A Karyometrical Study of Circulating Erythroblasts of Yolk Sac Origin in the Mouse Embryo*

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Summary. Circulating erythroblasts of embryonic mice were karyometrically examined by light microscopy.

In the erythroid cells in embryonic blood vessels, mitoses were encountered from 9 to 12 days of gestation. At 9 days, the circulating blood cells consisted of proerythroblasts and less mature cells. The nuclear diameter ranged from 4.8 to 9.8 μm , the majority ranging between 6 and 8 μm . At 11 days, hemopoietic cells with a nuclear diameter larger than 6 μm disappeared from embryonic circulation, and more than 90% had a nuclear diameter of less than 5 μm . Between 12 and 16 days of gestation, the smallest orthochromatic erythroblasts measuring 3.4 μm nuclear diameter showed the highest peaks.

The progress of primitive erythropoiesis in embryonic circulation is discussed in comparison with that of definitive erythropoiesis.

It is well known that in early embryonic life erythroblasts, which are derived from angioblastic cords in the yolk sac, enter peripheral circulation as primitive erythroblasts. One of the characteristics in the morphology of primitive erythroblasts is that the end cells in yolk sac erythropoiesis are nucleated (GILMOUR, 1941; RUSSELL and BERNSTEIN, 1968). Nuclear morphology can be employed for the classification of erythroblasts (ROSSE and TROTTER, 1974). Diminution in nuclear size is known to be a reliable criterion for determining erythroblastic maturation (DACIE and WHITE, 1949; WEICKER, 1959). In the present investigation, we have karyometrically examined the circulating erythroid cells of yolk sac origin to clarify the process of the differentiation of erythroblasts released from the yolk sac during embryonic life.

MATERIAL AND METHODS

Thirty-three dd-mice embryos at 8, 9, 10, 11, 12, 13, 14, 15, 16 and 18 days of gestation were used in this study. On each date, at least three embryos were used. Adult virgin females were mated overnight with males and the morning following mating was designated as Day 0. Pregnant females were anesthetized with chloroform, and the uterus was rapidly removed. From 8 to 13 days of gestation, each segment of the uterus containing an embryo was fixed in Zenker-formalin-acetic acid (20:2:1).

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Fig. 1. Nine days of gestation. Stained with hematoxylin and eosin. **a.** Yolk sac. A small hemopoietic focus (*H*) is present in the visceral yolk sac (*ys*). *ec* Extraembryonic coelom, *ysc* yolk sac cavity. $\times 830$. **b.** A section of an embryo. Blood vessels are formed in the embryo, and cells migrating from the yolk sac can be seen in the lumen of the vessels (*v*). *ec* Extraembryonic coelom, *ys* yolk sac, *ysc* yolk sac cavity. $\times 80$. **c.** Circulating cells in an embryonic vessel. The cells have large and pale nuclei with prominent nucleoli. $\times 830$

Embryos from 14 to 18 days of gestation were carefully removed from the uterus under a stereomicroscope and then immersed in the same fixative. Fixation took place for 6 hours. The tissues were embedded in paraffin and sectioned serially at a thickness of $5.5\ \mu\text{m}$. The sections were stained with hematoxylin and eosin.

Quantitative analysis

Karyometry: The nuclear diameters of yolk sac hemopoietic cells at 9 days and circulating erythroblasts in large vessels, mainly the aorta, from 9 to 16 days of gestation were measured. The circulating cells were randomly photographed with an oil-immersion objective and printed at a $1,650\times$ magnification. The nuclear areas in the microphotographs were measured with a computer-coordinating area-curvimeter (Ushikata, X-PLAN 360), and the nuclear diameters then calculated. On each gestational day, at least 500 cells were examined.

Mitotic index: The mitotic figures of blood cells in the large vessels were counted to obtain the mitotic index. More than 1,000 nucleated cells were observed under oil-immersion at each gestational stage.

RESULTS

At 8 days of gestation, angioblastic cords had formed in the connective tissue of the visceral yolk sac. Then the majority of the angioblasts differentiated into hemopoietic cells, while the others formed the endothelium of the capillaries in the yolk sac. Hemopoietic cells became free in the vessels in the yolk sac, and began to migrate from the yolk sac through the vitelline vessel to the embryo at 9 days (Fig. 1a, b). Hemopoietic cells in the yolk sac had a large and pale nucleus with prominent nucleoli. The nuclear diameter distribution of hemopoietic cells located in the yolk sac at 9 days of gestation is presented in Figure 4. The nuclear diameter ranged from 4.4 to 9.2 μm , and hemopoietic cells with nuclear diameters larger than 8 μm constituted 4.0%. The majority ranged from 6 to 8 μm ; erythroblasts measuring smaller than 5 μm nuclear diameter were quite scarce. At 10 days, the vascular plexus on the yolk sac developed and embryonic circulation was established by the migration of hemopoietic cells from the yolk sac. The hemopoietic cells which had left the yolk sac proliferated and differentiated into the final erythroblastic stage in embryonic circulation.

At 9 days of gestation, hemopoietic cells, which could be seen in the large embryonic vessels, possessed a basophilic cytoplasm and a round or ovoid nucleus poor in

Fig. 2. Ten days of gestation. Stained with hematoxylin and eosin. **a.** A cross section of embryo. Numerous circulating blood cells are present in the vessels (arrows). $\times 80$. **b.** Circulating cells. Among the numerous erythroblasts with pachychromatic nuclei, a hemopoietic cell with pale and large nucleus is seen (arrow). $\times 830$

Fig. 3. Circulating erythroblasts at 12 days of gestation. Stained with hematoxylin and eosin. The majority of erythroid cells have a dark and small nucleus. $\times 830$

Fig. 4. Nuclear size distribution of hemopoietic cells in the yolk sac at 9 days.

shown in Figure 5. The nuclear diameter ranged from 4.8 to 9.8 μm , and the majority ranged between 6 and 8 μm . Approximately 7% were larger than 8.0 μm in nuclear diameter, and 1% smaller than 5.0 μm . The mean nuclear diameter was $6.8 \pm 0.8 \mu\text{m}$ at 9 days.

At 10 days, polychromatic and orthochromatic erythroblasts which had an eosinophilic cytoplasm increased in number in the embryonic circulation (Fig. 2a, b). Their nuclei were small and dark, and the chromatin was so condensed that the nucleoli were less distinguishable. The mitotic index of the circulating erythroblasts at 10 days was 4.7%. The nuclear diameter ranged from 3.8 to 8.2 μm (Fig. 5). Cells having a nucleus larger than 8.2 μm disappeared from embryonic circulation, the majority ranging between 4 and 7 μm . Of these, 33% were erythroblasts having a nucleus smaller than 5 μm .

At 11 days, polychromatic and orthochromatic erythroblasts accounted for the vast majority of the blood cells, and hemopoietic cells with a pale nucleus were close to negligible. The mitotic index was 2.6% and the nuclear diameter ranged from 2.4 to 5.8 μm . Hemopoietic cells with a nucleus larger than 6 μm in diameter disappeared from circulation (Fig. 5). Erythroblasts displaying a 3–5 μm nuclear diameter constituted approximately 90% of the circulating erythroblasts.

From 12 to 18 days of gestation, most of the circulating erythroblasts were orthochromatic erythroblasts with a small and pachychromatic nucleus (Fig. 3). At 12 days, enucleated erythroid cells began appearing in small numbers in the circulation, and after 14 days they showed a marked increase in number. The mitotic index of circulating erythroblasts was 1.6% at 12 days of gestation and 0% at 13 days. After 13 days, no mitotic figures could be seen in the peripheral blood. At 12 days, the nuclear diameters ranged from 2.4 to 6.0 μm with more than 80% having nuclei smaller than 4 μm (Fig. 5). In the erythroblasts ranging between 3 and 4 μm in nuclear diameter, two peaks could be observed at 3.4 and 3.9 μm . Between 12 and 16 days of gestation, a gradual decrease in erythroblasts which had a nucleus larger than 4 μm was observed, and cells with a nuclear diameter of 3.4 μm showed the highest peak. At 14 days, cells measuring larger than 5 μm nuclear diameter disappeared from circulation and cells with a nuclear diameter of 3.8 μm decreased in frequency. At 16 days the nuclear diameter ranged between 2.2 and 4.0 μm with a single high peak at 3.4 μm . Between 13 and 16 days, the mean nuclear diameters were 3.3–3.4 μm . At 18 days there still remained a small number of erythroblasts with strongly pyknotic nuclei in the peripheral blood.

heterochromatin (Fig. 1c). Large round or somewhat elongated nucleoli were frequently contained in contact with the nuclear membrane, and, from their nuclear morphology, the circulating cells in the 9-day embryos consisted mainly of proerythroblasts, less mature cells, and a few basophilic erythroblasts. Neither polychromatics nor orthochromatics were observed. At 9 days, mitotic figures were often seen in the circulating blood cells within the vessels, and the mitotic index was 3.2%. The nuclear size distribution of circulating hemopoietic cells at 9 days of gestation is

Fig. 5. Nuclear size distribution of circulating cells from 9 to 16 days of gestation.

DISCUSSION

As in all mammals, mouse fetal hemopoiesis develops in three stages: the yolk sac, hepatic and myeloid; the hemopoietic stem cells of yolk sac origin are known to be concerned with the migratory phenomenon of the embryonic hemopoiesis (review: METCALF and MOORE, 1971). Hemopoietic stem cells produced in the mouse yolk sac

were detected in the circulation at 9–10 days of gestation (MOORE and JOHNSON, 1976). Therefore, they are included in the cells of yolk sac hemopoietic foci and circulating cells at 9 days in embryonic vessels. Electron microscope studies have revealed that, at 9 days, the majority of hemopoietic cells in the yolk sac can be classified as proerythroblasts, and the yolk sac contains a small number of large cells resembling angioblasts (SASAKI and KENDALL, 1985). The 9-day nuclear size distribution of yolk sac hemopoietic cells and circulating cells showed that, in addition to cells with a nuclear diameter of 6 to 8 μm , there was a small population of hemopoietic cells with a nuclear size larger than 8 μm . At the beginning of embryonic hepatic erythropoiesis, the most immature hemopoietic cells in liver cords had the nuclear and nucleolar characteristics of angioblasts (SASAKI and MATSUMURA, 1986). The hemopoietic stem cells which migrated from yolk sac and initiated the hepatic hemopoiesis might be included among cells with nuclear diameters larger than 8 μm at 9 days of gestation. Such large cells disappeared from embryonic circulation at 10 days.

Marked changes in the cellular composition of circulating erythroids occurred between 9 and 11 days of gestation. The cell population of immature erythroids with a nuclear diameter between 6 and 8 μm formed the majority of the 9-day circulating cells, but at 11 days, when the liver hemopoiesis started, these large erythroid cells disappeared from circulation. After 12 days, capillaries in the yolk sac contained mature types of erythroblasts (SASAKI and MATSUMURA, 1986), and the cellularity was the same in yolk sac and embryonic peripheral circulation. Mouse hemopoietic tissue in maturity, and splenic red pulp and bone marrow contained immature erythroid precursor cells displaying a larger than 7 μm nuclear diameter throughout their life, although their frequencies were very low. The immature cells maintained the splenic and marrow erythropoietic activity (SASAKI, MATSUMURA and ITO, 1982). The disappearance of the erythroid precursors results in the depletion of mitotic figures of circulating erythroids to then decrease the primitive erythropoietic activity during late embryonic life. As seen in the results of the mitotic index, the proliferation of the erythroids appeared most active at the 9- and 10-day stages. Then erythroblasts which had lost the ability to divide increased in number. At 13 days, the mitotic index became 0%, and the circulating erythroids matured to erythroblasts which were incapable of division. Between 13 and 16 days, erythroid cells measuring a larger than 4 μm nuclear diameter differentiated into the smallest cell population, which had nuclear diameters of 3.4 μm . Their nucleus reduced in size not by nuclear division but rather by karyopyknosis.

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