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Selective localization of gelatinase A, an enzyme degrading β-amyloid protein, in white matter microglia and in Schwann cells

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Abstract Gelatinase A is an enzyme capable of cleaving soluble β-amyloid protein (βAP), and may function as an α-secretase to produce secretory forms of amyloid precursor protein. We examined gelatinase A immunoreactivity in the brains and posterior roots of neurologically normal, lacunar stroke, Alzheimer disease (AD), amyotrophic lateral sclerosis, progressive supranuclear palsy and myasthenia gravis cases. The gelatinase A antibody stained only microglial cells in the white matter in all the brain tissues. In AD brain, the reactive microglia located in the center of classical senile plaques, as well as in other microglial cells in the gray matter, showed no immunoreactivity. Gelatinase A in white matter microglial cells may play a role in preventing local deposition of βAP. In the posterior root, Schwann cells had positive immunoreactivity. As with other metalloproteases, gelatinase A in Schwann cells may play an antiproliferative role.

Key words Gelatinase A · β-Amyloid · Microglia
Alzheimer's disease · Schwann cell

Introduction

Human gelatinase A, a 72-kDa protein (or 62-kDa activated form) also known as type IV collagenase or matrix metalloprotease 2 (MMP2), is a matrix enzyme capable of degrading gelatin as well as other collagens [20]. This and other collagenases are thought to be involved in various physiological and pathological conditions such as embryonic development, arthritis [14], tissue repair and tumor invasion and metastasis [16, 24]. Gelatinase A has been detected in various cell lines such as fibroblasts [18], melanoma cells [27], invasive or metastatic tumor cells [16, 24], bronchial epithelial cells [2] and macrophages [5]. Gelatinase A has also been found in the conditioned media of schwannoma [25], glioblastoma and neuroblastoma cell lines, and in human cerebrospinal fluid [12].

Recently one of us [12] reported that gelatinase A has amyloid precursor protein (APP) secretase-like activity, since it hydrolyzes the Lys_{16}-Leu_{17} bond within the βAP sequence. Two possible functions for gelatinase A were suspected: it may produce secretory APP on the plasma membrane and/or it may degrade soluble βAP in the extracellular matrix. In either case, it would act to prevent βAP aggregation. In the peripheral nervous system, Schwann cells have been thought to possess a metalloprotease-like activity [4, 13]. A role for this protease in regulating the Schwann cell surface content of low-affinity nerve growth factor receptor during development and regeneration has been proposed [4].

We report here that gelatinase A immunoreactivity was found in white matter microglia and in Schwann cells.

Materials and methods

In this study we examined the brains of three male and two female neurologically normal controls, aged 54–82 years, four males and five females with Alzheimer disease (AD), aged 67–82 years and two male lacunar stroke victims, aged 76 and 80 years. The diagnosis of AD was established using the criteria recommended by the National Institute on Aging [8]. Brains in all cases were obtained 2–15 h after death. We also examined the posterior root of three males with amyotrophic lateral sclerosis (ALS), aged 64–75 years, one female with progressive supranuclear palsy (PSP), aged 65 and one female with myasthenia gravis, aged 49 years. These roots did not show any evident pathology.

Small blocks from the temporal, parietal and midbrain regions and posterior root were dissected. They were fixed for 2 days in phosphate-buffered 4% parafomaldehyde. They where then transferred to a maintenance solution of 15% sucrose in 0.1 *M* phosphate-buffered saline (PBS), pH 7.4, and kept in the cold until used. Sections were cut on a freezing microtome at 20-μm thick-

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ness and stained by single or double immunohistochemical procedures using various primary antibodies. The antibodies used in this study and their dilutions were: anti-gelatinase A, 1 : 10,000 (rabbit polyclonal) and 1 : 1000 (mouse monoclonal); anti-leukocyte common antigen (LCA), 1 : 100 (mouse monoclonal, DAKO); antiβAP, 1 : 1000 (mouse monoclonal, DAKO). The polyclonal antibody to human gelatinase A was raised against the purified 72-kDa precursor. The monoclonal antibody was also raised against the purified 72-kDa precursor according to the procedure described by Zola and Brooks [26].

After treatment with the primary antibody for 48 h in the cold, the sections were treated for 2 h at room temperature with biotinylated secondary antibody (Vector), followed by incubation in the avidin-biotinylated horseradish peroxidase complex (Vector). Washing between steps was done with PBS containing 0.3% Triton X-100 (PBST). Peroxidase labeling was visualized by incubating with a solution containing 0.001% 3.3′-diaminobenzidine (DAB), 0.6% nickel ammonium sulfate, 0.05% imidazole and 0.0003% H_2O_2. When a dark purple product formed, the reaction was terminated. Sections were washed, mounted on glass slides, dehydrated with graded alcohols and coverslipped with Entellan.

Double immunostaining using anti-gelatinase A and either anti-LCA or anti-βAP antibodies was done. Sections were treated for 30 min with 0.5% H_2O_2 solution in PBST after the DAB reaction of the first cycle. The second cycle was carried out in a similar manner to the first, except that nickel ammonium sulfate was omitted from the DAB solution, yielding a brown precipitate in the second cycle. Further details on the immunohistochemical procedures have been published previously [11].

The specificity of the anti-gelatinase A antibodies was tested by immunoblot analysis using AD brain tissue. A sample of temporal white matter was homogenized in buffer (5 volumes of 20 m*M* TRIS-HCl, pH 7.4, 1 m*M* EGTA, 1 m*M* EDTA, 10 μ*M* leupeptin, 1 μ*M* leupeptin, 1 μ*M* pepstain and 0.3 μ*M* aprotinin). The homogenate was centrifuged at 15000 cpm for 30 min at 4° C, and the supernatant was collected as a crude cytosolic fraction. The pellet was re-dissolved in the homogenization buffer and used as a membranous fraction. Aliquots of both fractions containing 50 μg of protein were electrophoresed on sodium dodecyl sulfate/polyacrylamide gel (15% polyacrylamide gel; non-reducing conditions) and then transferred to a nitrocellulose membrane (25 m*M* TRIS-glycine buffer, pH 8.3, containing 20% methanol). The membrane was pretreated with 5% skim milk powder in 25 m*M* TRIS, pH 7.4, containing 150 m*M* NaCl (TBS), and then incubated with the polyclonal (1 : 10000) or monoclonal (1 : 1000) anti-gelatinase A antibody in 2% skim milk/TBS, for 18 h at 4° C. The membrane was extensively washed in TBS + 0.1% Tween 20 (TBST), and the bound antibody labelled with alkaline phosphate-conjugated anti-rabbit antibody (BRL, 1 : 5000, 2 h at room temperature) in TBST containing 1% skim milk. Following further washing, the membrane was developed in alkaline phosphatase substrate buffer [0.33 mg/ml nitroblue tetrazolium (BRL), 0.44 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BRL), 0.1 *M* NaCl and 50 m*M* $MgCl_2$ in 0.1 *M* M TRIS-HCl (pH 9.5)].

Results

One clear band of approximately 62 kDa was detected in the membranous fraction, while a weakly stained band with the same molecular mass was detected in the cytosolic fraction by both the polyclonal (Fig. 1 A) and monoclonal (Fig. 1 B) anti-gelatinase A antibodies.

Immunohistochemistry using the anti-gelatinase A antibody stained of cells in the white matter which had microglia-like morphology (Fig. 2 A, B, D). The staining was similar in pattern in all brains studied but seemed to be more intense in the parietal white matter in the AD than in non-neurological or stroke brains. Under high-power light microscopy, the immunoreaction products could be seen as fine granules (Fig. 2 B, D). Cortical gray matter showed no such positive structures in any brain (Fig. 2 C).

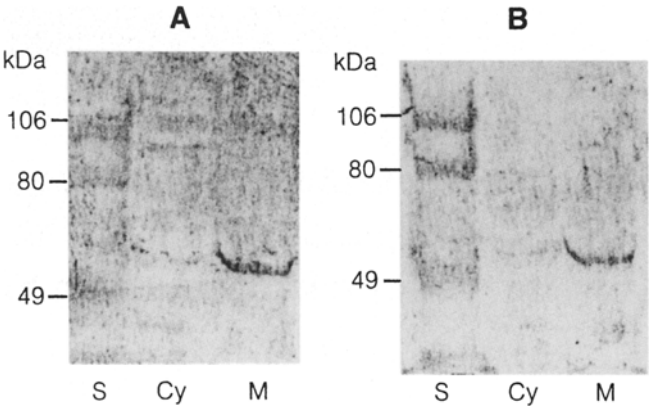

Fig. 1 A, B Immunoblot analysis using Alzheimer-brain tissue and the antibodies to gelatinase A. Both the polyclonal (**A**) and monoclonal (**B**) antibodies revaled a major band of approximately 62 kDa in the membranous *(M)* fraction, corresponding to the reported size for the active form of gelatinase A, with a weakly positive band of the same molecular mass being seen in the cytosolic *(Cy)* fraction. *S*: standard markers

The identification of the positive cells as microglia was confirmed by double immunostaining for gelatinase A and LCA; all LCA-positive microglia in the white matter had gelatinase A immunoreactivity (Fig. 2 D). Double immunostaining for gelatinase A and βAP in AD sections showed that reactive microglial cells located in the center of classical senile plaques had no immunoreactivity for gelatinase A (Fig. 2 C).

In the root areas, positive immunoreactivity to gelatinase A was seen in the Schwann cells in all samples studied. Clearly identifiable examples are shown in Fig. 3 A, B. Positive immunoreactivity was found surrounding the nuclei (probably in the cytoplasm) and extending 100–500 μm in the wrapping axons.

Discussion

The major results of this study was that the 62-kDa active form of gelatinase A was found immunohistochemically in brain microglial cells and Schwann cells. The distribution of gelatinase A immunoreactivity in brain is particularly interesting because of its exclusive localization to the white matter. Such a preferential localization to microglia in the white matter of human brain has also been reported for the c-met proto-oncogene product, a receptor for hepatocyte growth factor [23]. Such a restricted localization of gelatinase A was found in AD as well as control brains, but the staining of white matter microglia appeared more intense in AD tissue than in the other samples examined.

An elevated level of metallprotease activities, capable of degrading tissue matrix components, has been reported

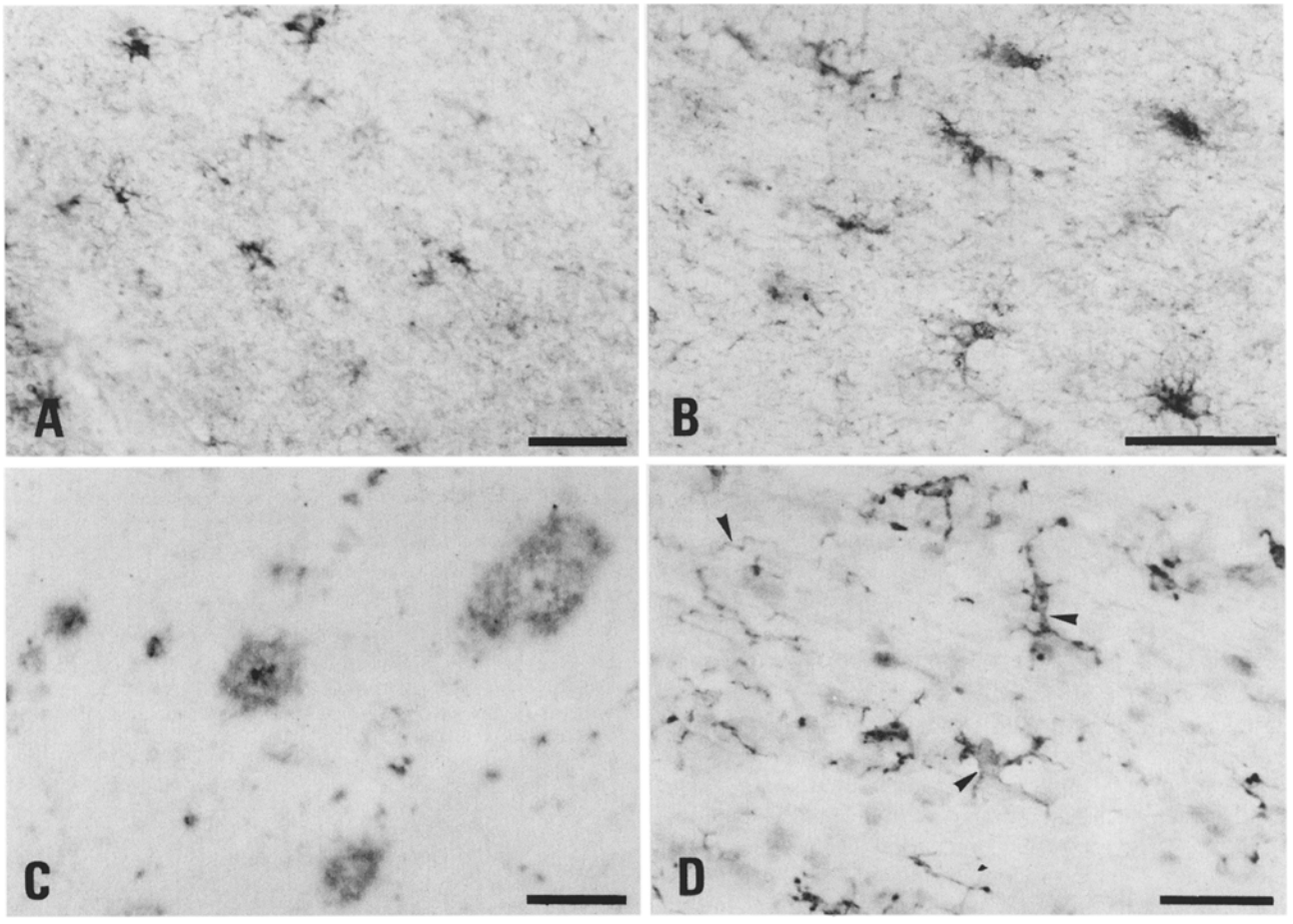

Fig. 2 Immunostaining with the antibody to gelatinase A in neurologically normal (**A**), Alzheimer (**B, C**) and lacunar stroke (**D**) brain tissues. **A** Gelatinase A staining in parietal white matter shows weak positive staining in cells having a microglia-like morphology. **B** Intense gelatinase A staining in similar structures is seen in the parietal white matter in AD. The immunoreaction product can be seen as fine granules scattered over the perikarya and processes. **C** Double immunostaining in parietal gray matter with anti-gelatinase A and anti-βAP showed no gelatinase A-positive microglial cells, even in the center of classical senile plaques. In the original slide, the gelatinase A immunoproduct appeared purple, and that of βAP brown. Only brown colored staining of the senile plaques was seen. **D** Double immunostaining with anti-gelatinase A and anti-LCA antibodies showed that all LCA-positive microglia in the white matter were also gelatinase A-positive. In the original slide, the LCA immunoproduct appeared brown *(lighter staining, arrowheads)*, and that of gelatinase A purple *(darker staining)*. *Each bar* = 50 μm

in the hippocampus in AD [1]. Furthermore, some human metalloproteases have been proposed as candidate α-secretases for APP degradation [10, 12]. However, the cells that synthesize the APP molecules that give rise to amyloid deposits in aged and AD brain tissue are still unknown. Neuronal [11, 15] and astroglial [6] production of APP has been reported. On the other hand, Haas et al. [7] reported that microglial cells in culture can synthesize full-length APP and that it is located in internal membranous vesicles, which may be responsible for βAP production. Several groups have described an intimate association between microglia and senile plaque amyloid [3, 9, 10, 21], and some have hypothesized that microglial cells do not phagocytose the amyloid fibrils, but rather process APP and make βAP [3, 22]. Recently, Wisniewski and Weigel [21] reported that the cells involved in βAP formation in the vessel wall and neuropil are the perivascular cells, perivascular microglia and neuropil and satellite microglia. Sapirstein et al. [17] reported that APP transported down the axons was deposited at sites in the axolemma as well as periaxolemmal myelin, and that coated vesicles isolated from the white matter contained levels of APP comparable to that found in axolemma and periaxolemmal myelin. Recently, Tokuda et al. [19] reported that a considerable amount of APP is carried by axonal transport, during which some of the APP are processed to their secretory form. Senile plaques in AD are almost always in the gray matter and are rare in white matter. Summing up these data, we could speculate that gelatinase A in white matter microglial cells may be playing a major role for the degradation of any APP or soluble βAP which might be originating from damaged axons or glia, and that the result is normal processing, with few or no local amyloid deposits. Future work is necessary to determine whether gelatinase A is an enzyme which helps to prevent the formation of insoluble βAP in vivo.

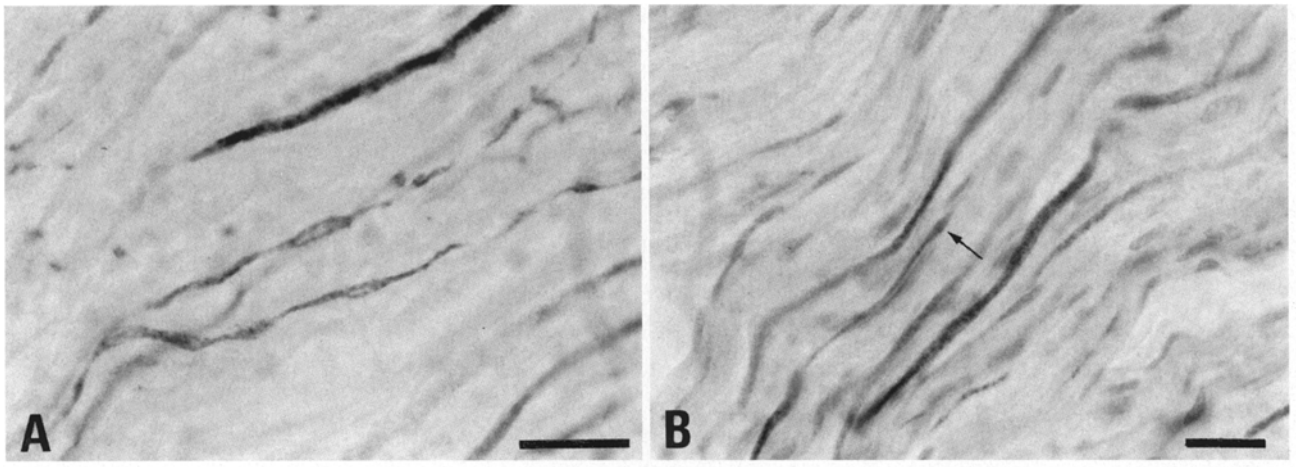

Fig. 3 Immunohistochemistry with the antibody to gelatinase A in the posterior roots in amyotrophic lateral sclerosis (**A**) and progressive supranuclear palsy (**B**). **A** Schwann cells are clearly stained except for their nuclear areas, showing that gelatinase A is localized in the cytoplasm. **B** Gelatinase A staining (blue color) counterstained with neutral red-give further confirmation that positive cells are Schwann cells. An *arrow* indicates the nucleus (red color) of a Schwann cell. *Bar* = 25 μm

The exact subcellular localization of gelatinase A has not been demonstrated in this study. However, the immunoblot analysis showed that the positive band of a size expected for the 62-kDa activated form of gelatinase A was predominantly in the membranous fraction. This suggests that the plasma membranes of microglial cells or the membranes of cytoplasmic organelles, such as secretory vesicles, may contain this molecule.

Metalloprotease-like activity has been found in Schwann cell and schwannoma cell line conditioned media [4, 13, 25]. One of the metallproteases in schwannoma has been identified as gelatinase A [25]. In addition we found this molecule in human Schwann cells. The metalloprotease in Schwann cells has been suspected to play a role in antiproliferative activity, similar to that of the 55-kDa neural antiproliferative protein. A balance between proteolytic and protease inhibitory activity is crucial for both normal neuronal development and for neuronal response to injury. Further studies using damaged peripheral nerves are needed to clarify the exact role of gelatinase A in Schwann cells.

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