FULL PAPER *Anatomy*

No Apoptotic Cell Death of Erythroid Cells of Erythroblastic Islands in Bone Marrow of Healthy Rats

Toshifumi YOKOYAMA[1)], Hiroshi KITAGAWA[1)]*, Takashi TAKEUCHI[2)], Shinji TSUKAHARA[1)] and Yasuyuki KANNAN[1)]

[1)]Department of Life Science, Graduate School of Science and Technology, Kobe University, Kobe 657–8501 and [2)]Institute of Experimental Animals, Shimane Medical University, Shimane 693–8501, Japan

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ABSTRACT. A possibility of apoptotic cell death in erythropoietic regulation was examined by means of detailed light microscopical histoplanimetry, electron microscopy, the *in situ* nick-end labeling method, and an immunohistological method in the rat bone marrow. Serum erythropoietin concentrations were shown at normal levels. The erythroid series on a mature process presented several morphological features of apoptosis, i.e. the shrinkage of both nuclei and cytoplasm and the chromatin condensation. In the light microscopical histoplanimetry, however, morphological signs of final apoptotic cell death were never found in any erythroid cell within the erythroblastic islands. This finding was also supported by detailed ultrastructural observation: No erythroid cell bodies were trapped and degraded by the central macrophages of the erythroblastic islands, while the denucleated nuclei with small amount of cytoplasm of late erythroblasts were often trapped and degraded in the macrophages. Nuclear DNA fragmentation was not detected in any erythroblasts, but was detected in the lysosomes of the central macrophages. These findings suggest that erythropoiesis is regulated by other regulatory mechanisms than apoptotic cell death. An additional ultrastructural finding shows that the reticulocytes anchored to the central macrophages are transported into the peripheral blood circulation.

KEY WORDS: apoptosis, denucleation, erythroblast, erythropoiesis.

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In erythropoiesis, a hematopoietic pluripotent stem cell differentiates to burst-forming unit-erythroid and subsequently to colony-forming unit-erythroid (CFU-E). CFU-E further divides and matures into acidophilic erythroblasts through proerythroblasts, basophilic erythroblasts, and polychromatophilic erythroblasts in this order. Acidophilic erythroblasts extrude their nuclei, and the denucleated reticulocytes then enter the blood circulation alone. It is still unknown whether reticulocytes positively transverse the sinus epithelium or whether the cells are passively driven across the epithelium wall by pressure between the sinusoids and the parenchyma as mentioned by Bull and Breton-Gorius [4].

Erythropoiesis is modulated by many cytokines, stem cell factor, interleukin-3, granulocyte-macrophage colony-stimulating factor, erythropoietin (EPO), etc. Especially, EPO influences and supports the differentiation and maturation of the progenitors of erythroid cells [31]. The serum EPO produced mainly by the kidney is physiologically regulated by oxygen concentration in the peripheral blood circulation [20, 21]. CFU-E is more sensitive to EPO than erythroblastic series in erythropoiesis [10]. EPO is clinically applied to some anemia patients and it is widely know that it effectively improves them [15]. However, the regulatory mechanisms of erythropoiesis by EPO still have not been completely elucidated.

EPO dose not only effect the differentiation and proliferation of erythroid cells directly, but also participates in their survival. *In vitro*, many erythroid cells incubated with a physiological concentration of EPO express DNA cleavage and finally die, but this apoptotic cell death is avoidable with the application of a high concentration of EPO [1]. This evidence leads to a hypothesis that many erythroid cells die *in vivo* with the physiological concentration of serum EPO, although erythroid cells are protected from apoptotic cell death with sufficient serum EPO to produce more erythrocytes, so that the acute anemia is overcome [28]. This hypothesis, however, has not been investigated *in vivo*.

In the present study, we examined the possibility of apoptotic cell death in erythroid cells of the bone marrow in rats with normal serum EPO concentration by means of detailed light microscopical histoplanimetry and electron microscopy. Based on these results, the involvement of apoptosis in erythropoiesis was discussed. Additionally, we also examined the migration mechanism of mature erythrocytes from the parenchyma into the sinusoids.

MATERIALS AND METHODS

Animals: Ten male Wistar rats aged 6 weeks (SLC, Japan) were used according to the guidelines for the care and use of experimental animals, in Rokkodai Campus, Kobe University. They were permitted free access to food and water. The animal facility was maintained under conditions of a 12 hr light/dark cycle at 21 ± 1°C and 50–60% humidity. No sign of hematological disorder was confirmed by clinical and pathological examinations in all animals. Serum EPO

* CORRESPONDENCE TO: KITAGAWA, H., Division of Relational Biosystems, Department of Life Science, Graduate School of Science and Technology, Kobe University, Kobe 657–8501, Japan.

concentrations were at the normal level (17.5 ± 7.0 mIU/m*l*).

Light and electron microscopy: Immediately after euthanasia with inhalation of ethyl ether, 5 animals were perfused with periodate-lysine-paraformaldehyde (PLP) fixative. Small tissue blocks of the femoral bone marrow and the ileum were extracted and immersion-fixed in the same fixative for 24 hr at 4°C. The blocks were then dehydrated and embedded in paraffin. Paraffin sections of 3 μm-thickness were stained with hematoxylin and eosin. The rest of the blocks were frozen and used for the detection of DNA-fragmentation.

The other 5 animals were perfused by 2.5% glutaraldehyde-2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4, PB). The bone marrows were immersion-fixed in the same fixative for 24 hr at 4°C. After postfixation with 1.0% OsO_4 in 0.1 M PB for 1.5 hr at room temperature, the small specimens were embedded in an epoxy resin (Epon 812 mixture). For light microscopy, semithin sections of 1 μm-thickness were cut using an ultramicrotome (Sorvall MT-1, U.S.A.) and stained with 0.05% toluidine blue in 0.01 M PB (pH 7.4). The ultrathin sections contrasted with both uranyl acetate and lead citrate were observed by using a transmission electron microscope (Hitachi H-7100, Japan) at an accelerating voltage of 75 kV.

Quantitative histology: Histoplanimetry was carried out on the 1 μm-thick epoxy sections as mentioned above under a light microscope. In brief, the appearance frequencies of the erythroblastic islands were counted in an area of 5 mm^2 on the sections of each animal. The means of the frequencies were represented as the number per mm^2. The appearance frequencies of proerythroblasts, basophilic erythroblasts, polychromatophilic erythroblasts, and acidophilic erythroblasts were counted in 3,000 erythroid cells of the erythroblastic islands for each animal, followed by calculation of the average of each erythroblast. The erythroid cells with morphological signs of apoptotic cell death, the phagocytized erythroid cells, and the erythroblasts with small globules protruding from the nuclei were counted in a 5 mm^2 area on the sections of each animal, followed by calculating the average of each animal. The means of the frequencies were represented as the number per mm^2. For each cell type of erythroblast, the longitudinal and transversal diameters were measured on the centrally-sectioned nuclei and the cell bodies of 20 cells. The average diameters of the nuclei and cell bodies were calculated from the means of the longitudinal and transversal diameters. Unpaired Student's *t*-test was employed for detection of statistical significances with the *p* value being less than 0.05.

In situ nick-end labeling method: The bromodeoxyuridin triphosphate (BrdUTP) method was applied to detect the final features of apoptotic erythroid cells according to a modified method described by Li and Darzynkiewicz [23]. Briefly, the blocks were snap frozen in liquid nitrogen with reference to an embedding method described by Barthel and Raymond [2]. Five-μm-thick frozen serial sections were cut using a Coldtome HM505E (Carl Zeiss, Germany) and placed on slide glasses precoated with 0.2% 3-aminopropyltriethoxysilane (Shinetsu, Japan). Four sections were prepared from each rat (Sections 1, 2, 3, and 4). Sections 1 and 2 were used as positive controls for Section 3. Section 4 was used as a negative control. The sections were pretreated with 1.5 μg/m*l* proteinase K solution (Sigma, U.S.A.) for 15 min at 37°C. After treatment with absolute methanol and 0.5% H_2O_2, Sections 1 and 2 were incubated with 1.5 mg/m*l* DNase I solution (Boehringer Mannheim, U.S.A.) for 10 min at 37°C. Following immersion in a terminal deoxynucleotidyl transferase (TdT) buffer (Boehringer Mannheim), Sections 1 and 3 were incubated with 30 μl of TdT reaction buffer (Boehringer Mannheim) containing BrdUTP solution (0.1 nM BrdUTP in 4 μl distilled water; Sigma) for 120 min at 37°C. All sections were then incubated with 1% normal bovine serum, and were treated with anti-BrdUTP mouse monoclonal antibody (diluted at 1:100; Bio-Science Introducts, Japan) for 18 hr at 4°C. Following incubation with anti-mouse IgG rabbit IgG (diluted at 1:50; Jackson, U.S.A.) for 1 hr at room temperature, the sections were treated with mouse peroxidase-anti-peroxidase-complex (PAP) (diluted at 1:50; Seikagaku, Japan). Finally, these sections were incubated with 3,3'-diaminobenzidine (DAB) containing 0.03% H_2O_2, and were counterstained with methyl green.

Immunohistochemistry: Frozen sections were treated with absolute methanol followed by incubations with 0.5% H_2O_2 and with 1% normal bovine serum. Thereafter, the sections were treated with anti-single strand DNA mouse monoclonal antibody (diluted at 1:100; DAKO U.S.A.) for 18 hr at 4°C. Following incubation with anti-mouse IgG rabbit IgG (diluted at 1:50; Jackson) for 1 hr at room temperature, the sections were treated with mouse PAP (diluted at 1:50; Seikagaku). These sections were then incubated with DAB containing 0.03% H_2O_2, and were counterstained with methyl green.

RESULTS

Light microscopical observations on erythroid maturations: On the light microscopic sections of the femoral bone marrow, erythroid cells were clustered in erythroblastic islands. The appearance frequency of cells was 50.4 ± 7.8 per mm^2 of section. An erythroblastic island consisted of a central macrophage and about 10 peripheral erythroid cells (0.06 ± 0.02 proerythroblasts, 1.1 ± 0.4 basophilic erythroblasts, 7.7 ± 0.9 polychromatophilic erythroblasts and 1.1 ± 0.3 acidophilic erythroblasts).

Proerythroblasts had the largest cell bodies and nuclei among all erythroblasts. The diameters of the cell bodies and nuclei significantly decreased during the erythroid maturation (Table 1). The ovoid nuclei of proerythroblasts had fine chromatins arranged radially, and were slightly lighter than those of basophilic erythroblasts. The cytoplasms of proerythroblasts were strongly basophilic, but slightly lighter than those of basophilic erythroblasts. Basophilic erythroblasts were characterized by the most basophilic cytoplasm and had largest cell bodies next to

Table 1. Sizes of erythroblasts in rat femoral bone marrow

Cell type	Cell body		Nucleus	
				μm
Pro-EB	8.7 ± 0.7[a)]	(7.1–10.2)	7.1 ± 0.3	(6.1–7.7)
Baso-EB	6.8 ± 0.5	(6.1–7.1)	5.7 ± 0.5	(5.1–6.1)
Poly-EB	5.6 ± 0.5	(5.1–6.1)	4.2 ± 0.4	(3.6–5.1)
Acid-EB	5.0 ± 0.4	(4.1–5.5)	3.4 ± 0.4	(3.1–4.1)

a) mean ± SD (min - max). Pro-EB, proerythroblast; Baso-EB, basophilic erythroblast; Poly-EB, polychromatophilic erythroblast and Acid-EB, acidophilic erythroblast. Each value differs significantly from those in other cell types (P<0.01).

those of proerythroblasts. In polychromatophilic erythroblasts, the nuclear chromatins were more condensed and the cytoplasms were stained diversely from basophilic to acidophilic. Small globules protruding from the nuclei were found in polychromatophilic erythroblasts or acidophilic erythroblasts (Table 2, Fig. 1). Acidophilic erythroblasts had more condensed nuclei and acidophilic cytoplasm stained relatively weaker than those of matured erythrocytes. The sole nuclei having highly condensed chromatins and no cytoplasm were often found within the cytoplasm of central macrophages (Table 2, Fig. 2). However, no erythroblasts were engulfed by the central macrophages of the erythroblastic islands in this detailed observation (Table 2).

The transference of erythrocytes, reticulocytes, or acidophilic erythroblasts from the parenchyma into the blood stream was occasionally observed in the sinusoidal endothelium (Fig. 3).

Detection of nuclear DNA fragmentation: DNA fragmentations were detected in a few nuclei of single cells scattered over the parenchyma of the bone marrow by using the *in situ* nick-end labeling method. However, these cells were different from erythroid cells in terms of some cytological criteria. DNA fragmentations were also detected in the lysosomes of the central macrophages of the erythroblastic islands, but the fragmentation was not detected in any erythroid nuclei around the central macrophages (Fig. 4a). The same results were further obtained from the immunohistochemistry employing anti-single strand DNA monoclonal antibody. In the experimental controls of the ileum, which were stained simultaneously with the bone marrows, the DNA fragmentations of villous epithelial cells were detected in the apical regions of the intestinal villi by means of the *in situ* nick-end labeling method. The reaction products that increased gradually going upward to the villous tips was confirmed as the optimal sensitivities to the detection of nuclear DNA fragmentation (Fig. 4b). In the positive

Table 2. Appearance frequencies of erythroblasts with morphological characteristics of apoptosis in rat femoral bone marrow

Cell type	Erythroblasts with nuclear globules	Phagocytized nuclei of erythroblasts	Apoptotic cell death of erythroblasts
			Cell number/mm^2
Pro-EB	0	0	0
Baso-EB	0	0	0
Poly-EB	5.1 ± 2.3[a)]	0	0
Acid-EB	2.4 ± 1.3	19.3 ± 5.6	0

a) mean ± SD. Pro-EB, proerythroblast; Baso-EB, basophilic erythroblast; Poly-EB, polychromatophilic erythroblast and Acid-EB, acidophilic erythroblast.

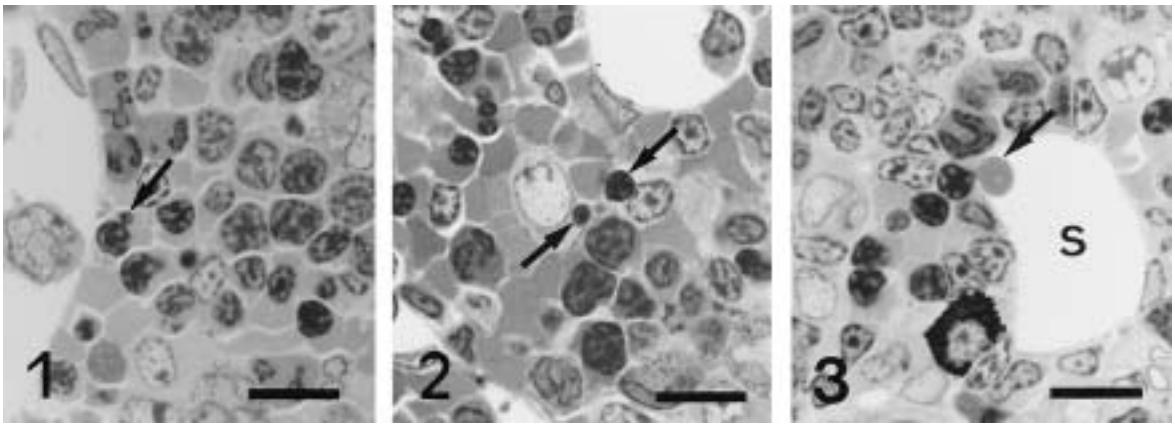

Fig. 1. A small globule (arrow) protruding from the nucleus of a polychromatophilic erythroblast. Toluidine blue staining. Bar=10 μm.
Fig. 2. Sole nuclei without any cytoplasm engulfed by a central macrophage (arrows). Toluidine blue staining. Bar=10 μm.
Fig. 3. An acidophilic erythroblast (arrow) passes through the endothelial wall of the sinusoid (S). Toluidine blue staining. Bar=10 μm.

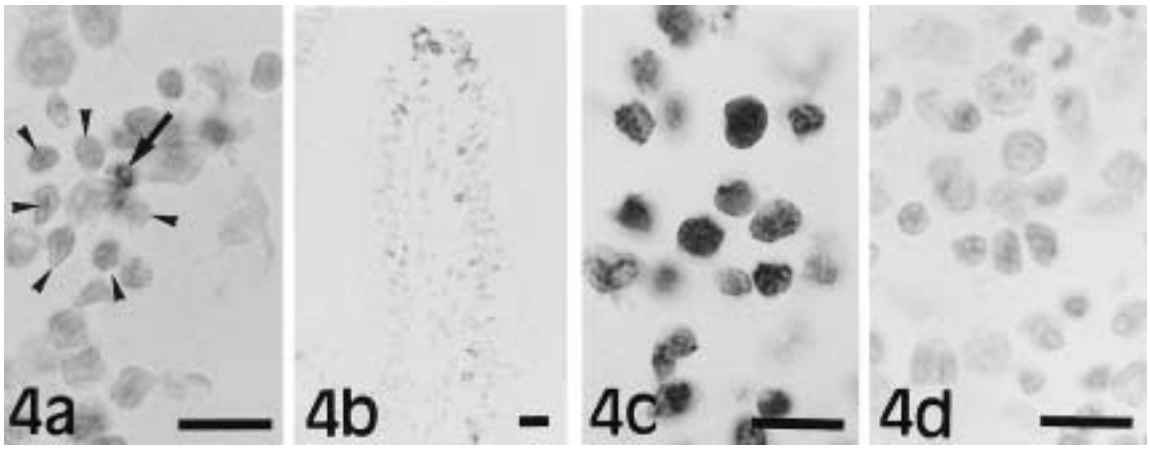

Fig. 4. a: DNA fragmentation is visible in a lysosome (arrow) of the central macrophage of the erythroblastic island (arrowheads), but no fragmentation is detected in the erythroid nuclei. Bar=10 μm. b: In the ileum, villous epithelial cells were DNA-fragmented in the apical regions of the intestinal villi, and the reaction products increased gradually going upward to the villous tips. Bar=10 μm. c: A positive control of the bone marrow. Bar=10 μm. d: A negative control of the bone marrow. Bar=10 μm. Counterstained by methyl green.

controls of the bone marrows, DNA fragmentations were detected in most nuclei (Fig. 4c). On the other hand, no positive reaction products were detected in the negative controls (Fig. 4d).

Ultrastructures of erythroblastic islands: Erythroid cells partially adhered to the cell body or slender cytoplasmic processes of a central macrophage in the erythroblastic island. As the erythroblast's maturation process proceeds, erythroblastic nuclei and cytoplasm increased in the electron densities and more shrunk. Cellular identification of proerythroblasts, basophilic erythroblasts, and polychromatophilic erythroblasts was easily performed. However, it was difficult to discriminate between polychromatophilic erythroblasts and acidophilic erythroblasts under an electron microscope, because the morphological changes from polychromatophilic erythroblasts to acidophilic ones were continuous. Furthermore, the signs of denucleation were found not only in typical acidophilic erythroblasts but also in polychromatophilic erythroblasts.

The cellular components of the erythroblastic islands were not uniform. It was seldom observed that the erythroblastic islands were composed of all types of erythroid series. The dispositions of erythroblasts in an early maturation stage, a middle maturation stage or a late maturation stage were frequently observed in each erythroblastic island. A peculiar erythroblastic island, which consisted of a central macrophage, peripheral reticulocytes and no erythroblast, was rarely found. The single layer of reticulocytes possessing no blebbing-like processes were anchored with many slender cytoplasmic processes of macrophages. This island migrated into the sinusoidal lumen through the sinusoidal endothelium (Fig. 5).

Mitosis was often observed in polychromatophilic erythroblasts, basophilic erythroblasts, and proerythroblasts, but never in typical acidophilic ones (Fig. 6). Small and electron-dense globules were also observed on the nuclear surface of polychromatophilic erythroblasts and acidophilic erythroblasts. The globules were precisely confirmed to be fragmented nuclei, since they contained many heterochromatins and little euchromatins (Fig. 7).

The denucleation of erythroblasts was initiated by the eccentric situation of their nuclei. The condensed nuclei were gradually pushed out of the constriction rings between the nuclear regions and the broad cytoplasms. The protruded nuclei were accompanied with little cytoplasm. The mitochondria, centrosomes and some vacuoles accumulated together near the constriction in the cytoplasm. Other organelles were not changed: The polysomes scattered over the cytoplasm and small invaginations were found on the entire cell surface. These characteristic arrangements of the organelles remained in the reticulocytes for a short time after the denucleation events. The blebbing-like cytoplasmic processes were often localized at the constrictions of denucleating erythroblasts. Such processes were never found on the denucleating nuclei (Fig. 8a). Another denucleation pattern was accompanied without cytoplasmic processes at the constriction site, but the morphological changes of the cell oraganelles were similar to those of the former pattern (Fig. 8b).

All nuclei engulfed by central macrophages were ultrastructurally confirmed to be the denucleated nuclei, whereas erythroblasts were never found in their lysosomes (Fig. 9).

DISCUSSION

It is well-known that individual matured erythrocytes migrate into the blood stream through the sinusoidal endothelium in the bone marrow [1]. The present study demonstrated the self-traffic of erythrocytes as one of the transportation mechanisms. In addition, we further suggest

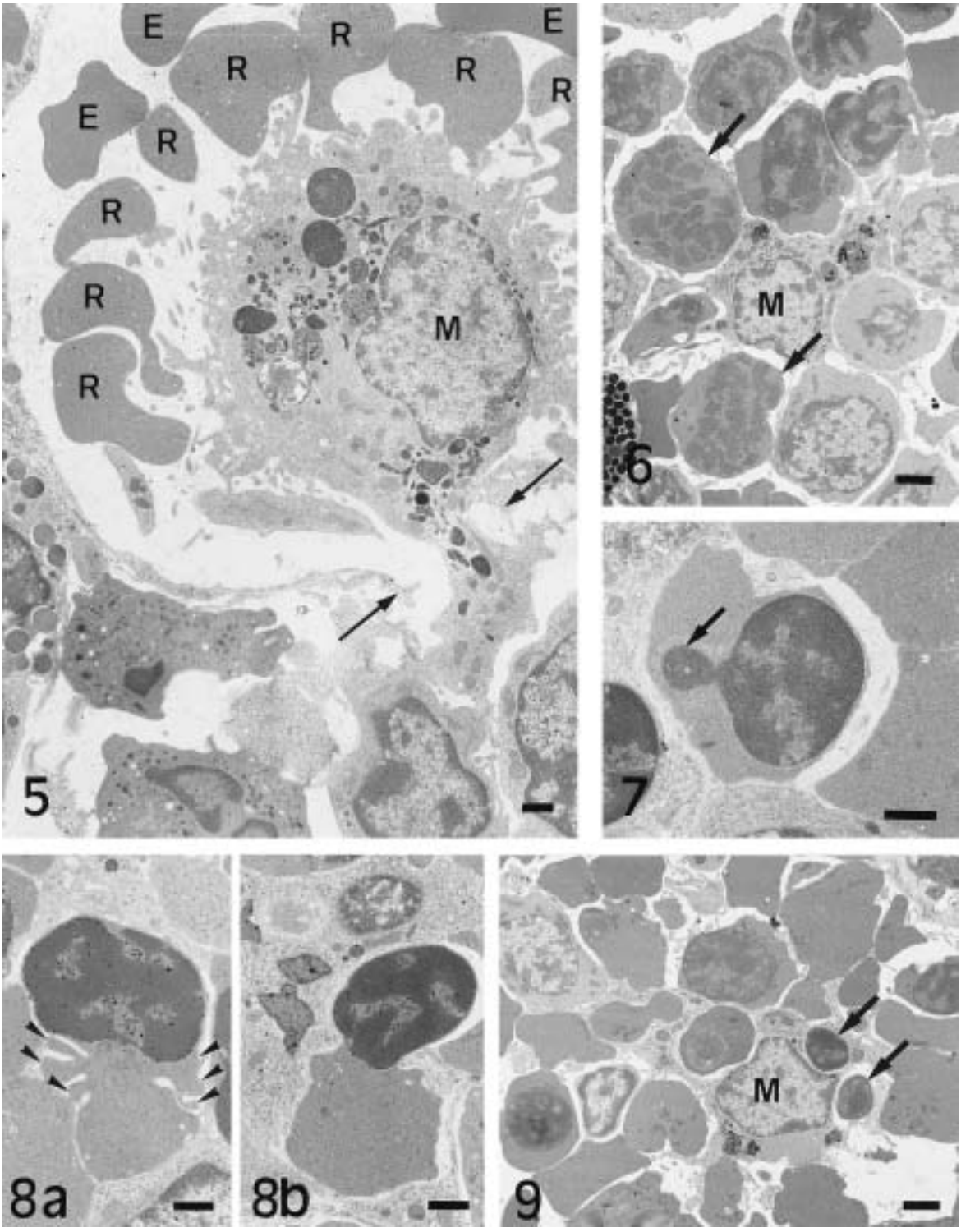

Fig. 5. An erythroblastic island bearing reticulocytes (R) migrating into the sinusoidal lumen through the pore of the sinusoidal endothelium (between arrows). E, matured erythrocyte; M, macrophage. Bar=1 μm.

Fig. 6. Mitosis of polychromatophilic erythroblasts (arrows). M, macrophage. Bar=2 μm.

Fig. 7. A small globule (arrow) from the nucleus in an acidophilic erythroblast. Bar=1 μm.

Fig. 8. Two patterns of denucleations of acidophilic erythroblasts. a: The blebbing-like cytoplasmic processes (arrowheads) appear at the constriction. No cytoplasmic process is visible on the nucleus. Bar=1 μm. b: No cytoplasmic process is accompanied. Bar=1 μm.

Fig. 9. Denucleated nuclei (arrows) in the cytoplasm of a central macrophage (M). Bar=2 μm.

another possiblility for the transportation of immature reticulocytes into the blood stream by the central macrophages. This mode of transportation has been never reported and its functional significance is still unknown. The maturation of immature erythrocytes is reported to be generally performed in the spleen [8]. Therefore, this type of the erythroblastic island may play a significant role in the delivery of the reticulocytes to the spleen and might contribute to the maturation of erythrocytes in the organ.

The typical morphological criteria for apoptotic cells have been defined as the shrinkage of both cell bodies and nuclei, the accumulation of nuclear chromatin, the nucleosomal fragmentation of DNA, blebbing on the cell surface, and fragmentation of the cell bodies and nuclei into apoptotic bodies [6, 11, 12, 36]. In mammalian erythropoiesis, some similar morphological characteristics have been also observed. The maturing stages of erythroid cells show cell shrinkage and the condensation of nuclear chromatins [4], and Howell Jolly bodies as the expression of nuclear fragmentation [35]. In our ultrastructural observations, it was also shown that the cell shrinkage and the fragmentation of the nuclei of polychromatophilic erythroblasts were found *in vivo* under normal serum EPO concentrations. In fact, basophilic erythroblasts express Fas on the cell surfaces, whereas polychromatophilic erythroblasts and acidophilic erythroblasts possess Fas-L on their cell surfaces [9]. This Fas-Fas-L binding, which is considered a feedback mechanism to a steady state from an accelerated state of erythropoiesis, does not always induce apoptotic cell death in erythroid cells *in vitro* [9]. The molecules of the Bcl family that prevent apoptotic cell death come out into acidophilic erythroblasts [13, 32]. These reports suggest that the maturation of erythroblasts progresses with modulation by offensive and defensive actions of apoptotic processes.

Intracytoplasmic activation of the caspase family generally causes the destruction of actin filaments [25] and fodrin [24], followed by further apoptotic changes in the cytoplasm and the nucleus which include DNA fragmentation [22]. In some cells, however, these changes do not always occur simultaneously [38]. That is, apoptotic thymocytes exhibit cytoplasmic and nuclear shrinkages without nuclear DNA fragmentation *in vivo* [27]. The externalization of phosphatidylserine prior to nuclear DNA fragmentation has been flowcytometically clarified in thymocytes and T-cell lines *in vitro* [5, 37]. In contrast, the fragmentation of nuclear DNA and the appearance of DNase occur before the cytoplasmic and nuclear shrinkage and the exfoliation of the columnar epithelial cells in the rat small intestinal villi [38] and in the chicken cecal villi [33, 34]. Thus, the initiation of apoptotic changes have a variety of patterns among the variously differentiated cells. Furthermore, the apoptosis-induced cells do not always eventually die, because the apoptotic lens fiber cells lose the cytoplasmic organelles, disintegrate the DNA, and finally remove the nuclei but do not die [3, 14].

Apoptosis-induced cells express phosphatidylserine on their external surfaces during the early stage before DNA fragmentation, and they are recognized and phagocytized by resident macrophages [5]. In the macrophage-rich tissues, therefore, apoptosis-induced cells are immediately removed by scavengers. Consequently, the nuclear fragmentation and the apoptotic bodies could not be detected. In fact, the scavengers recognize the changes in the cell membrane of thymocytes and engulf them before the nuclear fragmentation of thymocytes in thymus [6]. However, in the bone marrow, a scavenger-rich tissue, the present detailed light and electron microscopical observations indicated that the degraded cellular elements in the lysosomes of central macrophages included the denucleated nuclei of erythroblasts exclusively but not the entire cell bodies of erythroblasts.

In vitro under normal serum EPO concentrations, the fragmentation of the nuclear DNA occurred in cultured erythroblasts [17, 19, 32], suggesting a possibility that apoptotic cell death is involved in erythropoiesis [7, 19]. Excess erythroblasts are thought to be killed by the induction of apoptosis in a physiological condition, but the rapid production of numerous erythrocytes may be partly due to the reduction of apoptosis in the case of acute anemia [29]. In our detailed histoplanimetry, however, the fragmentation of nuclear DNA and apoptotic cell death were not detected in the *in vivo* erythroid cells under the exposure of a normal serum EPO level, although the degraded nuclei were often detected in central macrophages. We believe that the present method of detecting DNA fragmentation is appropriate, because more weak signals of the fragmentation were detectable in the villous epithelial cells of the small intestines. Thus, it is reasonable to consider that erythroid cells show some apoptotic feature but do not eventually die. In mammals, the induction of apoptosis in erythroblasts may be associated with the mechanisms of erythroid maturation, such as erythroid denucleation. Further experiments are needed to clarify the involvement of apoptosis in erythroid denucleation.

In general, surplus cells are removed by apoptosis in ontogenetic morphogenesis [26, 30]. However, in adulthood, the apoptotic removal of surplus cells was not reported with any functional significance. The proliferations of cells are regulated in various tissues by neural and humoral factors [16, 18]. The present findings might suggest the existence of other regulatory mechanisms in erythropoiesis besides the apoptotic removal of surplus erythroid cells.

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