THE ANATOMICAL RECORD 251:290–296 (1998)

Effects of Colchicine on the Enucleation of Erythroid Cells and Macrophages in the Liver of Mouse Embryos: Ultrastructural and Three-Dimensional Studies

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ABSTRACT

Enucleation is the last event in the development of a definitive erythroid line, and extruded nuclei are phagocytosed by macrophages. Both colchicine and cytochalasin have been known to exert a great influence on the enucleation process, but the relationship between enucleation and these agents has not yet been clearly revealed in vivo. Our aim was to clarify the significance of the enucleation in liver erythropoiesis and macrophage phagocytosis by colchicine and cytochalasin administration to embryonic mice.

Pregnant mice were intraperitoneally injected with colchicine or cytochalasin at 13 days of gestation. Embryonic livers were removed at intervals of 3, 6 and 12 h after injection for processing for light and electron microscopy, and, to obtain three-dimensional morphology of erythroids at enucleation, computer-aided reconstructions were performed by light microscopy.

Colchicine injections had cytolytic effects on hepatocytes and macrophages, and numerous erythroblasts were observed in the process of enucleation after colchicine injection. However, the extruding nuclei were irregularly shaped, and some erythroblasts at mitosis showed extreme peripheralization of their chromosomal masses and cell membrane constriction. Enucleation behavior could also be observed in immature erythroblasts. Liver macrophages engulfed extruded nuclei and erythroblasts in mitosis. Cytochalasin injections, on the other hand, had no significant effect on embryonic livers. The progress of erythroblast mitosis was clearly stopped by colchicine injection, and numerous erythroblasts at mitosis were extruding their nuclear compartment. Following colchicine injection, erythroid enucleation also took place in immature erythroblasts, and mitotic erythroids were phagocytosed. In enucleation, more attention should be paid to hematopoietic environmental factors than to hemopoietic cell factors. Anat. Rec. 251:290–296, 1998.

Key words: **colchicine; enucleation; erythroblast; liver; macrophage; mouse**

During mammalian ontogeny, the first erythroid cells are found in the yolk sac, and the end cells of yolk sac erythropoiesis, primitive erythrocytes, possess a nucleus. Later in fetal liver and bone marrow erythropoiesis, cells of the final erythroblast stage undergo nuclear expulsion, and the end production is nonnucleated. Enucleation is a unique behavior during definitive type erythroid maturation (Dessypris, 1993), and the extruded nuclei are immediately taken up by macrophages. Colchicine, a microtubule-disrupting substance, and cytochalasin, an inhibitor of actin polymerization, have been known to exert a great influence on the enucleation process (Skutelsky and Danon,

Grant sponsor: Kawasaki Medical School; Grant number: 9–805; Grant sponsor: Japanese Ministry of Education, Science, Sports and Culture; Grant number: 10770013.

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Received 21 October 1997; Accepted 11 February 1998

1970; Chasis et al., 1989; Koury et al., 1989). However, little information is available regarding the morphological aspects of the effect of both colchicine and cytochalasin on fetal erythropoiesis and macrophage phagocytosis in vivo. The fetal liver is the first intraembryonic hemopoietic organ where enucleation in definitive erythropoiesis occurs. The present investigation was therefore undertaken to examine ultrastructural and three-dimensional changes in erythroid enucleation after colchicine and cytochalasin administration, with special reference to the functional significance of the nucleus in the end stage of erythroid maturation.

MATERIALS AND METHODS

A total of 60 ICR mouse embryos were used in this study. ICR mice were purchased from Japan Clea Laboratory (Tokyo, Japan). Adult female mice were mated overnight with males, and the next morning was taken as day 0 of gestation. At 13 days of gestation, the mice were divided into the following three groups: colchicine-injected, cytochalasin-injected and control.

Colchicine-Injected Group

Pregnant mice were intraperitoneally injected with 50 µg colchicine (Wako Pure Chemical Industries, LTD., Osaka, Japan) in 1 ml of phosphate buffered saline (PBS). Mice were sacrificed with excessive chloroform at intervals of 3, 6 and 12 h after injection, and 15 embryos were evaluated.

Cytochalasin-Injected Group

Pregnant mice were intraperitoneally given cytochalasin B or D (Wako) in 1% dimethylsulphoxide. Two mice received a single injection of cytochalasin B, 50 µg/mouse, and five mice cytochalasin D, 100 µg/mouse. Mice were sacrificed at 3 and 6 h after the injection, and 20 embryos were evaluated. Within 3 h after cytochalasin D injection, three of five pregnant mothers died.

Control Group

This group consisted of ten embryos from an untreated pregnant mother and 15 embryos from three pregnant mothers given 1 ml of PBS without colchicine.

Electron Microscopy

Embryos were quickly removed, and embryonic livers were excised under a dissecting stereomicroscope and then immediately immersed in 4% paraformaldehyde with 5% glutaraldehyde in 0.1 M cacodylate buffer (Karnovsky's fluid). After 15 min, the livers were cut into small blocks (1 mm × 1 mm) and immersed in the same fluid for 3 h. The tissues were postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 h. Afterwards, they were washed in the buffer, dehydrated in graded ethanols and embedded in Epon 812. Ultrathin sections, 90 nm thick, were cut with a diamond knife on a Leica Ultracut S (Leica, AG., Wien, Austria) and were mounted on formvar film-coated 100 mesh copper grids. After double staining in uranyl acetate and lead citrate, observations were carried out on a JEM-2000 EX II operating at 80 kV.

Three-Dimensional Reconstruction

To obtain three-dimensional morphology of erythroid nuclei and cell profiles at enucleation, we performed computer-aided reconstructions on images from semithin serial Epon sections by light microscopy. Semithin sections, 0.3–0.5 µm thick, were cut and stained with toluidine blue. Serial sections were selected and televised onto a monitor at a magnification of ×5,000. Cellular and nuclear outlines of erythroblasts traced on plastic sheets were transposed with a conventional image scanner into the software Voxel View (Vital Image, Inc., Fairfield, USA) installed on a Power Macintosh 9500/132 computer. The processing resulted in stacks of 13–21 images containing the transferred object shape, and rotational operations in 3-D yielded the optimal orientation of the image stacks visualized on a monitor or plotted on paper. Nine series of serial sections were finally superimposed to obtain accurate cell profiles.

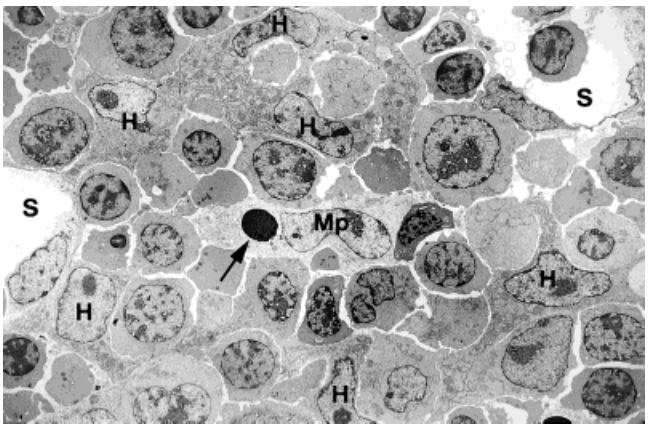

Fig. 1. Normal embryonic liver at 13 days of gestation. Numerous immature erythroid cells are scattered among hepatocytes (H), and a macrophage (Mp) contains a large phagosome (arrow) derived from the extruded nucleus of an erythroblast. S, sinusoids. ×1,100.

RESULTS

Cellularity in Normal 13-Day Embryonic Livers and Enucleation of Hepatic Erythroblasts

At 13 days of gestation, embryonic livers consisted of immature hepatic parenchymal cords interspersed with extended sinusoids. The sinusoids were separated by thin-walled endothelium from the hepatic cell cords, and the lumina contained not only circulating primitive erythrocytes derived from the yolk sac but also nonnucleated erythrocytes produced in situ. Besides erythroids, megakaryocytes were diffusely scattered among hepatocytes, but other hemopoietic cell lines (i.e., granuloids and lymphocytes) were seldom observed. Numerous macrophages were found in sinusoids. The macrophages showed active phagocytosis, and the cytoplasm was occupied by phagosomes filled with aging primitive erythrocytes and nuclei extruded from erythroblasts of definitive erythropoiesis. The hepatic cords, on the other hand, consisted of hepatocytes and hemopoietic cells. Definitive erythroid proliferation occurred within hepatic cords, erythroblastic islands appeared in small numbers, and central macrophages of the island began to form cell sockets on their cell surface for developing erythroblasts (Fig. 1).

Maturation and the differentiation process of erythroids in the hepatic cords. Definitive erythropoiesis, in which the end cells were nonnucleated erythro-

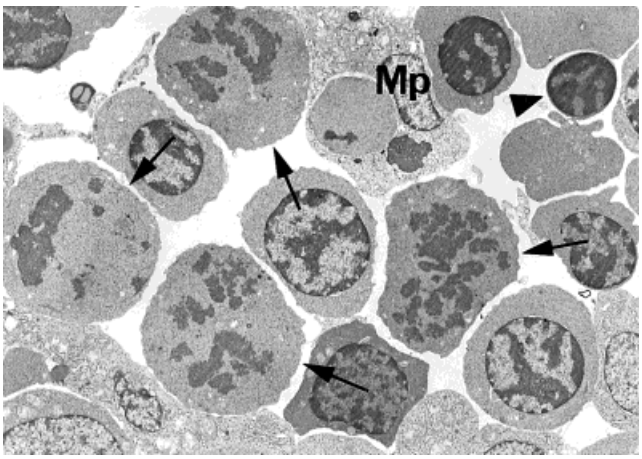

Fig. 2. Erythroblasts in the hepatic cell cord at 13 days of gestation. Four mitotic figures of erythroids (arrows) are observed among erythroblasts with light nuclei. The arrowhead indicates an erythroid nucleus just expelled. Mp, macrophage. ×2,800.

cytes, proceeded within the hepatic cords. As erythroids, hepatic cords contained proerythroblasts larger than 8 μm in diameter, polychromatophilic erythroblasts having a spherical nucleus and a slight darkly stained cytoplasm, orthochromatic erythroblasts having a small nucleus with extremely condensed heterochromatin and an electron-dense cytoplasm due to a high concentration of hemoglobin, reticulocytes which had just expelled their nuclei, and mature nonnucleated erythrocytes. In the liver at gestational day 13, the majority of erythroblasts were in the basophilic or polychromatophilic stage, and numerous mitotic figures of erythroids were also observed (Fig. 2).

Orthochromatic erythroblasts on the sections were round or oval, approximately 4–6 μm on their long axis, and possessed a small spherical nucleus 3–4 μm in diameter. The condensed heterochromatin formed thick strands, and the nucleoli were inconspicuous. The orthochromatic erythroblasts expelled their nuclei through the following four steps; 1) extreme peripheralization of the condensed nucleus, 2) beginning of nuclear deformation in the cell periphery, 3) constriction in the cell membrane as well as the nucleus and 4) complete separation of cytoplasmic and nuclear compartments. The nuclear component was completely surrounded by a thin rim of cytoplasm and plasma membrane, and the detached nuclei were engulfed and removed by macrophages in sinusoids and hepatic cords (Fig. 3).

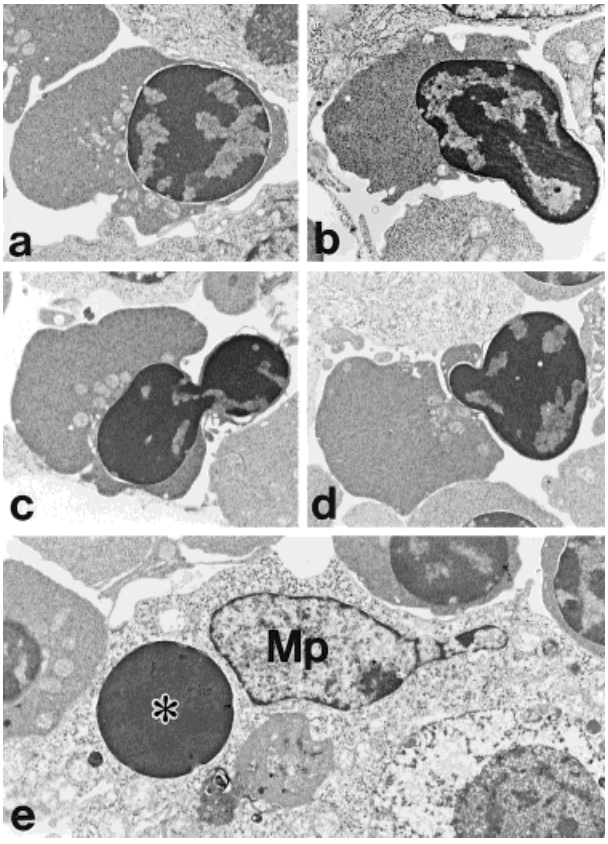

Fig. 3. Nuclear expulsion process of erythroblasts in control embryonic liver. ×4,500. **a:** First step. Extreme peripheralization of the condensed nucleus. **b:** Second step. Beginning of nuclear deformation. **c:** Third step. Constriction in the cell membrane and the nucleus. **d:** Fourth step. Separation of cytoplasmic and nuclear elements. **e:** A macrophage (Mp) containing the extruded nucleus (*).

Embryonic Livers After Colchicine Injection

Under a dissecting microscope, the embryonic livers of both normal and control groups were vividly reddish in color, and serous membrane covered the soft and pliable parenchyma. The hepatocytes generally had a pale and large nucleus with well-marked nucleoli on the inner nuclear membrane, and the cytoplasm contained large mitochondria, spherical or rod-shaped, abundant rough endoplasmic reticulum, and several spherical lipid drops with a diameter of 3–4 μm (Fig. 4a). At 3 h after colchicine injection, the liver became deep dark red in color, and the parenchymal consistency became markedly looser. Colchicine injections into pregnant mothers caused massive necrotic changes in embryonic livers identified light- and electron-microscopically by cytolytic effects mainly on both hepatocytes and macrophages. At 3 h after the injection, hepatocytes became expanded, and both the mitochondria and the rough endoplasmic reticulum became remarkably swollen, after which the cell membrane partly dissolved. At 6 h after the injection, together with the cytoplasmic changes, their nuclei also showed irregular condensation of heterochromatin. Rupture of the nuclear envelope occurred (Fig. 4b). Due to membrane dissolution, the cytoplasmic and nuclear contents were discharged (Fig. 4c). In the case of macrophages, swelling of cytoplasmic organelles occurred later, at 6 h after the injection, and in particular the lysosomes expanded and collapsed. At 12 h after the injection, the majority of macrophages contained large-sized phagosomes, showing progressive dissolution of the whole cell structure (Fig. 4d). At 3 h after the injection, megakaryocytes also showed swelling of cytoplasmic organelles, and then their cell membrane partly dissolved. At 6 h after the injection, their cell membrane completely dissolved. In erythroids, on the other hand, acute destructive changes were inconspicuous. After the injection, large immature erythroblasts, such as proerythroblasts, decreased in number, but small erythroblasts, such as polychromatophilic and orthochromatic erythroblasts, remained almost intact even at 12 h after injection, and several remarkable changes could be observed in the morphology of the erythroblast nuclei.

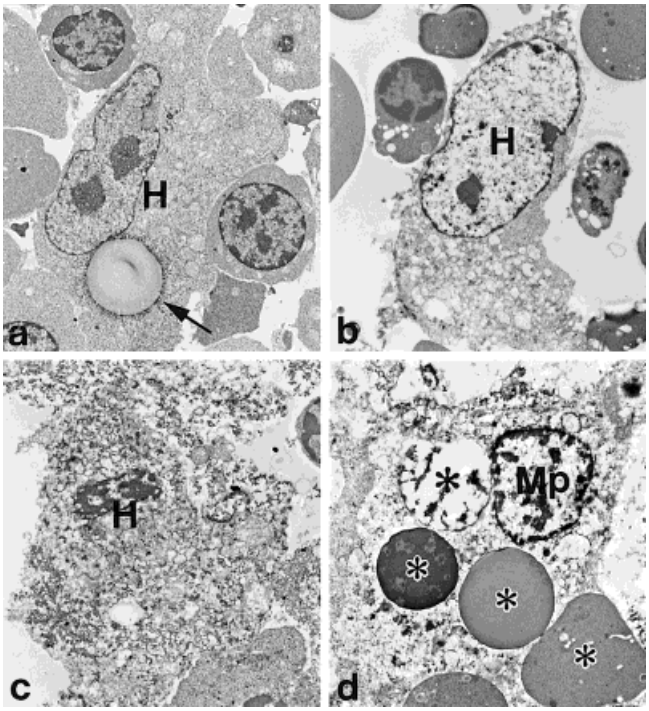

Fig. 4. Hepatocytes and macrophages after colchicine injection. ×2,700. **a:** An intact hepatocyte (H) of the control liver. A light nucleus has two prominent nucleoli, and the cytoplasm is filled with abundant rough endoplasmic reticulum. The arrow shows a spherical lipid drop. **b:** A hepatocyte (H) at 6 h after injection. Both the nucleus and cytoplasmic organelles have expanded. **c:** A degenerating hepatocyte (H) at 12 h after injection. Due to rupture of the plasma membrane, cytoplasmic content is discharged. **d:** A macrophage (Mp) at 12 h after injection. The destroyed cytoplasm contains several large phagosomes (∗).

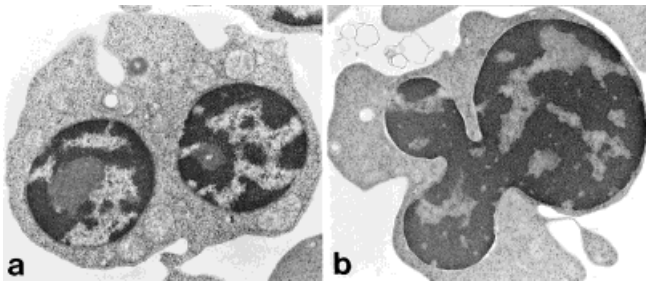

Fig. 5. Erythroblasts in liver at 3 h after colchicine injection. ×6,200. **a:** A binucleated erythroblast. **b:** An erythroblast with a lobulated nucleus.

Nuclear morphology of the erythroblasts in the colchicine-injected liver. The hepatic cords contained not only numerous erythroblasts in the metaphase of mitosis but also erythroblasts with irregularly shaped nuclei. In sections, small erythroblasts often appeared either binucleate (Fig. 5) or multinucleate. A computerized three-dimensional reconstruction of the erythroblasts having irregularly shaped nuclei showed that they had lobulated nuclei with condensed chromatin. The lobulated nuclei usually had two lobules with dumbbell-shaped forms, and three or four lobulated nuclei were rarely observed. In addition to lobulated nuclei, a few erythroblasts possessed two or three separate nuclei with a diameter of 3–4 μm.

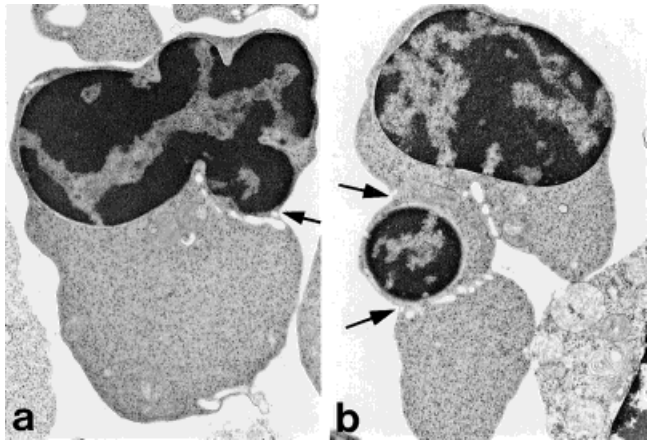

Fig. 6. Erythroblast nuclear expulsion after colchicine injection. ×6,000. **a:** An erythroblast with a four-lobulated nucleus showing extreme peripheralization and constriction in the cell membrane (arrow). Three hours after injection. **b:** An erythroblast expulsing two distinct nuclei separately. The erythroblast is dividing into three different cell compartments. The arrows show cell membrane constriction. Six hours after injection.

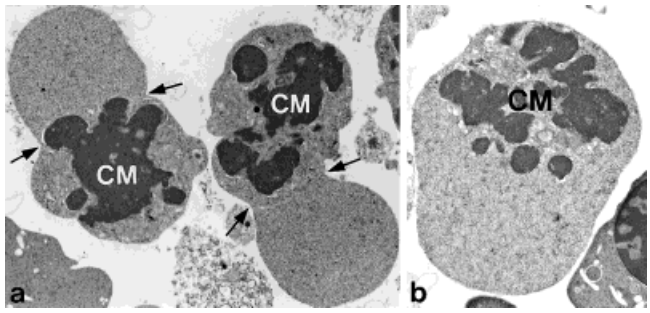

Fig. 7. Chromosomal mass expulsion in erythroblasts at 6 h after colchicine injection. ×3,200. **a:** Two erythroblasts in mitosis show extreme peripheralization of chromosomal mass (CM) and distinct constriction in the cell membrane (arrows). **b:** An immature erythroblast with electron-lucent cytoplasm appears to be expulsing chromosomal mass (CM).

Enucleation of Erythroblasts and Phagocytosis of Macrophages after Colchicine Injection

Numerous erythroblasts in the process of enucleation were observed in colchicine-injected livers. The extruding nuclei were lobulated or dumbbell-shaped (Fig. 6), and, from 6–12 h after the injection, the chromosomal mass in the metaphase of mitosis often showed extreme peripheralization in the cytoplasm under electron microscopy (Fig. 7a). Computerized three-dimensional reconstructions showed that the chromosomal mass appeared to be extruded at the metaphase of mitosis, and the visualized images corresponded to the first to third steps in the normal enucleation process (Fig. 8). The extruding chromosomal mass could also be observed in immature erythroblasts having a more electron-lucent cytoplasm than orthochromatic erythroblasts (Fig. 7b).

Macrophages in the colchicine-injected livers contained variously sized phagosomes, and six to ten extruded nuclei

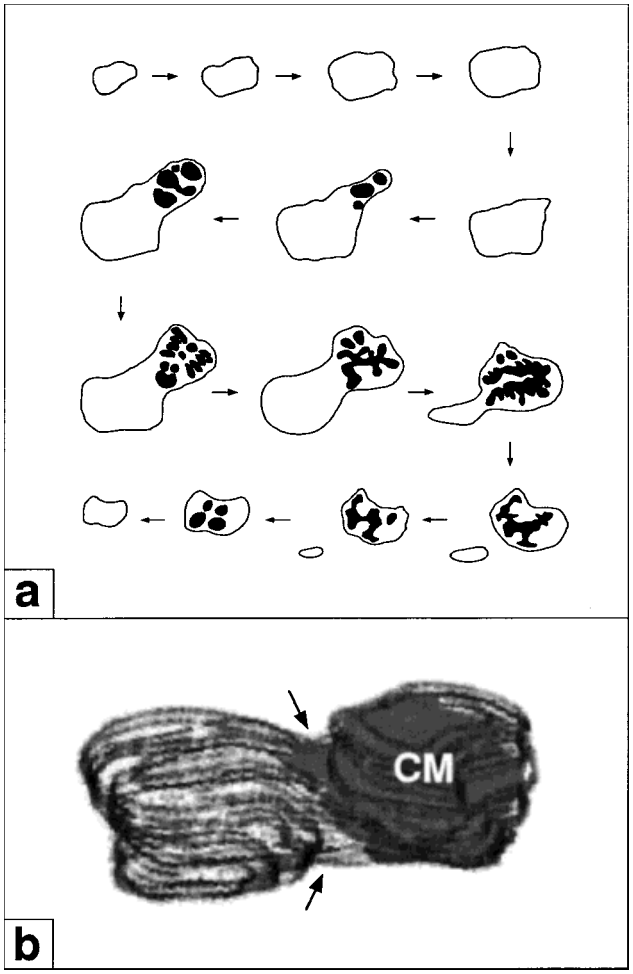

Fig. 8. A computerized three-dimensional reconstruction of an erythroblast expulsing a mitotic nucleus. **a:** Fourteen serial sections, 0.5 μm in thickness, from an erythroblast at 6 h after colchicine injection. Black areas show chromosomal profiles. ×1,600. **b:** A visualized image from the serial sections. Cell membrane constriction (arrows) can clearly be recognized in the reconstructed image, and the erythroblast is divided into two different elements: a cytoplasmic element and a chromosomal mass element (CM). ×4,800.

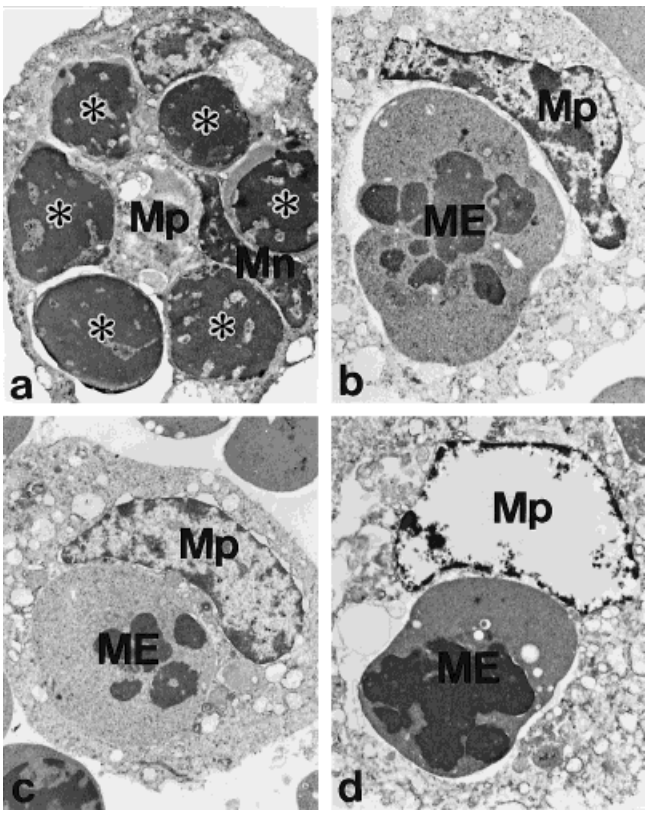

Fig. 9. Liver macrophages at 6 h after colchicine injection. ×3,300. **a:** A macrophage (Mp) phagocytosing six extruded nuclei (*). Mn, macrophage nucleus. **b:** A macrophage (Mp) phagocytosing a mitotic erythroblast (ME). **c:** A mitotic erythroblast (ME) with electron-lucent cytoplasm is also engulfed by a macrophage (Mp). **d:** A macrophage (Mp) containing a mitotic erythroblast (ME) showing peripheralization of the chromosomal mass.

were occasionally observed in a single macrophage (Fig. 9a). Besides extruded nuclei, macrophages often contained erythroblasts in mitosis and with lobulated nuclei (Fig. 9b). Large erythroblasts with an electron-lucent cytoplasm were also phagocytosed at the metaphase of mitotic division (Fig. 9c). Macrophages also engulfed mitotic erythroblasts whose chromosomal mass showed extreme peripheralization like the first step of the normal nuclear expulsion process (Fig. 9d). Central macrophages in erythroblastic islands also became remarkably swollen, and erythroblasts were detached from the cell socket of the macrophages. The island structure became obscure after colchicine injection.

Embryonic Livers after Cytochalasin Injection

At 3–6 h after cytochalasin injection, the embryonic livers were vividly reddish in color, the same as in the control group. The hepatocytes appeared to be fully intact, and the hepatic cords contained numerous immature erythroblasts, orthochromatic erythroblasts, and extruded nuclei. However, extruded nuclei didn't appear to be increased in number, and neither enucleation during mitosis nor extrusion of lobulated nuclei could be observed.

DISCUSSION

In normal bone marrow erythropoiesis, erythroblasts undergo four cell divisions during the maturation pathway from the proerythroblast stage to the orthochromatic stage (Staring and Rosse, 1976). Orthochromatic erythroblasts extrude their nuclei to become young reticulocytes, so enucleation gives rise to two different erythroid elements: one the nuclear element surrounded by a narrow rim of cytoplasm and the other the cytoplasmic element accumulating hemoglobin (Skutelsky and Danon, 1967; Dessypris, 1993). Since the plasma membrane surrounds two erythroid elements, enucleation can be included in the category of cell division rather than exocytosis, which can be seen in gland secretory cells. In this regard, nuclear expulsion is considered to be the last cell division without nuclear division during the final maturation stages of definitive erythropoiesis. There has been conflicting evidence concerning the influence of colchicine on erythroid enucleation. Colchicine injection into rats has resulted not

only in a decrease in the number of freely extruded nuclei but also in an increase in the number of orthochromatic erythroblasts at the stage of nuclear extrusion (Chasis et al., 1989). Therefore, it was concluded that colchicine inhibited erythroid enucleation in vivo. Since colchicine has been known to be a potent inhibitor of cell division through its effects on spindle microtubule assembly (Sluder, 1991), microtubules might functionally be essential to enucleation. On the other hand, Koury et al. (1989) showed that cytochalasin D caused a complete inhibition of enucleation and that F-actin played an important role in the enucleation of Friend virus–infected mouse spleen erythroid cells, but colchicine had no influence on the enucleation in vitro. Based on the increased number of erythroblasts in mitosis after colchicine administration, our results showed that progress of erythroblast mitosis was clearly stopped only by colchicine injection. However, numerous erythroblasts at the first and second steps in the nuclear expulsion process were also observed, and, in addition, some chromosomal masses at the metaphase of mitosis were detaching from the cytoplasmic compartment just as in ordinary enucleation. Therefore, colchicine had little effect on the initiation of enucleation in vivo, although the chromosome movement in erythroid mitosis is clearly inhibited in mice given colchicine.

Of great interest, as shown in the results, is confirmation that enucleation does not always take place at the end of erythroid maturation. After colchicine injection, compared with hepatocytes and macrophages, erythroid cells during the late maturation stages remained nearly intact in embryonic livers. In normal definitive erythropoiesis, the nuclei of erythroblasts become condensed with the progress of maturation, and enucleation usually occurs only at the orthochromatic stage (Dessypris, 1993). However, in colchicine-injected livers, 3-D reconstructive images showed that some erythroblasts at the metaphase of mitosis were about to extrude their chromosomal masses, and enucleation also could be recognized in immature erythroblasts with an electron-lucent cytoplasm. Taken together, the present observations suggested that possibly neither the progress of erythroid maturation nor chromatin condensation is associated with enucleation in definitive erythropoiesis. In general, pyknosis and nuclear shrinkage have been reported to be early morphological signs of programmed cell death (Harmon and Allan, 1996). In yolk sac primitive erythrocytes, the nuclei showed massive chromatin concentration and were partly dissolved. These primitive erythrocytes kept their useless nuclei in their cytoplasm and circulated in embryonic blood soon to be removed by liver macrophages (Sasaki et al., 1997). In definitive erythropoiesis in embryonic liver, the nuclei extruded from orthochromatic erythroblasts were still larger than those of aging primitive erythrocytes, having irregular thick strands of heterochromatin (Sasaki et al., 1997), and definitive erythrocytes which pushed their nuclear elements wholly out had a much longer life span in blood circulation, 40–43 days in mice, than primitive erythrocytes (Van Putten, 1958). The possibility that the useless nuclei may have unfavorable effects on erythrocyte life span cannot be ruled out. In cell cultures in which enucleation did not occur, enucleations of definitive erythroblasts could be induced by adding stromal cells to the culture medium (Yanai et al., 1989). Therefore, in erythroid enucleation, more attention should be paid to hematopoietic environmental factors than to hemopoietic cells themselves.

At the beginning of liver hemopoiesis, liver sinusoid macrophages actively phagocytosed primitive erythrocytes, nuclei extruded from the erythroblasts of definitive erythropoiesis, and the cell fragments derived from primitive erythrocytes (Sasaki, 1990; Sonoda et al., 1996). With the development of liver hemopoiesis, scavenger macrophages migrated from sinusoids into the hepatic cords to become central macrophages of erythroblastic islands, and erythroblasts tended to gather around the macrophages (Sasaki et al., 1993; Iwatsuki et al., 1997). As mentioned in the results, colchicine injections have a cytolytic effect on macrophages, but the phagocytosis of macrophages was not inhibited early after colchicine injections. In normal fetal liver, macrophages phagocytosed aged primitive erythrocytes and nuclei extruded from the erythroblasts of definitive erythropoiesis but did not take up any reticulocytes which had just expelled their nuclei (Sasaki et al., 1993). As shown in our results, at 6 h after colchicine injection, the phagocytotic targets of macrophages appear to be considerably different from the normal environment. Macrophage cytoplasms often contained erythroblasts in mitosis or erythroblasts with lobulated nuclei, and the nuclei showed extreme peripheralization. Iwatsuki et al. (1995) reported that the phagocytotic selectivity of macrophages to erythroid elements was related to desialization on the cell surface. Since a decrease in sialic acid on the surface of circulating erythrocytes has been known to be related to the removal of erythrocytes in the reticuloendothelial system (Aminoff, 1988), sialic acids on the surface of erythroids appear to be important not only for phagocytosis of macrophages but also for erythrocyte life span. It is possible that colchicine administration caused changes in sialic acid on the surface of erythroids in addition to the morphological abnormality of erythroblast nuclei. Using extruded erythroblast nuclei and macrophages isolated from adult mouse bone marrow in vitro, Qiu et al. (1995) reported that the protein receptor on the surface of macrophages was important not only for phagocytosis but also for recognition of the ligand on the surface of the extruded nucleus. Phagocytotic changes after colchicine administration should be further examined with respect to recognition mechanisms as well as disruption of microtubules in macrophages.

As shown in our results, colchicine had marked influences on erythroid enucleation. Cytochalasin, on the other hand, had no obvious effect on the inhibition of erythroid enucleation in embryonic livers. In this study, histological examinations on embryonic livers were made by intraperitoneal injections into pregnant mothers. Therefore, to examine cytochalasin influence on the erythropoiesis of embryonic liver, direct injections into embryos need to be considered.

ACKNOWLEDGMENTS

The authors thank Mr. K. Uehira and Mr. T. Suda for their skillful technical assistance. This work was supported in part by a research project grant from Kawasaki Medical School (9–805, 1997) and a Grant-in-Aid for Scientific Research from the Japanese Ministry of Education, Science, Sports and Culture (No. 10770013, 1998).

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