NOTE *Anatomy*

Immunohistochemical Detection of Phosphatidylserine and Thrombospondin on Denucleating Erythroblasts in Rat Bone Marrow

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ABSTRACT. The hypothesis that apoptotic factors play some roles in the denucleation of erythroblasts has been confirmed by the immunohistological detection of both phosphatidylserine and thrombospondin as phagocytosis-inducing factors in general apoptotic events. Both phosphatidylserine and thrombospondin were detected on the surface of cell membrane of mature erythroblasts, while thrombospondin was also detected in more immature erythroblasts. The intensities of their immune reactions increased as the erythroids matured. During denucleation, the positivities of both phosphatidylserine and thrombospondin were restricted on the surface of the cell membrane surrounding the protruding nuclei. Thus, the apoptotic process involves denucleation of erythroblasts and phosphatidylserine, and thrombospondin acts as phagocytosis-inducing factors in the denucleation event.

KEY WORDS: apoptosis, denucleation, erythropoiesis, phosphatidylserine, thrombospondin.

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Apoptosis is a gene-regulated cell death process induced by death signals, lack of growth factor, DNA damage, endoplasmic stress and so on. Caspases play important roles in the execution of apoptotic events. However, not all of apoptosis-induced cells performed cell death in a short period. For example, lens fiber cells [13], megakaryocyte [8], keratinocyte [20], and monocyte [19] express caspases during their differentiation or maturation. Several apoptotic factors such as caspase-3, Bcl-2 family proteins, Fas, and Fas-L, are involved in erythropoiesis [4–5, 9, 15, 23]. We have also mentioned the various morphological features of apoptosis in erythroblast maturation, including cell shrinkage, and nuclear fragmentation [21].

In general, apoptosis-induced cells shrink in volume and become fragmented into small particles called "apoptotic bodies", which are recognized and engulfed by resident macrophages [7, 16, 18]. This recognition is performed by phagocyte-recognizing signals such as phosphatidylserine or thrombospondin [16, 18]. Phosphatidylserine generally helps macrophages in recognizing protruded and denucleated erythroid nuclei *in vitro* [22]. However, the contribution of other phagocyte-recognizing signals to the recognition of denucleated erythroid nuclei is unknown. Therefore, we performed immunohistochemical analysis and examined the relationship between the distribution of phagocyte-recognizing signals and denucleation of erythroids.

Animals: Eight male Wistar rats aged 6–7 weeks (Japan SLC, Hamamatsu, Japan) were used according to the Kobe University Animal Experimentation regulations (Permission number: 14–09–01). The animals had free access to food and water. The animal facility was maintained under conditions of alternating 12 hr light and dark cycle at 21 ± 1°C and 50–60% humidity. None of the animals showed any signs of hematological disorder in both clinical and pathological examinations. Serum erythropoietin concentrations were normal (14.1 ± 4.4 mIU/m*l*).

Detection of phosphatidylserine: TACS Annexin V-Biotin Apoptosis Detection Kit (R&D Systems, Minneapolis, MN, U.S.A.) was used to detect phosphatidylserine on the cell surface. We followed the manufacturer's instructions but with a slight modification in the composition of the reaction buffer and temperature so as to prevent artificial cell death. Briefly, immediately after euthanasia under deep anesthesia with diethyl ether, tissue blocks (0.125–1 mm^3) of femoral bone marrow were extracted from 3 animals. After rinsing with RPMI-1640 (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (10% FBS RPMI-1640) at 4°C, the specimens were incubated with 200 μl annexin V solution (10% FBS RPMI-1640 containing 10 μg biotinylated annexin V, 2 mM $MgCl_2$ and 2 mM $CaCl_2$) for 40 min at 4°C. As experimental control, the remaining specimens were incubated under the same conditions except for no annexin V. After a single rinse with 10% FBS RPMI-1640 at 4°C, the specimens were immersed in periodate-lysine-paraformaldehyde (PLP) fixative for 4 hr at 4°C, followed by incubation with peroxidase-conjugated streptavidin (diluted at 1:30; DAKO, Glostrup, Denmark) for 40 min at 4°C, and incubated again with 3,3-diaminobenzidine (DAB) containing 0.01% H_2O_2. The specimens were refixed with the PLP fixative for 18 hr at 4°C, and then dehydrated and embedded in paraffin, after

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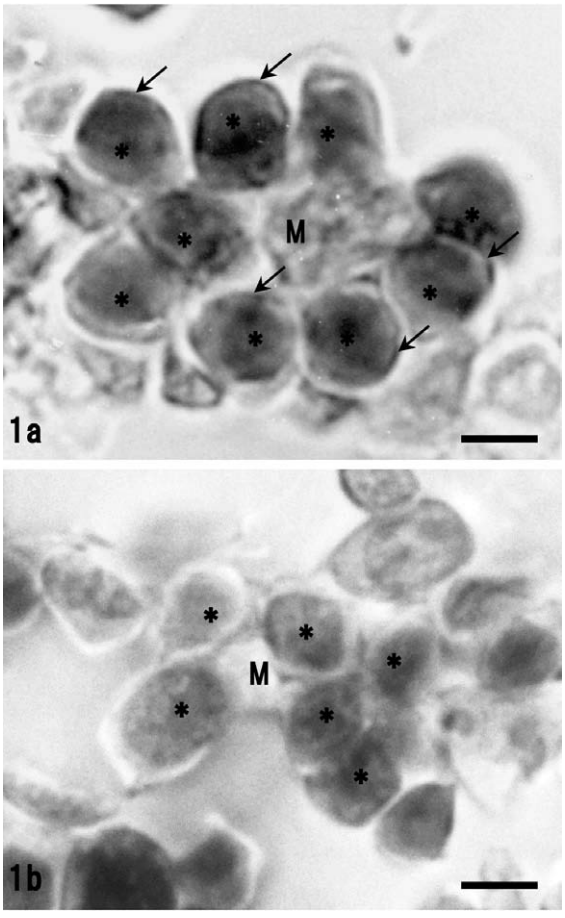

Fig. 1. Light micrographs of phosphatidylserine in an erythroblastic island of rat bone marrow. Phosphatidylserine is detected in polychromatophilic erythroblasts and orthochromic erythroblasts (arrows, 1a). No positive signal is detected in the experimental control (1b). M, macrophage; asterisks, erythroblasts (Scale bar=5 μm).

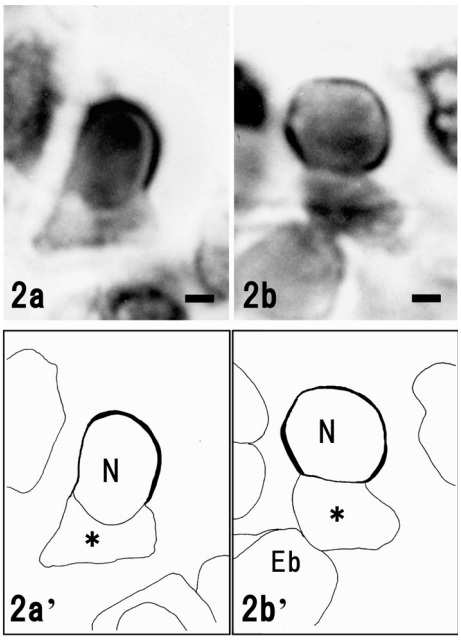

Fig. 2. Light micrographs of phosphatidylserine located on denucleating erythroblasts in rat bone marrow. The pattern diagrams are schema of the above micrographs. High positivity of phosphatidylserine is detected on the cell surface of cell membrane around the protruded nuclei of denucleating erythroblasts (bold lines in the schemata). Eb, erythroblast; N, nuclei of erythroblasts; asterisks, cytoplasm of denucleating erythroblasts (Scale bar=1 μm).

which 4-μm-thick sections were cut and weakly counterstained with hematoxylin. The maturation stage of erythroblast was determined on the basis of the sizes of cytoplasm and nucleus, and the pattern of nuclear chromatin.

Detection of thrombospondin: Immediately after euthanasia under deep anesthesia with diethyl ether, 5 animals were intracardially perfused with the PLP fixative. Small tissue blocks of the femoral bone marrow were extracted and immersed in the same fixative for 24 hr at 4°C. The blocks were snap-frozen in liquid nitrogen, according to the embedding method described by Barthel and Raymond [1]. Five-micrometer-thick frozen serial sections were cut using a Cryostat HM505E (Carl Zeiss, Oberkochen, Germany), placed on glass slides precoated with 0.2% 3-aminopropyltriethoxysilane (Shin-Etsu Chemical, Tokyo, Japan), and treated with absolute methanol followed by incubations with 0.5% H_2O_2 and then with 1% normal wild bullfrog serum (prepared in our laboratory). Thereafter, the sections were treated with anti-thrombospondin 1/2 goat antibody (diluted at 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 18 hr at 4°C, followed by incubation with peroxidase-conjugated mouse anti-goat IgG (diluted at 1:50; Chemicon International, Temecula, CA, U.S.A.) for 1 hr at room temperature. These sections were incubated with DAB containing 0.01% H_2O_2. Control sections were incubated with phosphate buffered saline instead of the primary antibody.

Phosphatidylserine was detected on the cell surfaces of matured erythroblasts such as polychromatophilic erythroblasts and orthochromic erythroblasts (Fig. 1a). During denucleation, phosphatidylserine was concentrated on the surfaces of the cell membrane surrounding the protruded nuclei. However, no positive signal was detected around the cell body (Fig. 2), or in the experimental control (Fig. 1b).

Thrombospondin was detected on the cell surfaces of maturing erythroblasts from the proerythroblast to orthochromic erythroblast stage, and the amount of thrombospondin increased with erythroblast maturation (Fig. 3a). During denucleation, high thrombospondin immunoreactivity was detected on the surfaces of the cell membrane surrounding the protruded nuclei (Fig. 4), whereas no immunoreactivity was found in the control sections (Fig. 3b).

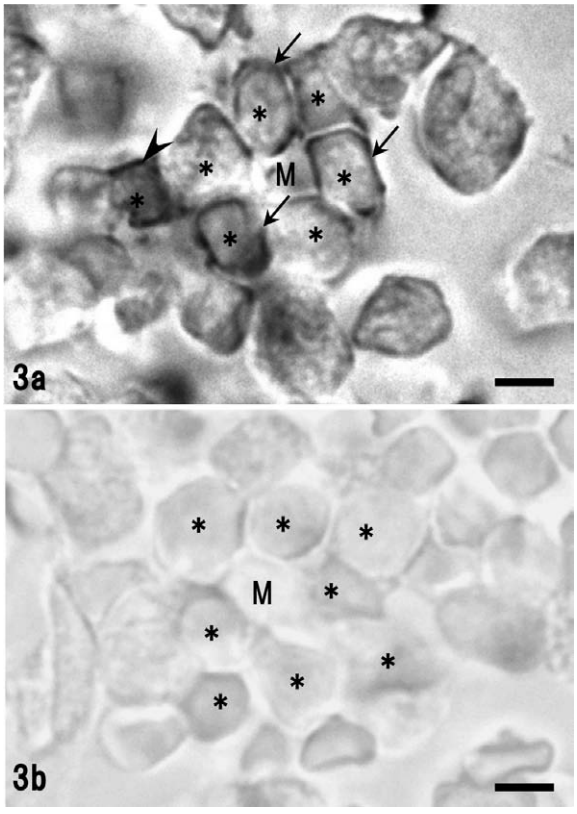

Fig. 3. Light micrographs of immunohistochemical staining of thrombospondin in an erythroblastic island of rat bone marrow. High positivity thrombospondin are detected in matured erythroblasts (arrows) and protruded nucleus of denucleating erythroblast (arrowhead) in erythroblastic island (3a). No immunoreactivity is detected in the control section (3b). M, macrophage; asterisks, erythroblasts (Scale bar=5 μm).

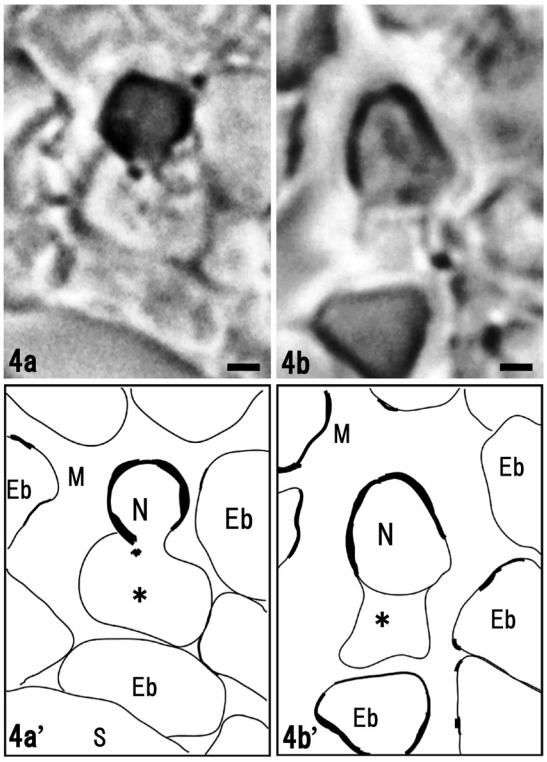

Fig. 4. Light micrographs of immunohistochemical staining of thrombospondin on denucleating erythroblasts in rat bone marrow. The pattern diagrams are schema of the micrographs above. Thrombospondin is strongly detected on the surface of the cell membrane surrounding the protruded nuclei of denucleating erythroblasts (bold lines in the schemata). Eb, erythroblasts; M, cytoplasm of macrophages; N, nuclei of erythroblast; S, sinusoid; asterisks, cytoplasm of denucleating erythroblasts (Scale bar=1 μm).

Erythroid differentiation and maturation was regulated by the microenvironment constructed by many cells such as macrophages and endothelial cells and so on [6, 17]. However, denucleating process occurred without these cells *in vitro* [10, 11, 24]. Therefore, it was considered that the denucleation of erythroblasts is regulated by an intrinsic pathway [10]. Prior to the erythroid denucleation, cytoskeletal molecules such as intermediate filaments and microtubules disappeared [14] perhaps due to the activity of caspases, because erythroid maturation was suppressed by the inhibition of caspase activity *in vitro* [5, 24].

During erythroid maturation, many apoptotic morphological features were observed [21], and the expression of anti-apoptotic molecules such as Bcl-xL peaked in orthochromic erythroblasts [12]. Detection of phosphatidylserine exposure and thrombospondin attachment around the protruded erythroid nuclei suggests that apoptosis may play a role in erythroid maturation.

Phosphatidylserine, a major phagocyte-recognizing signal, is located in the inner layer of the cell membrane under normal conditions and is translocated to external surfaces during early apoptosis [18]. Phosphatidylserine is related to macrophage recognition and engulfment of denucleated erythroid nuclei *in vitro* [22]. In the present study, the strong exposure of phosphatidylserine in mature erythroblasts, especially during denucleating stage in erythroblastic islands, suggests a possible contribution of phosphatidylserine to the recognition of denucleated erythroid nuclei *in vivo*.

Thrombospondin is an adhesion molecule located between apoptosis-induced cells and macrophages [18]. In addition, the accumulation of thrombospondin or carbohydrate chain is important for the recognition of the dying cells [18]. Therefore, the detection of thrombospondin concentration on the cell membrane surrounding the protruded erythroid nuclei suggests that thrombospondin also contributes to the recognition of erythroid nuclei.

Thrombospondin contains at least 5 interactive domains for a wide variety of adhesive protein and cell receptors, and is involved in numerous biological process such as cell adhesion, proliferation, migration, and angiogenesis [2]. Cell adhesion between erythroblasts and macrophages via

VCAM-1, VLA-4, ICAM-4, or thrombospondin [3, 6, 17], starts at the stage of colony–forming–unit erythroids, and enhances the survival and proliferation of erythroblasts [6, 17]. Therefore, it was assumed that the detection of thrombospondin in immature erythroblasts may be related to the formation of an erythroblastic island and regulation of erythropoiesis, in addition to the recognition of denucleated nuclei of erythroblasts.

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