Note

Existence and Differential Changes of Peptidylarginine Deiminase Type II in Mouse Yolk-Sac Erythroid Cells

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Peptidylarginine deiminase (PAD) catalyzes the conversion of arginyl residues in proteins to citrullyl residues in the presence of Ca\textsuperscript{2+}. Recently, we obtained a monoclonal antibody, EH7, which reacted only with mouse PAD type II. Here, we describe immunohistochemical findings on the cellular localization of PAD type II in mouse fetus by using the monoclonal antibody. PAD type II is expressed in yolk-sac erythroid cells and the level of the enzyme in these cells decreases as the cells differentiate.

Peptidylarginine deiminase (protein-L-arginine iminohydrolase, EC 3.5.3.15) (PAD) is a post-translational modification enzyme that catalyzes deamination of arginine residues of proteins in the presence of calcium ions.\textsuperscript{17} Although this enzyme is found widely in various tissues of vertebrates,\textsuperscript{2,18} its physiological role is still largely unknown. The findings that the head domain of an intermediate filament protein was specifically deaminated by PAD in vitro, causing a change in the filament protein’s polymerization activity,\textsuperscript{20} and the existence of citrullinated intermediate filament proteins in tissues,\textsuperscript{20} suggest that the enzyme is involved in cell differentiation. We found that there are three types of PAD in mouse tissues and proposed designating them PAD types I, II, and III according to their elution order on anion-exchange column chromatography.\textsuperscript{21} These enzymes showed similar catalytic properties, but their molecular weights and substrate specificities were different.\textsuperscript{21} Recently, we obtained a monoclonal antibody EH7 (MAbEH7) that is specific to mouse PAD type II and indicated that its specific epitope was localized in the eight-residue segment at the amino-terminal portion of the enzyme.\textsuperscript{21} Since an

Fig. Immunohistochemical Stain of PAD Type II in Mouse Yolk-Sac Chorion.
Section of mouse fetus on day 13 after gestation was immunostained by using MAbEH7 as described in the text. Bar indicates 0.02 mm. The upper direction of photography shows amniotic cavity. Eh, erythroblast; EdC, endodermal cell; EtC, endothelial cell.

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Abbreviations: PAD, peptidylarginine deiminase; MAbEH7, monoclonal antibody EH7; YSEC, yolk-sac erythroid cells; PBS, phosphate buffered saline; PBSS, phosphate buffered saline containing 0.01% CaCl\textsubscript{2} and 0.01% MgCl\textsubscript{2}.
immunocross activity test of MAβEH7 with PAD type II from several vertebrates and other types of PAD from mouse showed that the monoclonal antibody reacted only with mouse PAD type II, the antibody should be useful for further studies of PAD type II, especially tissue and cellular localization by immunohistochemistry. Here, we describe the immunohistochemical findings, based on MAβEH7 reactivities, that PAD type II is expressed in yolk-sac erythroid cells (YSEC) and that the level of the enzyme in YSEC decreases as the cells differentiate.

Ddy sexually mature female mice were paired with males of proven fertility in the evening, and the morning on which a vaginal plug or spermatozoa was found in the vagina was designated as day 1 of gestation. From days 11 to 17 of gestation, females were anesthetized with ethyl ether, killed by bloodletting from the heart, and then the fetuses were stripped from the uterus. For immunohistochemical studies, the fetuses were fixed in Bouin’s fixative. Tissues fixed in Bouin’s fixative were dehydrated with a graded series of ethanol solutions, infiltrated with xylene, and embedded in paraffin wax. The sections (4 μm thick) were deparaffinated with xylene, treated with PBS containing 3% hydrogen peroxide to block endogenous peroxidase activity, and then rinsed with phosphate buffered saline (PBS). After incubation with 1:70 diluted normal horse serum (Vector Laboratories Inc.) to block nonspecific binding, the sections were incubated with purified MAβEH7 (30 ng/ml). The sections were then treated with Vectastain ABC kit (Vector Laboratories Inc.) according to the supplier’s instructions. The presence of PAD type II in the section was detected by the development of peroxidase staining using 3,3-diaminobenzidine and hydrogen peroxide. The sections were counterstained with Mayer’s hematoxylin. Figure shows the immunohistochemical staining of the yolk-sac chorion of the fetus from day 13 after gestation. The erythroblasts in the yolk-sack blood island were heavily stained, but endothelial cells, the amnion, and endodermal cells were not stained. During mammalian ontogeny, erythropoiensis begins in the yolk-sack blood islands. As development progresses, erythropoiensis shifts to the fetal liver, fetal spleen, and bone marrow. To confirm the existence of PAD in yolk-sac-derived erythroid cells, we isolated YSEC by centrifugation through discontinuous density gradients of Percoll and measured PAD activity. Mouse fetuses on day 11 (before the development of fetal liver) were removed surgically from amniotic sacs, rinsed in PBS to remove the majority of maternal erythrocytes, and gently disaggregated in PBS containing 0.01% CaCl₂ and 0.01% MgCl₂ (PBSS) to form a single-cell suspension by using pipettes of 2-mm diameter aperture. After removing unbroken tissues by filtering with nylon gauze, the cells were pelleted at 150 x g for 5 min, washed twice with PBSS, and pelleted again. A three-step Percoll gradient was produced by the method of Harrison et al. The cells resuspended in PBSS were put on the top of the gradient and centrifuged at 3000 x g for 30 min. Each cell fraction was banded at the interfaces of the gradient. The cell fractions were collected with a Pasteur pipette and the cell concentration was calculated from counting of a diluted sample in a hemocytometer. To remove the Percoll, the cell fractions were diluted 20-fold with PBSS and the cells were then pelleted by centrifugation at 300 x g for 5 min. For measurement of PAD activity, cells suspended in ice-cold 20 mM Tris·HCl buffer, pH 7.6, containing 10 mM EDTA, 10 mM dithiothreitol, and 0.43 mM phenylmethylsulfonyl fluoride were disrupted by sonication with a Cosmo-Bio cell rupturer. The cell debris was removed by centrifugation at 20,000 x g for 30 min, and the supernatant was used to measure PAD activity. The activity of PAD was assayed by measuring the formation of citrulline residues in Bz-L-Arg-O-Et as described previously. Table shows the morphological profiles and PAD activities of each cell fraction banded at the interfaces of the gradient and sedimented at the bottom. The cells banding at the 40%/70% interface were found to consist predominantly of proerythroblasts. The fractions at the 70%/80% interface and at the bottom contained mainly erythroblasts and reticulocytes, respectively. As shown in the Table, YSEC showed a significant level of PAD activity. Furthermore, it is evident from the distribution of PAD activity that the enzymatic activity was highest in the proerythroblast fraction and lowest in the reticulocyte fraction. These results suggest that the level of PAD in YSEC changes during cell differentiation.

The erythroid lineage has been extensively studied. The committed erythroproietic stem cell divides repeatedly in response to erythropoietin, to form clusters of rapidly differentiating proerythroblasts and erythroblasts. During the final stage of erythroid cell differentiation, the erythroblasts become smaller and their nuclei disappear to form reticulocytes and finally erythrocytes. We also examined the immunohistochemical staining of YSEC at different stages of gestation. The stainings declined as the differentiation of YSEC progressed, and staining of the erythrocytes in the fetus from day 17 after gestation was negligible (data not shown). Nagata et al. reported PAD activity in rat and mouse hemopoietic cells. They demonstrated that bone marrow cells had a significant activity of PAD, in contrast to no activity or a trace of activity in erythrocytes. Our results thus agree with these findings. We then asked whether the other types of PAD also exist in YSEC. Since specific antibodies against types I or III are not available at present, the above question is unanswered.
References