma membrane seemed to have no definite direction in their development, differing from the P-type megakaryoblast.

c) Megakaryoblasts showing tubular invaginations (T-type megakaryoblast) (Fig. 4, 7): Still other megakaryoblasts contained minute circular or short paired membranous profiles, indicating the profiles of narrow tubules. These tubular structures were continuous to the plasma membrane and distributed randomly in the periphery of the cells. The connecting sites of these membranous profiles to the plasma membrane were multitudinous. The figures sometimes gave evidence of the tubular profiles' branching out and increasing in number in accordance with their maturation.

The membrane invagination of these three types of megakaryoblasts was observed frequently in the areas neighboring the hepatocytes.

Almost all the megakaryoblasts of the 12-day embryos were of the P-type. A small number of L-type cells did intermingle with them. At 13 days, the L-type cells were observed intermingling with a small number of the P-type cells, without any T-type cells. At 14 days, most immature megakaryoblasts were of the T-type, while the P- and L-type cells were scarce. After 15 days, all of these immature types of megakaryoblasts could be found only rarely.
Fig. 2. P-type megakaryoblasts in embryonic liver of a 12 day embryo. The cells include the bilobed nucleus, some specific granules, abundant polysomes. Osmium-tannic acid method. A. Long, paired membrane structures extend to the intermediate cytoplasmic area. Arrows show the invaginating portion of the plasma membrane. *Hc* hepatocyte. Uranyl acetate. × 4,850. B. Long, paired membrane structures enclose the nucleus doubly or triply. Uranuyl acetate. × 6,200
2) Promegakaryocytes (Fig. 5, 7)
Some of the cells of the megakaryocytic series showed a large cell size (2-3 times as large as megakaryoblasts) and a lobulated nucleus with an irregular profile at 12-15 days of gestation. These had a well-developed Golgi apparatus and specific granules increasing in number, size and electron density. The demarcation membrane formed a reticular mass around the nucleus in the intermediate cytoplasmic area. It was difficult with promegakaryocytes to guess as to which of the above-mentioned types of megakaryoblasts the cells might represent. These cells frequently extruded many bleb-like extrusions containing none of the ordinary cell organelles, and touching with the surrounding cells. An osmium-tannic acid reaction was observed in the demarcation membrane and the plasma membrane. A DAB reaction was found only in the cavities of this membrane system.

3) Megakaryocytes (Fig. 6)
Hepatic megakaryocytes were generally small in size (50-70 μm) and were frequently observed not only in the parenchyme but also in the perisinusoidal spaces and sinuses as well. The demarcation membrane visualized by the DAB and tannic acid reactions formed networks, segregating the cytoplasm into platelet areas. Some of these matured to the discharging stage.

Cytochemical observations
In the controls of the tannic acid and DAB reactions under the HRP treatment, no reaction products were found in the plasma membrane or the demarcation membrane.
Fig. 4. T-type megakaryoblast of a 14 day-embryonic liver. Arrows show tubular invagination at multiple sites. Hc hepatocyte. Tannic acid method. Uranyl acetate. × 4,200

Fig. 5. Promegakaryocyte in liver of a 14-day embryo. The demarcation membrane develops and forms a reticular mass in the intermediate cytoplasmic zone. Arrows show the connections of the plasma membrane and the demarcation membrane. Uranyl acetate. × 5,200
Studies on the differentiation of blood cells have demonstrated the occurrence of the biosynthesis of colony-stimulating factors (CSFs) (Burgness et al., 1980; Nabel et al., 1981a, b; Gasson et al., 1984) and the possibility of inhibitory systems by hematopoietic microenvironment (Wright and Lord, 1978, 1979; Eastment et al., 1982; Allen, 1984). On fetal hematopoiesis, the effect of CSF-α and -β derived from placenta (Nicola et al., 1979) and the relationship between hematopoietic stem cells and surrounding cells in livers (Emura et al., 1984) have been examined. The mechanisms of embryonic hematopoiesis are obscured by many small details undoubtedly vital to this process.

The present report demonstrated the cells of the megakaryocytic series surrounded by hepatocytes at every stage of maturation, along with a high frequency of membrane invagination in megakaryoblasts in those areas making contact with the hepatocytes. Therefore, we considered that the formation of the demarcation membrane might be stimulated by hepatocytes. It was impossible to examine the relationships of other morphological characteristics of the megakaryocytic cells (nuclear lobulation and the formation of specific granules) to the activities of hepatocytes. A physiologically significant attachment between the cells of the erythrocytic series and various types cells in fetal livers could not be found in light and electron microscopic observations. This result differs from the report by Emura et al. (1984).

The morphological classification of megakaryocytic cells has been discussed by many investigators. They are roughly classified into megakaryoblasts, promegakaryocytes and megakaryocytes according to cell size, nuclear lobulation and the number of specific granules. The definition of the megakaryoblast has been long indefinite, since...
morphologically undifferentiated blast cells showing the activities of platelet peroxidase (Breton-Gorius and Guichard, 1972) and anti-platelet antibody (Fedorko, 1978; Honma, 1985) have been reported. The present study demonstrates that morphologically differentiated immature megakaryocytic cells have blastic cell characteristics (a single, oval or slightly bilobed nucleus, dispersed polysomes, and small numbers of ordinary cell organelles) and resemble proerythroblasts except for their having a few characteristics of the megakaryocytic series. It seems illogical if one restricts the definition of megakaryoblasts to cells showing platelet peroxidase and anti-platelet antibody activities without taking into account their morphological characteristics. We consider that the most immature cells having the characteristics of the megakaryocytic series are megakaryoblasts. We agree with the suggestion by Breton-Gorius et al. (1982) that morphologically undifferentiated blast cells should be called promegakaryoblasts, in order to avoid the confusion between undifferentiated blast cells and the megakaryoblasts.

Concerning the classification of the cells of the megakaryocytic series, observations on the shapes of the demarcation membrane at each maturation stage have been reported by Yamada (1957). We showed that the three types of megakaryoblasts gradually increased in cell size and in the number of specific granules to them send off branches of the demarcation membrane in every direction inside the cell. As immature megakaryocytic cells matured, the early formation patterns of the demarcation membrane were broken. With this in mind, we would suggest defining cells which lose the initial formation pattern of this membrane system as promegakaryocytes, and cells with the network of this membrane system segregating the cytoplasm into platelet areas as megakaryocytes, regardless of cell size.

The present electron cytochemical studies on the demarcation membrane have indicated that cavities of this membrane system are connected with the outer environment throughout all the stages of the maturation, in agreement with the views by

![Fig. 7. Diagrams of the development of the demarcation membrane in the three different types of megakaryoblasts (MKBs).](image)
BEHNKE (1968, 1969), MACPHERSON (1972), FEDORKO et al. (1976) and INZUMI et al. (1977). Furthermore, we examined the three types of megakaryoblasts with reference to the following three characteristics: 1) the distribution of the connecting portions between the demarcation membrane and the plasma membrane; 2) the extent of the connecting area; and 3) the direction of the development of the demarcation membrane. As for the first factor, in the case of the P- or L-type cells the sites of membrane invagination were few and localized, whereas in the T-type cells they were multitudinous and non-localized. As for the second aspect, each junction area was obviously larger in the P- or L-type cells than in the T-type cells, although we could not calculate the total area of connecting portions in each type of megakaryoblasts. As for the third item, the demarcation membrane of the P-type cells showed a definitely developed pattern enclosing the nucleus. These three characteristics suggest the existence of different inner elements and states inducing the formation of the demarcation membrane among the three types, excluding external stimulating factors. Recently, studies of the carbohydrate chains of glycoprotein on the plasma membrane have demonstrated differences in the structures of these chains between fetal blood cells and adult ones (FUKUDA et al., 1979, 1984), as well as specific chains on cells showing high differentiating ability (SHIRAISHI et al., 1982). We suspect that the localization or multiple dispersion of the membrane in invaginations of megakaryoblasts might reflect the differences in molecular structures of the plasma membrane at different embryonic stages. It seems that the membrane structure necessary for membrane invagination was more localized and crowded on megakaryoblasts at the early stages of liver hematopoiesis than at later stages. Membrane invagination in endocytosis or cytoplasmic division during mitosis is known to be related to changes in the cytoskeleton. We postulate that the difference between the P-type cells and the L- or T-type cells in the direction of the development of the demarcation membrane might be due to variations in the cytoskeleton including microtubules and microfilaments. This possibility must be examined in further studies.

REFERENCES


