Ultrastructural Identification of the Hemopoietic Inductive Microenvironment in the Human Embryonic Liver

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Summary. Reciprocal interaction between the hemopoietic organ stromal cells and the cells of the granulocytic, megakaryocytic and erythrocytic series in the human liver obtained from 109 embryos 28 to 49 days after ovulation and 76 fetuses from 8 to 22 weeks of gestation were investigated by light and electron microscopy.

The close association of stromal cells with immature cells of the three series was confirmed under the electron microscope and a presumptive HIM (hemopoietic inductive microenvironment) was visualized. A majority of immature erythroblasts intruded into the cytoplasm of the hepatocytes, so the presumptive hemopoietic stem cell types II and IV are undoubtedly differentiated into cells of the erythroid line by contact with hepatocytes at a certain stage of maturation. Granulopoiesis developed among the reticular cells around the ductus venosus—or large arteries in hepatic parenchyma—and the cells of the granulocytic series were enclosed by thin cytoplasmic projections of mesenchymal cells. Neither erythropoiesis nor megakaryopoiesis was noted here. Therefore the compartments composed of one or more reticular cells around the ductus venosus or large arteries seem to have a capacity to regulate the differentiation of the presumptive hemopoietic stem cells type IV into cells of the granulocytic series. This differentiation of presumptive hemopoietic stem cell types II and IV into the megakaryocytic series is believed to be induced by the presence of the microenvironments that consist of foci of a few reticular cells in the hepatic parenchyma, as immature cells of megakaryocytic lineage were encircled by the cytoplasmic projection of one or more reticular cells among hepatocytes.

Erythroblastic islets are concluded to be a kind of HIM, where erythroblasts loosely adhere to the central macrophages and undergo mitoses and maturation.

Experimental investigations have indicated that the differentiation of pluripotent hemopoietic stem cells into a single line may occur under the presence of distinct hemopoietic inductive microenvironments (HIM) (KNOSPE, BLOM and CROSBY, 1966; CURRY and TRENTIN, 1967a, b; CURRY, TRENTIN and WOLF, 1967; CURRY, TRENTIN and CHENG, 1967; FRIED et al., 1973). However, the mutual relationships between stromal cells and the immediate progenies of multipotential hemopoietic stem cells have not been demonstrated morphologically.

In our previous papers, we demonstrated the precise ultrastructures of the presumptive hemopoietic stem cells and cells of the granulocytic, megakaryocytic and erythrocytic series in human embryonic and fetal livers (EMURA, SEKIYA and OHNISHI, 1983a-d).
The present study was conducted to reveal the reciprocal relationships between the immature hemopoietic cells of the three lineages and hepatocytes or reticular cells in the human embryonic and fetal liver.

MATERIALS AND METHODS

The hepatic tissues examined were obtained from 109 human embryos 28 to 49 days after ovulation and 76 human fetuses 8 to 22 weeks of gestation by legal abortion from healthy women.

Light microscopy. Eighteen embryos between 4 to 24 mm crown rump (C. R.) length (estimated age: 30 to 49 days after ovulation) and hepatic tissue from 34 fetuses (50 to 154 days during gestation) were fixed in 10% neutral formalin and then embedded in paraffin. Every fifth serial section was stained with hematoxylin-eosin, and the rest were subjected to a silver impregnation (WATANABE's method, 1961), periodic acid-Schiff (PAS) reaction and naphtol AS-D chloroacetate esterase stain.

Transmission electron microscopy. Ninety-one embryos, including a 21 somite embryo of 3 mm C. R. length (estimated age, 27±1 days), and hepatic tissues from 76 legally aborted fetuses were fixed as soon as possible in 2% glutaraldehyde in 0.1 M phosphate buffer, pH 7.5, at 4°C for 2 hrs. In the fixative, the embryos and livers were divided into small pieces using a dissection microscope. The tissue pieces were then rinsed in a 0.1 M phosphate buffer solution, pH 7.5, and postfixed in 1% osmium tetroxide at 4°C for 2 hrs. All the specimens were dehydrated and embedded in Epon, with ten sets taken at intervals of 15-20 μm from a block of each case. Ultrathin sections were counterstained with uranyl acetate and lead citrate and examined with a Hitachi HS-9 electron microscope.

RESULTS

I. Granulocytic series around the ductus venosus

A. Light microscopical findings

A small number of hemopoietic cells were first disclosed around the ductus venosus of an embryo of 14 mm C. R. length (estimated ovulation age: 40 days), with granulopoiesis seemingly established here within a week (EMURA, SEKIYA and OHNISHI, 1983b). Clusters of cells of the granulocytic series were frequently observed. Neither erythropoiesis nor megakaryopoiesis was observed at this site.

During the early stages, the wall of the ductus venosus was composed of numerous reticular cells; a fine framework of reticular fibers was demonstrated here by silver impregnation. Granulopoiesis developed everywhere in the wall. With the development of the fetus, however, thick collagen fibers increased under the endothelium and granulopoiesis was restricted to the narrow area between this fibrous wall of the vessel and the hepatic parenchyma. Reticular cells and a fine framework of reticular fibers were shown in the above defined area (Fig. 1).

B. Electron microscope findings

Characteristically, the majority of myeloid progenitor cells and the occasional myeloblasts found 50 days after ovulation were encircled by the cytoplasmic projections of
one or more reticular cells (Fig. 2) (EMURA, 1978). Most of them were wrapped individually, but occasionally granulocytic cells at different maturation stages gathered closely and the cytoplasmic projections of reticular cells extended among them (Fig. 3).

Where the granulocyte- reticular cell contacted, the opposing cell membranes became parallel, and the intercellular space was narrowed. Pinocytotic vesicles were often found at the cytoplasmic membranes of granulocytic cells or reticular cells, but neither a communicating structure nor any apparent desmosome-like attachment could be detected.

Until 50 days of gestation, the cells of the granulocytic series coexisted with numer-

Fig. 1. Granulopiesis around a blood vessel in the liver of a human fetus, 20 weeks of gestation. Two serial sections: a. Clusters of the cells of granulocytic series are found between the wall of blood vessel and hepatic parenchyma (arrows), naphtol AS-D chloroacetate esterase stain ×100. b. Fine framework of reticulin fiber demonstrated according to the area, silver impregnation. ×100
Fig. 2. A myeloblast (M) around the ductus venosus of a human fetus, about 55 days after ovulation. The myeloblast is wrapped in the slender cytoplasmic projections of reticular cells (R). × 6,100

Fig. 3. A cluster of cells of the granulocytic series around the ductus venosus of a human fetus, about 8 weeks of gestation. The slender cytoplasmic projections of the reticular cell (R) extend out and enclose cells of the granulocytic series (G). × 4,100
ous reticular cells, but any ultrastructural images suggesting a mutual relationship between both kinds of cells could not be demonstrated.

C. Ultrastructure of the reticular cell

Cells hitherto described as wrap cells (Emura, 1978) and mesenchymal cells (Emura, Sekiya and Ohnishi, 1980) correspond to the reticular cells in this paper. These cells are irregular in contour and provided with dendritic cytoplasmic projections. Desmosomelike structures frequently occurred between these cells or between these and hepatocytes. They adhered to or embraced slender bundles of reticular fibers. A basement membrane was not found around them.

The nucleus was irregular in shape and contained one or two small nucleoli. Chromatin was finely dispersed in the nucleus of these cells in the early embryonic liver, whereas in later stages it underwent a slight reticular condensation. Moderately dilated and branched cisternae of rough endoplasmic reticulum containing an amorphous material were conspicuous in the cytoplasm. The Golgi apparatus was composed of lamellar cisternae and small vesicles. Free ribosomes were scarce, and the mitochondria had a frequency of 10 to 20 per section. Lysosome-like granules were occasionally found. Phagosomes were encountered only rarely.

II. Megakaryocytic and erythrocytic series in the hepatic parenchyma

A. Light microscopical findings

For embryos in the first month, no hemopoietic cells were found in the hepatic parenchyma, and all blood cells in the sinusoids were primitive erythroblasts of yolk sac origin.

Fig. 4. An early hepatic promegakaryocyte at stage I (Me) in the hepatic parenchyma of a human embryo about 6 weeks after ovulation. This cell is encircled by the thin cytopasmic projections of reticular cells (R). × 4,900
A small number of hemopoietic cells were first identified among hepatocytes in an embryo of 6.2 mm C. R. length (estimated ovulation age: 33 days) and in embryos at 40 days of ovulation, hemopoiesis in the liver seemed to be established (EMURA, SEKIYA and OHNISHI, 1983a, c, d). The majority of large immature erythroblasts were found among hepatocytes, whereas most of mature erythroblasts gathered in the subendothelial spaces of sinusoids.

Megakaryocytes were frequently found in the subendothelial spaces of sinusoids or in the sinusoidal lumina.

B. Electron microscopic findings

1. Megakaryocytic series

Megakaryoblasts and promegakaryocytes at stage I that were encircled by thin cytoplasmic projections of reticular cells were found among hepatocytes both in the early stage of hepatic hemopoiesis (Fig. 4) and the late stage (Fig. 5). Occasionally, immature megakaryocytes at different stages of maturation gathered to form compact cell clusters among the hepatocytes. Reticular cells extended their attenuated projections among these immature megakaryocytes (Fig. 6).

In some regions of megakaryocyte-reticular cell contact, the cell membranes were closely juxtaposed and the intercellular gap was narrowed. Pinocytotic vesicles often occurred among the cell membranes of either cell. Only rarely were desmosome-like attachments found between reticular cells and megakaryoblasts.

Mature forms of this series, with occasionally immature forms, were frequently

Fig. 5. a. A late hepatic megakaryoblast (Me) in the hepatic parenchyma of a human fetus, about 10 weeks of gestation. This cell is enveloped by the cytoplasmic projections of reticular cells (R). ×11,400. b. The earliest stage of demarcation membrane system formation (section from the same cell as that shown in Fig. 5a but at another level). ×10,000
observed in the subendothelial spaces or in the sinusoidal lumina, indicating that the cells of the megakaryocytic series coexist with reticular cells during the earliest stages of their maturation course.

As for the reticular cells encircling immature cells of megakaryocytic series, no morphological differences between the reticular cells associated with granulocytes and those surrounding megakaryocytes could be noted.

2. Erythrocytic series

The majority of basophilic erythroblasts, proerythroblasts and more immature erythroblasts found in the liver during both the early stage of hepatic hemopoiesis (Fig. 7) and in later stages (Fig. 8), entered the cytoplasm of hepatocytes or were enveloped by cytoplasmic projections of hepatocytes (EMURA, SEKIYA and OHNISHI, 1983c). Occasionally, two immature cells coexisted in a compartment composed of hepatocytes, but compact clusters of immature cells of erythrocytic series were hardly to be found. Although numerous microvilli usually occurred on the surface of the hepatocytes, they were rarely encountered on the surface facing the invaginating erythroblasts.

In regions of hepatocyte-erythroblast contact, desmosome-like attachments were found between the plasma membranes of adjacent cells. They were most frequently encountered between hepatocytes and proerythroblasts or more immature forms of erythroblasts. Slender cytoplasmic projections of hepatocytes adhered and even entered a short distance (0.2 \( \mu \text{m} \)) into the cytoplasm of the erythroid progenitor cells. Each of these projections contained a microtubule (0.03 \( \mu \text{m} \) in diameter) in its axis. On the plasma membrane of the erythroid progenitor cell opposite the tip of this projection, a coated pinocytotic pit was noticed. The hepatocyte projection often forked within the
Fig. 7. A proerythroblast detected in the hepatic parenchyma of an embryo, about 6 weeks after ovulation. The cell is invaginating into the cytoplasm of hepatocytes and desmosoma-like attachments are found between hepatocytes and the cell. ×6,400

Fig. 8. A proerythroblast in the hepatic parenchyma of a fetus, about 8 weeks of gestation. Desmosome-like attachments are observed between the cell and hepatocytes. ×6,300
erythroid cell; in this case, the pinocytotic invagination in the latter cell occurred in
duble, i.e., opposite each tip of the projection (Fig. 9). These structures suggested the
possible transfer of some material and a form of information which might influence
the development of the erythroblasts.

The central macrophage of erythroblastic islets was surrounded by a ring of ery-
throblasts, though sometimes two or more rings were found (Fig. 10). Moreover, many
erthroblasts gathered around hepatocytes (Fig. 11) or Kupffer cells, showing images
similar to the erythroblastic islet. Slender cytoplasmic processes of the macrophages,
hepatocytes or Kupffer cells were extended to enclose the surrounding erythroblasts.
In areas of central cell and erythroblast contact, the opposing cell membranes approach-
ed each other across a narrow space, but neither a desmosome-like attachment nor any
communicating structure between the cells could be observed.

C. Ultrastructure of the macrophages
The macrophages were stellate cells with fine cytoplasmic extensions. No mutual re-
lationship between the macrophage and bundles of collagen filaments could be detected.
However, at sites of macrophage-reticular cell contact, the opposing cell membranes
became parallel. The intercellular spaces of these cells decreased in width and filled
with an amorphous material containing a small number of filaments. The nucleus was
irregular in contour and possessed a small nucleolus. The chromatin showed slight
reticular condensation. The cytoplasm contained short narrow cisternae of rough
endoplasmic reticulum, mitochondria, a small Golgi apparatus and lysosomal granules.
Phagosomes were frequently found.
Fig. 10. An erythroblastic island. The central macrophage is surrounded by a ring of erythroblasts. × 4,400

Fig. 11. Erythroblasts gather around the hepatocyte (H) showing similar ultrastructures to the erythroblastic island. × 5,900
D. Ultrastructures of the hepatocytes

Hepatocytes in the active hepatic hemopoiesis stage underwent a marked differentiation in comparison with those in the liver where hemopoiesis did not develop.

During the first month, when the hepatic hemopoiesis was still not developed, the hepatocytes (Fig. 12) were irregular in size and shape. The plasma membrane possessed numerous microvilli. The oval nucleus had one or two distinctive nucleoli and its chromatin was finely dispersed. The nuclear pores were numerous. The cytoplasm contained abundant polyribosome, a moderate number of mitochondria and a small number of glycogen particles. The cisternae of rough endoplasmic reticulum were short and distributed in an irregular pattern. The Golgi apparatus consisted of several groups. Primary or secondary lysosomes and a small plexus of smooth surfaced reticulum were rarely found. Definitive bile canaliculi were equally as scarce.

In the stages of hepatic hemopoiesis, the nucleus of the hepatocyte contained prominent nucleoli and the chromatin was finely dispersed (Fig. 13). The hepatocytes exhibited a distinct polarity of the cytoplasm, and bile canaliculi were frequently observed. Mitochondria increased in number. The cisternae of rough endoplasmic reticulum became elongated and were arranged in stacks of 7 to 20 lamellae. Polyribosomes gradually decreased in number. The smooth surfaced reticulum consisted of a close-meshed plexus of anastomosing tubules. Primary and secondary lysosomes increased in number. Glycogen particles were occasionally found.

Fig. 12. A hepatocyte (H) and an undifferentiated mesenchymal cell (UMeC) in an embryo, about 30 days after ovulation. The polyribosomes were abundant in the cytoplasm and the cisternae of rough endoplasmic reticulum were short and distributed in an irregular pattern. ×6,000
III. Candidate progenitor cells for the three lineages

It was possible to detect cells that were encircled by the thin cytoplasmic projections of hepatocyte or entered the cytoplasm of the hepatocytes and had desmosome-like attachments between the plasma membranes of adjacent cells, both in the liver in the early stage of hepatic hemopoiesis (Fig. 14) and in later stages (Fig. 15). In the same way, some immature cells that were encircled by the cytoplasmic projections of reticular cells were found among hepatocytes both in both early (Fig. 16) and later (Fig. 17) stages of hepatic hemopoiesis. Small cells quite similar to small lymphocytes and were enclosed by the slender cytoplasmic projections of reticular cells (Fig. 18) were disclosed around the ductus venosus after 50 days of ovulation. But until 50 days of ovulation, hemopoietic cells were scattered among numerous reticular cells, though any ultrastructural images supporting a mutual relationship between the immature cells of granulocytic lineage and reticular cells were not able to be demonstrated ultrastructurally.

The cells in Figures 14 and 16 showed a remarkable resemblance. They were oval in shape and measured 14 μm in mean diameter. The large nucleus was round with a slight indentation and contained at least one prominent nucleoli. Chromatin was finely dispersed and the nuclear pores were numerous. The cytoplasm contained abundant polyribosomes and a moderate number of mitochondria. The cisternae of rough endoplasmic reticulum were short and small in number. The Golgi apparatus consisted of a few short cisternae and small vesicles. A pair of centrioles was observed in the central area of the Golgi apparatus.

The cells shown in Figures 15, 17 and 18 were identical in ultrastructure. The
The average diameter of these cells was 8 μm. The nucleus contained a small less prominent nucleolus. The chromatin showed a coarse accumulation and the nuclear pores were small in number. The narrow cytoplasm contained a few single ribosomes and mitochondria. Polyribosomes were only rarely found. The cisternae of rough endoplasmic reticulum were hardly seen, and the Golgi apparatus was small in size.

**DISCUSSION**

HIM are believed to play important roles in the directing of pluripotent hemopoietic stem cells into each of the several possible lines. But an ultrastructural detailing of the mutual relationships between stromal cells and the immediate progenitors of multipotential hemopoietic stem cells has yet to be shown. The present investigation revealed the interactions between stromal cells and immature cells of each of the three lineages in human embryonic liver.

Any ultrastructures by which we are able to classify the cells shown in Figures 14 to 18 as one of each of the three lineages could not be found in these cells. However, the cells shown in Figures 14 and 16 resemble the most immature forms of the erythrocytic or megakaryocytic lineages found in the liver in early stages of hepatic hemopoiesis (Emura, Sekiya and Ohnishi, 1983a, c) respectively. Similarly, the cells shown in Figures 15, 17 and 18 recall the most immature cells of the three lineages detected in the late stage (Emura, Sekiya and Ohnishi, 1983a–c). Moreover, we have demonstrated the mutual relationships between immature cells of the three lineages and stromal cells. It seems possible to conclude that: the cells shown in Figures 14 and 15
that intrude into the cytoplasm of hepatocytes are erythrocytic, the cells in Figures 15 and 17 that are detected among hepatocytes and are encircled by the cytoplasmic extensions of reticular cells are megakaryocytic, and the cell in Figure 18 found around the ductus venosus and encircled by cytoplasmic projections of reticular cells to be granulocytic.

The authors proposed that the cells of the granulocytic, megakaryocytic and erythrocytic lineages were differentiated from presumptive hemopoietic stem cell type II during the early stage of hepatic hemopoiesis and those of the three lineages from the presumptive hemopoietic stem cell type IV in the later stage of hepatic hemopoiesis (EMURA, SEKIYA and OHNISHI, 1983a-d).

The presumptive hemopoietic stem cell type II range in size from 10 to 15 \( \mu m \) and show a high nucleocytoplasmic ratio. The nucleus contains one or more prominent nucleoli and its chromatin is finely dispersed. Nuclear pores are numerous. Polyribosomes are abundant, and the 10 to 15 mitochondria per section tend to gather around the small Golgi apparatus. The cisternae of rough endoplasmic reticulum are rarely found. The cells in Figures 14 and 16 are quite similar to the presumptive hemopoietic stem cell type II in ultrastructure. In the same way, the presumptive type IV hemopoietic stem cells are round in shape and range from 7 to 8 \( \mu m \) in diameter. The nucleus possesses a small, less prominent nucleolus. Chromatin is coarsely accumulated and nuclear pores are seldom seen. The narrow cytoplasm contains a small number of single ribosomes and a few mitochondria. The Golgi apparatus is small in size and cisternae of rough endoplasmic reticulum are hardly found. A pair of centrioles occurs
near the Golgi apparatus. The cells shown in Figures 15, 17 and 18 morphologically resemble presumptive type IV hemopoietic stem cells.

These findings indicate 1) that the cells in Figures 14 and 16 are immediate progenitors of presumptive type II hemopoietic stem cells and the cells shown in Figures 15, 17 and 18 are those of presumptive hemopoietic stem cells type IV, 2) that presumptive hemopoietic stem cell types II and IV induce differentiation into cells of the erythroid line by contact with the hepatocytes that are in certain stages of differentiation, 3) that the compartments composed of one or more reticular cells around the ductus venosus have an ability to regulate the differentiation of the presumptive hemopoietic stem type IV cells into the cells of the granulocytic series and 4) that the differentiation of presumptive hemopoietic stem cell types II and IV into the cells of megakaryocytic series is due to the presence of a particular microenvironment that consists of foci of a few reticular cells in the hepatic parenchyma.

The hepatocytes into which the immature cells of the erythrocytic series enter show marked differentiation in comparison with the hepatocytes found in the liver where hepatic hemopoiesis has not appeared. Neither erythropoiesis nor megakaryopoiesis developed among the mesenchymal cells around the ductus venosus where granulopoiesis actively occurs, and in later stages, granulopoiesis does not occur in the fibrous walls of blood vessels, but rather develops in the narrow area between the hepatic parenchyma and the fibrous wall of blood vessels. It was possible to demonstrate reticular cells and a fine framework of reticular fibers in the above mentioned area. These findings seem to support our assertion, and our conclusions are summarized schematically in Figure 19.

As to the roles of the central macrophage of the erythroblastic islet, two main
Fig. 17. A small lymphoid cell in the cluster of cells of the megakaryocytic series in the hepatic parenchyma of a fetus, about 10 weeks after gestation. The cell is invaginating into the cytoplasm of reticular cell (R). Me A large cytoplasmic projection of megakaryocyte. ×11,700

Fig. 18. A small lymphoid cell around the ductus venosus of a fetus, about 9 weeks of gestation. The cell is encircled by the cytoplasmic extensions of reticular cell (R). ×10,800
Theories have been proposed: a scavenger role in ingesting extruded nuclei of erythroblasts, (Yoffy, 1973) and the transfer of ferritin with some form of information which may influence the development of neighboring erythroblasts (Bessis, 1959). We could not find any evidence that supported the transfer of ferritin or some form of information from the central macrophage to the surrounding erythroblasts. The central macrophage undoubtedly has a phagocytic activity, but the scavenger role does not seem to be the most intrinsic function of the central macrophage, because erythroblasts frequently encircle the hepatocytes showing ultrastructures similar to erythroblastic islets. Since the encircling erythroblasts are exclusively basophilic erythroblasts or more mature forms, and mitotic figures were frequently found among them, we surmise that the basophilic erythroblasts or more matured erythroblasts adhere to certain fixed cells and undergo maturation and mitoses, and that the erythroblastic islet is a kind of HIM.

The results of recent experimental investigations have indicated that T-lymphocytes play important roles on hemopoiesis (Goodman et al., 1972; Cerney, 1974; Zipori and Trainin, 1975; Sharkis et al., 1979). However, T-lymphocytes do not seem to influence the differentiation of the cells of erythrocytic, megakaryocytic and granulocytic lineages from presumptive hemopoietic stem type II cells, because cells similar to small lymphocyte in ultrastructure are not found either in the liver or the epithelial primordia of the thymus during the early stage of fetal development.

**Fig. 19.** A model of hematopoiesis in the human embryonic and fetal liver. HIM hemopoietic inductive microenvironment, UMeC undifferentiated mesenchymal cell, I presumptive hemopoietic stem cell type I, II presumptive hemopoietic stem cell type II, III presumptive hemopoietic stem cell type III, IV presumptive hemopoietic stem cell type IV, Myel. Pro. myeloid progenitor cell, My.Bl. myeloblast, Neut neutrophile, Mg.Pro.C. megakaryocytic progenitor cell, Mg.Bl. megakaryoblast, Plat. platelet, Ery.Pro.C. erythrocytic progenitor cell, Pr.Ery. Bl. proerythroblast, Ery. erythrocyte.
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