Differentiation of Red Pulp and Evaluation of Hemopoietic Role of Human Prenatal Spleen

Hiroshi ISHIKAWA

Department of Pathology (Prof. Y. OHNISHI), Niigata University School of Medicine, Niigata, Japan

Received December 27, 1984

Summary. Both the development of red pulp and hemopoietic activity in spleen obtained from 62 human embryos and fetuses between 30 days and 20 weeks after ovulation were investigated light and electron microscopically.

The spleen develops in the left-posterior portion of the dorsal mesogastrium at 35-40 days after ovulation. At the 8th week after ovulation, reticular cells formed a three-dimensional meshwork. Two types of reticular cells (dark and clear reticular cells) were observed in the splenic cords in the 12-13th week after ovulation.

Mature hemopoietic cells, mostly of the erythroblastic series, increased in number in the extravascular spaces with the development of the fetus. However, presumptive hemopoietic stem cells or “undifferentiated mononuclear cells” (FUKUDA, 1973a) did not appear in the spleen. Moreover, immature hemopoietic cells such as proerythroblasts, myeloblasts and megakaryoblasts could not be detected. Therefore, despite the occurrence of a well-developed reticular cell network, hemopoiesis was judged to have not taken place in human fetal spleen.

Macrophages appeared in the spleen at the 8th week after ovulation and increased in number with the development of the fetus. Phagocytosis of decrepit blood cells proved to be an essential function of the spleen.

With the light microscope, SABIN (1912) and ONO (1930) studied the development of the human spleen. They reported the differentiation of the red and white pulp, and conceded hemopoiesis in the spleen at the second trimester. Recently, some investigators (ZAMBI and WESTIN, 1964; WEISS, 1973; WEISS and CHEN, 1974) have researched the structural development of the human spleen under the electron microscope. They have hardly mentioned hemopoietic activity in the fetal spleen.

On the other hand, hemopoiesis in the spleen is one of the characteristic features of patients with myelofibrosis. It has been said that this extramedullary hemopoiesis in the adult spleen might be a reactivation of hemopoiesis in the fetal spleen. Many investigators have examined hemopoiesis in the fetal spleen using non-human mammals. KELEMEN and associates (1979), on the other hand, studied human splenic smears and concluded that the spleen contributed only in a small proportion to blood cell formation during the development of the fetus. WOLF and co-workers (1983) immunohistochemically investigated the human spleen in fetuses ranging from 12 to 40 weeks in gestational age, and reported that the human fetal spleen was not a hemopoietic organ.
In this paper, we ultrastructurally examine the role of the human fetal spleen in hemopoiesis and describe findings in reticular cells in the embryonic and fetal spleen.

**MATERIALS AND METHODS**

The spleens used in this study were obtained from 41 human embryos 30 to 56 days after ovulation and 21 human fetuses in the 9th to the 20th week of ovulation, all by legal abortions from healthy women (Table 1). The embryonic and fetal age was appraised from the crown-rump length (CRL), the characteristics of the body surface and the development of long bones (Moore, 1982).

*Light microscopy*: Twenty-seven embryos between 6 and 24 mm CRL (estimated age: 30 to 53 days after ovulation) and spleens of 23 embryos and fetuses (7th to 20th week of ovulation) were fixed in 10% formalin and then embedded in paraffin. Sections were treated with hematoxylin and eosin, silver impregnation method, Azan-Mallory staining, elastica and van Gieson staining, periodic acid-Schiff (PAS) reaction, diastase digested PAS reaction, naphthol-ASD-chloracetate esterase reaction and acid phosphatase reaction.

*Electron microscopy*: The spleens of 6 embryos and 16 fetuses were immediately fixed in 2% glutal aldehyde in 0.1 M phosphate buffer solution, pH 7.5, at 4°C for 2 hrs. In the fixative, the spleen was divided into small pieces. The tissue pieces were then rinsed in 0.1 M phosphate buffer solution, pH 7.5, and postfixed in 1% osmium tetroxide at 4°C for 2 hrs. All specimens were dehydrated and embedded in Epon. Ultra-thin sections were counterstained with uranyl citrate and lead citrate. The sections were observed with a Hitachi HS-9 electron microscope.

**RESULTS**

1. **Development**

*5th-6th week after ovulation*: The human splenic anlage appeared in the left-posterior portion of the dorsal mesogastrium in embryos of 9-11 mm CRL (estimated ovulation age: 35-40 days) (Fig. 1a). Undifferentiated mesenchymal cells proliferated in the anlage, and a fine network of reticular fibers was present among the mesenchymal cells. Some layers of columnar epithelia covered the surface of the anlage (Fig. 1b).
7th week after ovulation: Reticular cells proliferated in the splenic anlage which protruded into the abdominal cavity. It was easy to distinguish the tissue of the splenic anlage from that of the dorsal mesogastrium (Fig. 2). A few blood vessels were partially surrounded by processes of reticular cells, though a basement membrane was hardly detectable. A small number of unclassifiable immature cells (Fig. 8) appeared in the extravascular spaces. Several erythrocytes and/or orthochromatic erythroblasts occurred only in the blood vessels.

8th week after ovulation: Reticular cells with many cytoplasmic processes formed a three-dimensional meshwork (Fig. 3). Many collagen filaments occurred in the intercellular spaces. A few phagocytic macrophages were first discerned among the reticular cells.
Fig. 2. Reticular cells proliferating in the splenic anlage in the 7th week after ovulation, hematoxylin and eosin stain. ×50

Fig. 3. Reticular cells forming a three-dimensional meshwork in the spleen in the 8th week after ovulation. A phagocytic macrophage is seen (arrow). ×2,350
9th–10th week after ovulation: Blood vessels increased in number. Spindle shaped periendothelial cells (Zamboni and Westin, 1964) were found around the arteries. Several erythrocytes, polychromatic and orthochromatic erythroblasts, and megakaryocytes first appeared in the intercellular spaces of reticular cells. Phagocytic macrophages increased in number among the reticular cells.

11th week after ovulation: Numerous erythrocytes appeared in the extravascular spaces. Thrombocytes, polychromatic and orthochromatic erythroblasts increased in number. Granulocytic and megakaryocytic cells were rarely observed. Some mature erythroblasts occasionally formed small clusters. However, erythroblastic islands (Bessis, 1958) could not be detected. Reticular cells extended their long cytoplasmic processes among blood cells. The endothelium came to lie partially on the basement membrane.

12th–13th week after ovulation: Small cellular nodules (primordia of white pulp) were first demonstrated in this stage. Ultrastructurally, reticular cells could be classified into two groups (dark and clear reticular cells). Numerous blood cells and phagocytic macrophages occurred in the extravascular spaces. Lymphoid cells first appeared in the intra- and extravascular spaces except for around the arteries of the spleen.

14th–20th week after ovulation: Both red pulp and white pulp were recognized in the spleen and trabeculae were formed. A small number of large lymphocytes gathered around the central arteries. Lymphocytes increased in number with the development of the fetus. During this period, erythroblasts increased in number remarkably. Both the sinus lumina and extravascular spaces were filled with these erythroblasts as well as with cells of both the granulocytic series and megakaryocytic series (Fig. 4).

Time of appearance for each of the cells in the embryonic and fetal spleen is shown in the diagram (Fig. 5).

2. Hemopoietic cells and macrophages
Hemopoietic cells in the spleen first appeared in the extravascular spaces of an embryo.
in the 9th week after ovulation and increased in number with the development of the fetus. Most of them were of the erythrocytic series. One of the most immature erythroblasts in the extravascular spaces is shown in Figure 6. The nucleus of the cell was situated centrally, being oval in shape. The nucleolus was obscure. Chromatin clumped coarsely. The electron dense cytoplasm contained a moderate number of polyribosomes, a few mitochondria and several vacuoles. The Golgi apparatus was small in size. This cell was more mature than the basophilic erythroblast. Despite careful observation, we could not identify immature cells such as proerythroblasts, myeloblasts and megakaryoblasts in the extravascular spaces. Presumptive hemopoietic stem cells which correspond to the "undifferentiated mononuclear cells" first described by Fukuda (1973) were not detected in the spleen.

A few macrophages were first found among the reticular cells of an embryo in the 8th week after ovulation (Fig. 3, 7). The number of macrophages increased with the development of the fetus. Their nucleus was irregular in contour and possessed a small nucleolus. Lysosomal granules and phagocytized erythroblasts frequently appeared in the cytoplasm. Irregular villous cytoplasmic extensions were characteristic. A very small number of unclassifiable cells as shown in Figure 8 appeared among the reticular cells of an embryo in the 7th week after ovulation. The nucleus was irregular in shape and possessed one or more prominent nucleoli. Chromatin was finely dispersed in the nucleus. The cytoplasm was darker than the surrounding reticular cells and contained short slender cisterns of rough-surfaced endoplasmic reticulum (rER), ten or more mitochondria, a few lysosomal granules, and a moderate number of polyribosomes. Characteristically, bundles of microfilaments appeared in the cytoplasm. Many pinocytic vesicles and pits and microvilli were arranged along the cell membrane. 

**Fig. 5.** Diagram showing the time of appearance of each of the hemopoietic cells in the extravascular spaces of the prenatal spleen.
cells contained no secondary lysosomes. The cells could not be recognized in later stages.

3. Mesenchymal cells and reticular cells

The cells in the initial stage of the splenic anlage were morphologically indistinguishable from the mesenchymal cells in the dorsal mesogastrium (Fig. 1). We could not observe this early splenic anlage under the electron microscope. Reticular cells in the splenic anlage were distinguishable from the mesenchymal cells in the dorsal mesogastrium (Fig. 2) by the 7th week after ovulation. In later stages, these reticular cells were classified into two subtypes. In this paper, the nomenclatures of these cells are based on those used in the works of Weiss (1973) and Weiss and Chen (1974).

Reticular cells (Fig. 9): These cells were irregular in contour and ranged from 10 to 15 μm in long diameter. A slightly indented nucleus occupied the greater part of the cell and possessed a small but prominent nucleolus. Chromatin was finely dispersed. A pair of centrioles was found in the small Golgi apparatus. Mitochondria tended to gather around the Golgi apparatus. The narrow cytoplasm contained a small number of polyribosomes, microtubules, microfilaments, and short and slightly dilated cisterns of rER. Primary and secondary lysosomes did not appear in the cytoplasm, whose cells issued cytoplasmic processes and often cilia. Zonula adherens-like attachments occurred frequently between the plasma membranes of adjacent reticular cells.

Dark reticular cells (Fig. 10): These cells were characteristically dark in appearance, being mainly located along the sinuses (Fig. 11, 12). However, it was easier to

Fig. 6. One of the most immature erythroblasts found in the spleen in the 18th–19th weeks after ovulation (175 mm CRL). Nuclear chromatin clumps reticularly and polyribosomes are scattered in the cytoplasm. ×12,000
Fig. 7. A macrophage with a secondary lysosome (S) and several primary lysosomes found in the spleen in the 8th week after ovulation. ×7,300

Fig. 8. An unclassifiable immature cell occurring in the spleen in the 7th week after ovulation. Bundles of microfilaments are found in the cell (arrows). ×10,500
find these cells in the white pulp than in the red pulp. A basement membrane did not surround these cells. Amorphous materials containing collagen filaments filled the narrow spaces between these cells and adjacent cells, especially sinusoidal endothelial cells. The dark reticular cells were irregular in shape and issued many cytoplasmic extensions with zonula adherens-like attachments. Chromatin clumped coarsely in the irregular nucleus, and a nucleolus was rare. The cisterns of rER and the perinuclear cisterns were dilated. The Golgi apparatus was small in size. A few polyribosomes and mitochondria lay scattered in the cytoplasm. Numerous microfilaments were detected in the cytoplasm.

Clear reticular cells (Fig. 13): These cells, showing a low electron density, were situated mainly in the red pulp and formed a three-dimensional meshwork of the splenic cord. They adhered to or embraced slender bundles of collagen filaments. Neither a basement membrane nor amorphous materials were found around these cells. The cells were irregular in shape and possessed zonula adherens-like attachments. The oval nucleus contained chromatin showing reticular clumping to varied degrees. Perinuclear cisterns were narrow. The Golgi apparatus consisted of short cisterns and small vesicles. Small lysosomal granules were occasionally formed near the apparatus. A pair of centrioles were often present. The cytoplasm contained a few mitochondria, polyribosomes, microtubules, microfilaments and rER. The cisterns of rER dilated moderately and contained amorphous materials.

DISCUSSION

The human fetal spleen has commonly been regarded as an organ of hemopoiesis.
Fig. 10. A dark reticular cell extending its cytoplasm along the sinus in the spleen in the 17th–18th weeks after ovulation (175 mm CRL). ×6,000

Fig. 11. Dark reticular cells situated under the sinus endothelial cells in the spleen in the 18th–19th weeks after ovulation (175 mm CRL). ×2,150
Recently, however, a few investigators have reported that the spleen was not a significant organ of hemopoiesis in the human fetus (Kelemen, Calvo and Fliedner, 1979; Wolf, Luevano and Neiman, 1983). Attention was focused on this problem in the present study on the ultrastructures of hemopoietic cells and reticular cells in the human fetal spleen.

By the 8th week after ovulation, phagocytic or non-phagocytic macrophages appear in the extravascular spaces. Macrophages increase in number with the development of the fetus. Many of these macrophages phagocytize erythrocytes, mature erythroblasts or thrombocytes. Phagocytosis of decrepit blood cells has been believed to be a fundamental function of the spleen. There have been many discussions as to the origin of the precursor of macrophages. The immature cells shown in Figure 8 seem to be precursors of macrophages because they contain bundles of microfilaments in the cytoplasm, and pinocytic pits and vesicles on the cell margin. We could not detect any transitional forms in the stages of differentiation between macrophages and any other cells in the splenic pulp. Many macrophages were found in the yolk sac within 5 weeks of gestation (Fukuda, 1973b) and in the liver at 6 to 8 weeks of gestation (Enzan et al., 1983). On the other hand, hemopoiesis in bone marrow did not appear in the 8th week of ovulation (Kelemen, Calvo and Fliedner, 1979). Therefore it seems reasonable to assume that the precursor of macrophages in the spleen comes from a extrasplenic organ, excepting the bone marrow, via the blood stream.

Concerning the hemopoietic stem cells, there has long been discussion as to whether they come from the yolk sac via the blood stream or whether they originate in the liver. Recent experimental studies have demonstrated that avian stem cells arose...
within the embryo, and did not derive from the yolk sac (Dietlen-Lievre, Beaupain and Martin, 1979). In human embryonic liver, Fukuda (1973a) first described undifferentiated mononuclear cells which he considered hemopoietic stem cells. Emura and associates (1983d) reported that these undifferentiated mononuclear cells differentiated into the cells of the erythrocytic, megakaryocytic and granulocytic series during early stages of hepatic hemopoiesis.

A few immature cells (Fig. 8) were found in the extravascular spaces of the splenic anlage in an embryo in the 7th week after ovulation. Ultrastructurally, however, these cells differ from the undifferentiated mononuclear cells. They are considered to be an immature form of macrophages. They are also distinguished from the precursor cells of the erythrocytic, granulocytic and megakaryocytic series. Therefore, it seems most reasonable to conclude that presumptive hemopoietic stem cells (undifferentiated mononuclear cells) do not appear in the human embryonic spleen.

Hemopoiesis in the human fetal spleen has been said to begin early in the second trimester. Hemopoiesis, the process of the formation of blood cells, initiates with the differentiation of pluripotential hemopoietic stem cells into the committed stem cells of varied lineages. It is difficult to distinguish the committed stem cells from the pluripotential ones in tissue. Many erythrocytic, granulocytic and megakaryocytic cells in various stages of maturation have been found in the extravascular spaces in hemopoietic organs, such as bone marrow (Tanaka and Goodman, 1972) and fetal liver (Emura, Sekiya and Ohnishi, 1983a–c). According to these findings, if the human fetal spleen is truly a hemopoietic organ, then cells in successive stages of maturation ought to be found in the extravascular spaces. Despite careful observation, immature forms of the erythrocytic, granulocytic and megakaryocytic series, such as proerythroblasts,
myeloblasts and megakaryoblasts could not be detected in the extravascular spaces of the human fetal spleen. This indicates that the differentiation of pluripotential hemopoietic stem cells into the cells of each of the three lineages does not take place. We must conclude that hemopoiesis does not develop in the spleen of the human embryo and fetus. The same conclusion has been reached by a few investigators (Amano, 1948; Kelemen, Calvo and Fliedner, 1979; Wolf, Luevano and Neiman, 1983). Wolf and associates (1983) asserted that hemopoietic cells in the fetal spleen were those filtered out from the fetal blood. We agree with them in this context.

The reticular cells in the splenic anlage are probably derived from the mesenchymal cells of the dorsal mesogastrium. They are classified into two subtypes. Dark reticular cells, characterized by their dark cytoplasm, are situated under the sinusoidal endothelial cells. Amorphous materials mixed with many collagen filaments fill the spaces between these cells and adjacent cells. These cells surround the splenic sinuses and support them with their processes. Finding in these cells electron dense areas as seen in smooth muscle fibers, Fukuda (1981) regarded these cells as myofibroblasts. The dark reticular cells have abundant microfilaments in the cytoplasm, but we could not recognize the occurrence of electron dense areas in these cells. These cells are considered to be fibroblasts since the cisterns of rER and numerous microfilaments are developed. Clear reticular cells formed a three-dimensional meshwork of the splenic cords. This meshwork seems similar to that of the reticular cells in the adult spleen. Reticular cells in the adult spleen contain moderate amounts of microtubules and microfilaments (Chen and Weiss, 1972). The clear reticular cells in the fetal spleen contain a small amount of microfilaments and microtubules. This relation seems compatible with the view that the clear reticular cells are the precursor cells of the reticular cells in the adult spleen.

Experimental studies have suggested that pluripotential hemopoietic cells differentiate into a single cell line under a distinct hemopoietic inductive microenvironment (Trentin, 1970). Recent morphological studies demonstrated that immature cells of the granulocytic and megakaryocytic series were wrapped by the cytoplasmic projections of reticular cells in the human embryonic liver (Emura, Sekiya and Ohnishi, 1984). They emphasized that the compartments composed of one or more reticular cells possessed an ability to regulate the differentiation of the presumptive hemopoietic cells into cells of the granulocytic and megakaryocytic lineages. Morphologically, a hemopoietic inductive microenvironment of erythrocytic series has not been reported except for erythroblastic islands (Bessis, 1958).

Throughout the present observations, we remained unable to find either immature forms of the erythrocytic, granulocytic and megakaryocytic series, or an intimate interaction between reticular cells and immature granulocytic cells or megakaryocytic cells in the fetal spleen. Erythroblastic islands could not be found, either. Therefore, it seems safe to conclude that the reticular cells in the human fetal spleen do not possess an ability to regulate the differentiation of pluripotential hemopoietic stem cells into cells of some hemopoietic series, and moreover, it is not able to provide an appropriate microenvironment for the differentiation of immature hemopoietic cells of any lineage.

Remarkable extramedullary hemopoiesis, however, develops in the spleens of patients with myelofibrosis and some other disorders. Recent experimental studies revealed that both pluripotential hemopoietic stem cells and committed stem cells appeared in peripheral blood (Barr, Whang-Peng and Petty, 1975). It remains to question why extramedullary hemopoiesis can develop in the adult spleen. Does an inflow of hemopoietic cells into the spleen occur? And do the cells in the spleen acquire the
ability of an hemopoietic inductive microenvironment? This problem must be solved in future studies.

Acknowledgments. The author thanks Professor Yoshihisa Ohnishi for his critical reading of the manuscript and helpful comments, and Dr. Iwao Emura for his advice. The author also gratefully acknowledges Drs. G. Takahashi, Y. Takeyama and M. Hoshii, Section of Obstetrics and Gynecology, Takeyama Hospital, for providing cases. Excellent technical assistance was provided by Mr. T. Hasegawa, Mr. K. Sato, and Mr. S. Momozaki, Department of Pathology, Niigata University School of Medicine, and the author is accordingly grateful.

REFERENCES

Amano, S.: Basis of Hematology (In Japanese). Maruzen, Tokyo, 1948.

Barr, R. D., J. Whang-Peng and S. Petty: Hemopoietic stem cells in human peripheral blood. Science 190: 284-285 (1975).

Bessis, M.: L’ilot érythroblastique, unité fonctionnelle de la moelle osseuse. Rev. hematol. 13: 8-11 (1958).

Chen, Li-T. and L. Weiss: Electron microscopy of the red pulp of human spleen. Amer. J. Anat. 134: 425-458 (1972).

Dieterlen-Lievre, F., D. Beaupain and C. Martin: Potentialities and migrations of hemopoietic stem cells of yolk sac and intraembryonic origins, studied in avian chimeras obtained by blastoderm recombination. In: (ed. by) N. LeDouarin: Cell lineage, stem cells and cell determination, Elsevier/North-Holland, Amsterdam-New York, 1979 (p. 175-189).

Emura, I., M. Sekiya and Y. Ohnishi: Two types of immature megakaryocytic series in the human fetal liver. Arch. histol. jap. 46: 103-114 (1983a).

Emura, I., M. Sekiya and Y. Ohnishi: Two types of progenitors of the granulocyte series in the human embryonic liver. Arch. histol. jap. 46: 229-242 (1983b).

Emura, I., M. Sekiya and Y. Ohnishi: Two types of immature erythrocytic series in the human fetal liver. Arch. histol. jap. 46: 631-643 (1983c).

Emura, I., M. Sekiya and Y. Ohnishi: Four types of presumptive hemopoietic stem cells in the human fetal liver. Arch. histol. jap. 46: 645-662 (1983d).

Emura, I., M. Sekiya and Y. Ohnishi: Ultrastructural identification of the hemopoietic inductive microenvironment in the human embryonic liver. Arch. histol. jap. 47: 95-112 (1984).

Enzan, H., H. Hara, Y. Yamashita, T. Okkita and T. Yamane: Fine structure of hepatic sinusoids and their development in human embryos and fetuses. Acta pathol. jap. 33: 447-466 (1983).

Fukuda, T.: Undifferentiated mononuclear cell in human embryonic liver; presumptive hemato-poietic stem cell. Virchows Arch. Abt. B 14: 31-34 (1973a).

Fukuda, T.: Fetal hemopoiesis. 1. Electron microscopic studies on human yolk sac hemopoiesis. Virchows Arch. Abt. B 14: 197-213 (1973b).

Fukuda, T.: Perifollicular, perisinusal and trabecular myofibroblasts in the human fetal spleen. Virchows Arch. Pathol. Anat. 393: 1-8 (1981).

Kelemen, E., W. Calvo and T. M. Fliedner: Atlas of human hemopoietic development. Springer-Verlag, Berlin-Heiderberg-New York, (1979).

Moore, K. L.: The developing human. 3rd ed. W. B. Saunders, Philadelphia, 1982.

Ono, K.: Untersuchungen über die Entwicklung der menschlichen Milz. Z. Zellforsch. 10: 573-603 (1936).

Sabin, F. R.: The development of the spleen. In: (ed. by) F. Keibel and F. R. Mall: Mannual of human embryology. J. B. Lippincott, Philadelphia, 1912 (p. 745-751).

Tanaka, Y. and J. R. Goodman: Electron microscopy of human blood cells. Harper and Row Publishers. New York-Evanston-San Francisco-London. 1972
Trentin, J. J.: Influence of hematopoietic organ stroma (hematopoietic inductive microenvironments) on stem cell differentiation. In: (ed. by) A. S. Gordon: Regulation of hemopoiesis. Appleton-Century-Crofts, New York. 1970 (p. 161-186).

Weiss, L.: The development of the primary vascular reticulum in the spleen of human fetus. Amer. J. Anat. 136: 315-338 (1973).

Weiss, L. and Li-T. Chen: The differentiation of white pulp and red pulp in the spleen of human fetus. Amer. J. Anat. 141: 393-414 (1974).

Wolf, B. C., E. Luevano and R. S. Neiman: Evidence to suggest that the human fetal spleen is not a hematopoietic organ. Amer. J. clin. Pathol. 30: 140-144 (1983).

Zamboni, L. and B. Westin: The ultrastructure of the human fetal spleen. 1. One type of mesenchymal cell in the early stages of development of the spleen. J. Ultrastr. Res. 11: 469-493 (1964).

Dr. Hiroshi ISHIKAWA
Department of Pathology
Niigata University School of Medicine
1 Asahimachi-dori, Niigata
951 Japan