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Short communication

MICROGLIAL EXPRESSION OF PEPTIDYLARGININE DEIMINASE 2 IN THE PRENATAL RAT BRAIN

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Abstract: Peptidylarginine deiminases (PADs) are Ca^{2+}-dependant post-translational modification enzymes that catalyze the citrullination of protein arginyl residues. PAD type 2 (PAD2) is thought to be involved in some processes of neurodegeneration and myelination in the central nervous system. In this study, we found PAD2-positive cells in rat cerebra in 19- to 21-day old embryos, i.e. at a developmental stage well before myelination begins. Most of the cells were microglial marker-positive cells found mainly in the prospective medulla, and others were microglial marker-negative cells found mainly in the prospective dentate gyrus of the hippocampus. The former seemed to be in an activated state as judged by morphological criteria. The specificity of the enzyme activity, immunoblotting and reverse transcriptase-polymerase chain reaction analyses revealed that these cells expressed PAD2 and not PAD1, PAD3 or PAD4. Our data is indicative of microglial expression of PAD2 in the prenatal developing cerebrum.

Key words: Protein deimination, Post-translational modification enzyme, Microglial cells, Central nervous system, Gene expression

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Abbreviations used: BAEE – benzoyl-L-arginine ethyl ester; BzArg – benzoyl-L-arginine; GAPDH – glyceraldehyde-3-phosphatedehydrogenase; GFAP – glial fibrillary acidic protein; KA – kainic acid; MAP2 – microtubule associated protein 2; mAb – monoclonal antibody; MBP – myelin basic protein; PAD – peptydylarginine deiminase; PAD2 – peptidylarginine deiminase type 2; RT-PCR – reverse transcriptase-polymerase chain reaction; SDS – sodium dodecyl sulfate

INTRODUCTION

Peptidylarginine deiminases (PADs, EC 3.5.3.15) are a family of post-translational modification enzymes that convert protein arginine residues to citrulline residues in a Ca^{2+}-dependant manner [1, 2]. The type 2 form (PAD2) is expressed in the central nervous system [3-6], localized mainly in the astrocytes of the normal adult mammal brain [5, 7, 8].

We demonstrated that PAD2 activates and citrullinates various cerebral proteins under hypoxic conditions [7] and during kainate-evoked neurodegeneration [5]. Furthermore, we recently found abnormal accumulation of citrullinated proteins in hippocampal extracts from patients with Alzheimer's disease [9]. Thus, protein citrullination is thought to be involved in the process of neurodegeneration.

On the other hand, the apparent presence of citrullinated myelin basic protein (MBP) in minor myelin subfractions [10, 11] and PAD2-positivity in primary cultures of immature oligodendrocytes from rats [12] imply that myelinating oligodendrocytes express PAD2 and that this protein is responsible for citrullinating MBP. In addition, Pritzker *et al.* [13] indicated relatively strong PAD activity in the non-compacted, loose myelin fraction of the mouse brain during postnatal development, suggesting a requirement for PAD in the process of myelination. They also detected PAD transcripts and PAD protein in the brains of mice as young as five days old. However, little is known about PAD expression in the developing nervous system preceding the stage of myelination, especially in prenatal stages.

In prenatal brain development, there are many cellular events of research interest, such as the proliferation and differentiation of neuronal and glial cells, the movement of neurons, nerve network formation, and the programmed cell death and scavenging of unwanted neurons. In this study, we examined rat brains in prenatal stages of development by indirect immunostaining using a specific antibody [3] which recognizes PAD2 but not other types of PAD. We found PAD2-immunoreactive cells in the cerebrum of embryos at day 19-21 of development (E19-E21). Most such cells also displayed microglial marker-positivity, whereas other cells showed microglial marker-negativity. In addition to this immunocytochemical data, we also present here the results of the analyses of the specificity of enzyme activity, Western immunoblotting and reverse transcriptase-polymerase chain reaction (RT-PCR) to confirm the type of PAD. This is the first report demonstrating microglial expression of PAD2 in the prenatal brain.

MATERIALS AND METHODS

Experimental animals

The Wistar rats used here were housed in an environmentally controlled room and fed *ad libitum*. The Animal Care and Use Committee of the Tokyo

Metropolitan Institute of Gerontology approved the experimental protocol used. The developing cerebra were obtained at E18-E21 after Caesarean operation and decapitation of the embryos. The adult (4-month old) cerebra and epidermis were obtained after systemic perfusion with PBS to remove blood cells from the brain, and decapitation.

Histological preparations

Each cerebrum was quickly excised and sagittally cut into halves. One half of the cerebrum was fixed with ice-cold 4% paraformaldehyde in PBS and embedded in O.C.T. compound 4583 (Miles Inc.) before being cut into 4-μm thick cryosections.

Immunocytochemical staining

Indirect immunoperoxidase staining was performed using Vectastain Elite ABC kit (Vector Laboratories, Inc.). Staining for PAD2 was done with rabbit anti-rat PAD2 polyclonal antibody [3] as the primary antibody. Endogeneous peroxidases were inactivated by incubation in 1% hydrogen peroxide in methanol after the incubation with the primary antibody.

Double fluorescence staining

Double staining was performed via the indirect fluorescence method using rabbit anti-PAD2 polyclonal antibody [3] and mouse monoclonal antibody (mAb) to each marker. The mouse antibodies used were mAb to glial fibrillary acidic protein (GFAP; as an astrocyte marker [14], Boehringer Mannheim), mAb to vimentin (clone V9, PROGEN Biotechnik), and mAb to microtubule-associated protein 2 (MAP2, as a neuronal marker [15], Chemicon International).

For the double staining of PAD2 and each marker protein, the sections were incubated, first with a mixture of rabbit anti-rat PAD2 and mAb to each marker, and second with a mixture of FITC-conjugated goat anti-rabbit IgG and biotinylated goat anti-mouse IgG. The sections were then incubated with streptoavidin-labeled Cy3 conjugate (Sigma).

For double staining of PAD2 and alfa-D-galactose (a microglial marker [16]), the sections were incubated, first with a mixture of anti-rat PAD2 and biotinylated isolectin B4 of *B. simplicifolia*, and second with FITC-conjugated goat anti-rabbit IgG and streptoavidin-labeled Cy3.

Determination of enzyme activity

The remaining half of each cerebrum was homogenized with ice-cold 0.1 M Tris-HCl buffer (pH 7.4) containing 1 mM ethylenediaminetetraacetic acid and 1 mM phenylmethanesulfonyl fluoride. For the determination of PAD activity, the homogenates were incubated with benzoyl-L-arginine ethyl ester (BAEE) or benzoyl-L-arginine (BzArg) as described [3]. One unit was defined as the amount of enzyme catalyzing the formation of 1 mol of citrulline derivative in 1 h at 50ºC. Protein concentrations were measured by the method of Lowry *et al.* [17].

Gel electrophoresis and immunoblotting

For the analysis of proteins, sodium dodecyl sulfate (SDS) and 2-mercapto-ethanol were added to the homogenates to give final concentrations of 2% (w/v) and 5% (v/v), respectively. These preparations were sonicated and boiled for 4 min. Each sample (containing 20 μg proteins) was subjected to SDS-polyacrylamide gel electrophoresis [18], and the resolved proteins were Western blotted onto nitrocellulose membranes. Immunoblotting was performed by using anti-PAD2, and bound antibodies were indirectly visualized by chemiluminescence.

RT-PCR analysis

Total RNA was isolated from the rat cerebra and epidermis using ISOGEN (Nippon gene). The RT-PCR was performed with a Takara mRNA Selective PCR Kit (Takara). PAD1 primers (nt 2937 to 2961 and nt 3545 to 3567) [19], PAD2 primers (nt 2409 to 2433 and nt 2812 to 2836) [20], PAD3 primers (nt 2391 to 2413 and nt 3016 to 3038) [21], and PAD4 primers (nt 2023 to 2047 and nt 2203 to 2227) [19] were designed in the 3' non-coding region of the cDNA. Glyceraldehyde-3-phosphatedehydrogenase (GAPDH) primers (nt 75 to 96 and nt 840 to 862) [22] were designed in the coding region of the cDNA. PCR products were visualized by agarose gel electrophoresis with ethidium bromide staining.

RESULTS AND DISCUSSION

First, we performed immunocytochemical analyses using the anti-PAD2 antibody. The PAD2 profiles of the cerebrum at E18 showed no positive cells (Fig. 1A), whereas the PAD2 profiles at E19-E21 showed scattered localization of positive cells in the cerebrum (Fig. 1B-F). The positive cells with strong intensity were mainly distributed in the prospective medulla. Positive cells with weak but significant intensity were also detected in the prospective dentate gyrus of the hippocampus. Fig. 1C shows the typical PAD2 profile of the coronal section at E20.

At least two kinds of PAD2-positive cells were observed at E19-E21. Those mainly in the prospective medulla had relatively large cell bodies with a round or ameboid morphology (Fig. 1E, arrows). A second type of the cells was located mainly in the hippocampal prospective dentate gyrus, and these were relatively small with short processes (Fig. 1F). Occasionally, such smaller cells were also observed in the other area (Fig. 1E, arrowhead).

To substantiate the identity of the PAD2-positive cells, we performed double fluorescence staining. PAD2-positivity did not coincide with the presence of the astrocyte marker (GFAP) or neuron marker (MAP2) in the developing brain at E19-E21 (data not shown). However, in the relatively large, amoeboid- or round-shaped cells, we found that the presence of PAD2 coincided with that of alfa-D-galactosyl, a microglial marker recognized by isolectin B4 of

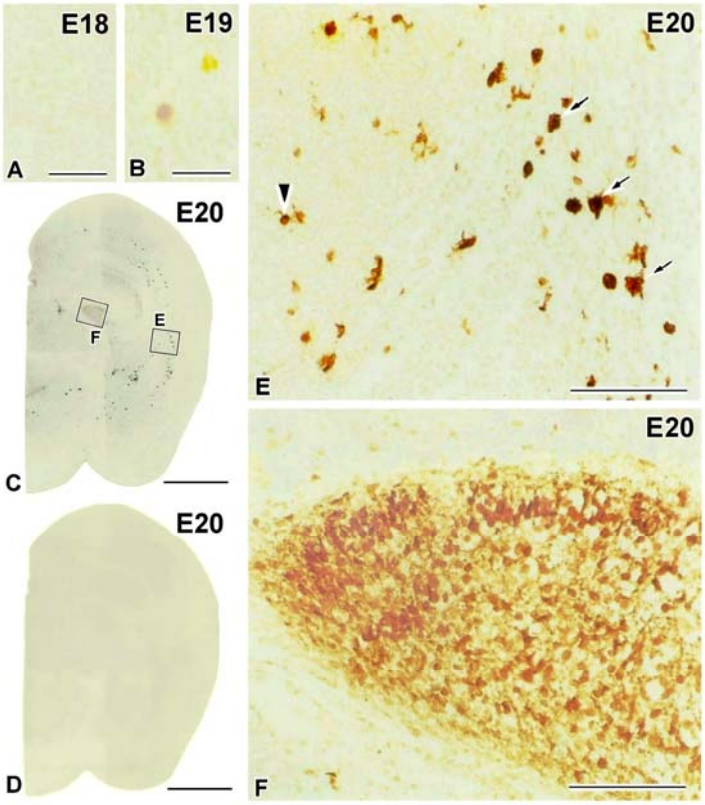

Fig. 1. Immunoperoxydase-stained PAD2 in histological sections of the developing brain at E18-20. A – Photomicrograph at E18 stained with anti-PAD2 antiserum. B – Photomicrograph at E19 stained with anti-PAD2. C – Macroscopical photographs of the coronal section at E20, stained with anti-PAD2. D – Macroscopical photographs of the same section stained with non-immune serum as a negative control. The two enclosed regions in C are magnified and represented in E and F. The arrows and arrowhead in E indicate the two kinds of cell as described in the text. Bars indicate 10 μm (A, B), 200 μm (C, D) , or 20 μm (E, F).

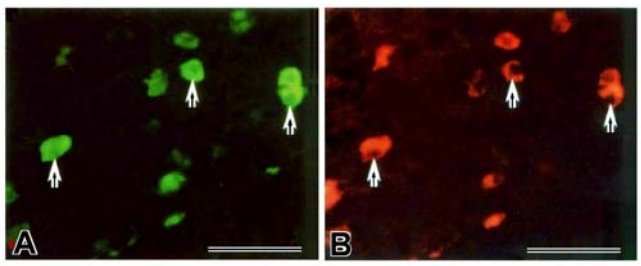

Fig. 2. Paired photomicrographs of the double fluorescence stain showing PAD2 (green) and the microglial marker (red) in the prospective medulla at E20. The colonal sections were first incubated with a mixture of anti-rat PAD2 and biotinylated isolectin B4 of *B. simplicifolia*, and then with FITC-conjugated goat anti-rabbit IgG and streptoavidin-labeled Cy3. The arrows indicate some coincident positions. The scale bars are 10 μm.

B. simplicifolia (Fig. 2A, B). In addition, all the PAD2-immunoreactive cells in the developing cerebra were also vimentin positive (data not shown).

To examine whether this PAD2 immunoreactivity reflected the enzyme's physiological presence, we measured PAD activity and performed immunoblotting using anti-PAD2 of the homogenates from developing (at E20, n = 5) and adult (4-month old, n = 4) cerebra. When BAEE was used as a substrate, the activity (mean ± SD) of the developing cerebrum was 5.1 ± 0.7 mU/mg protein, whereas that of the adult cerebrum was 35.8 ± 3.6 mU/mg protein. When BzArg was used as the substrate, the PAD activity of the developing cerebrum was 0.9 ± 0.1 mU/mg protein, whereas that of the adult was 6.8 ± 0.6 mU/mg protein. Thus, not only the adult but also the developing cerebra showed about 5-fold higher PAD activities towards BAEE than towards BzArg. This is a characteristic of PAD2 [3]. Therefore, this data seemed to reflect a difference in PAD2 content revealed as anti-PAD2-immunoreactive band intensity on the immunoblot of the corresponding extracts, as shown in Fig. 3A.

To confirm type 2-specific expression of PAD in the prenatal cerebra, we performed RT-PCR analysis. As expected, developing (at E20) and normal adult cerebra specifically expressed the type 2 enzyme, but none of the other types tested (Fig. 3B, lanes 1 and 2). In the rat epidermis (lane 3), used as a positive control, all the types of PAD were expressed, as previously demonstrated [5, 6, 23]. To the best of our knowledge, this is the first report on PAD2 in the prenatal brain.

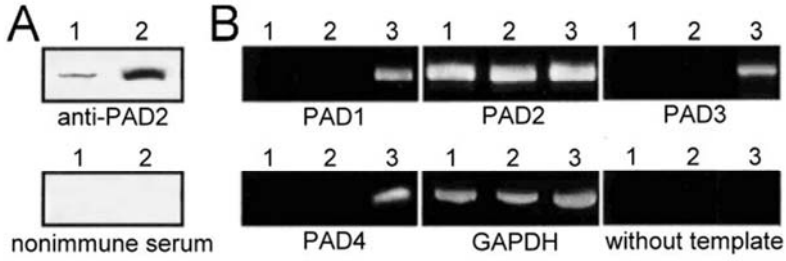

Fig. 3. Immunoblotting and RT-PCR analyses. A – Immunoblot with anti-PAD2 and non-immune serum as the negative control. Lane 1, extract from the prenatal (E20) cerebrum; lane 2, extract from the adult (4-month-old) cerebrum. B – RT-PCR analysis. Total RNA was isolated, and RT-PCR was performed with primers for PAD1, PAD2, PAD3, PAD4, and GAPDH, or without primers as a negative control. Lane 1, PCR products from the prenatal (E20) cerebrum; lane 2, PCR products from the adult cerebrum; lane 3, PCR products from the adult epidermis as a positive control.

The major finding in this study is the microglial expression of PAD2 in the developing cerebrum. At present, the functional role(s) of PAD2 in the microglia remain unknown. No correlation between PAD2 immunoreactivity and the microglial marker in normal adult brains had been observed [5, 8, 23]. However,

we noted abundant expression of PAD2 in activated microglia after KA-evoked neurodegeneration [23]. As shown in Fig. 1B and D, PAD2-positive microglia also showed a round or ameboid morphology. This is a characteristic of activated but not resting microglia. Such activated microglia might be the cells responsible for removing the debris of dead neurons by programmed cell death during development of the cerebrum. Presumably, PAD2 performs a physiological function in microglia during their activated state, as suggested previously [23].

We failed to identify PAD2-positive cells in the prospective dentate gyrus of the hippocampus, since they reacted with anti-vimentin but not with the other probes tested. Such cells showed morphological features distinct from the microglial cells regardless of their activation state. According to Vincent *et al.* [8], many PAD2-positive/GFAP-positive astrocytes were distributed in the hippocampal dentate gyrus in adult rats. We also observed such PAD2-positive astrocytes at that site in adult rats [5, 7, 23]. Therefore, PAD2-positive small cells in the prospective dentate gyrus might be astrocyte precursors expressing PAD2. This idea in no way contradicts our finding that all PAD2-positive cells showed vimentin immunoreactivity, since vimentin is expressed not only in the microglia but also in astrocyte precursors [24].

In addition to the four types of PAD tested in this study, an egg- and embryo-abundant PAD-like protein (ePAD; PAD6) was reported [25]. PAD6 is expressed in the oocyte and early-stage embryo [25], but not in the adult human brain [26]. Further studies are needed on PAD6 expression in relation to cerebral development.

In summary, this report revealed the expression of PAD2 by microglia in the prenatal cerebrum at E19-E21. Combined with earlier data, these results are consistent with our previous observations and suggest of a role for the PAD enzyme during normal brain development and in neurodegeneration [23]. Identification of the target protein molecule(s) of PAD2 in the microglia will be of great importance in elucidating the biological significance of PAD2 in the cells.

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