Localization of Tissue Inhibitor of Matrix Metalloproteinases in Alzheimer's Disease and Normal Brain

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Abstract. Based upon the hypothesis that metalloproteinases and their inhibitors might be involved in the pathogenesis of Alzheimer’s disease, we studied brain samples of eight cases of Alzheimer’s disease, six other pathological entities and three elderly controls for tissue inhibitor of metalloproteinase (TIMP) immunoreactivity. Specificity was supported by a loss of immunoreactivity following antigen preabsorption of antisera. Areas studied included ependyma, choroid plexus, frontoparietal, hippocampal and cerebellar cortex, n. basalis of Meynert, basal ganglia, midbrain,pons, and medulla. TIMP positivity was localized to neuritic senile plaques, neurofibrillary tangles and Purkinje cells. The pattern of TIMP plaque staining was similar to that observed with anti tau and SP18 antibodies. It differed from that observed with anti SP40, HAM 56 and GFAP antibodies. The selective localization of TIMP to the neuritic lesions of Alzheimer’s disease in a codistribution with the amyloid precursor protein and abnormally phosphorylated and truncated tau supports a possible role for metalloproteinases and their inhibitors in the evolution of these lesions.

Key Words: Alzheimer’s disease; Amyloid; Beta protein; Metalloproteinase inhibitors; Neurofibrillary tangles; Senile plaques.

INTRODUCTION

The β-amyloid precursor protein, APP, a large membrane spanning protein, is processed in the Golgi system in a constitutive secretory pathway and by degradative lysosomal enzymes. Cleavage by secretase, in the secretory pathway, splits the beta protein region yielding a small membrane-associated fragment and a large, NH2 terminally derived, secreted fragment (1–3). This means of degradation does not generate intact the essential portion of beta protein required for amyloid formation. Lysosomal processing, by multiple proteases, results in the production of COOH terminally derived, potentially amyloidogenic fragments, some of which contain the entire beta region (4–7). These fragments can be degraded within lysosomes by the cysteine proteases, cathepsins B and L (8). In addition, varied systemic and neuroepithelial cells including neurons, astrocytes and microglia release intact soluble beta protein which appears identical to fibrillar amyloid beta protein (9–11). Based upon insensitivity to lysosomal blockers, it appears that the secretory pathway may also be responsible for this APP product (12). Conversion of soluble beta protein into a fibrillar non-soluble amyloid form may occur in Alzheimer’s disease. The mechanisms which govern the production of soluble beta protein and its conversion into non-soluble fibrillar amyloid are unknown. One hypothesis in Alzheimer’s disease proposes an imbalance between proteinases and proteinase inhibitors. Supporting data consists of the demonstration that amyloid plaques contain not only serine and aspartic lysosomal proteinases (cathepsins B and D), but also serine proteinase inhibitors (13–18).

Recent studies have suggested that metalloproteinases participate in the generation of β-amyloid protein (19, 20). Both metalloendopeptidases involved in neuropeptide cleavage and matrix metalloproteinases (MMP) of the collagenase family have been implicated. Matrix metalloproteinases and tissue inhibitor of metalloproteinase (TIMP) have been recognized to play an important role in diseases associated with excessive connective tissue breakdown (21). In the nervous system, neurite outgrowth within an extracellular matrix involves MMP activity and can be inhibited by a synthetic inhibitor of MMP activity (22). Both MMP and TIMP have been identified in astrocytes (23), Schwannoma cells (24), and other neuronal cells (25); these proteins are also readily identified in cerebrospinal fluid (Zucker, Gijbels, unpublished). The role of these proteinases and inhibitors in central nervous system (CNS) disease remains to be elucidated.

It has recently been demonstrated that the dominant CNS matrix metalloproteinase, gelatinase A (72 kDa type IV collagenase, MMP-2), has an APP secretase-like activity which hydrolyses a crucial bond in the β-amyloid sequence (19). Elegant studies by Roberts et al suggest that secretase is an integral membrane metalloendopeptidase that colocalizes with APP but has a different inhibitory profile from the MMP family of enzymes (20).

Based on the hypothesis that a MMP might be involved in the production of β-amyloid protein, the favorable kinetics for binding of TIMP to activated matrix metalloproteinases (26) and the demonstration of TIMP in the nervous system (27), we initiated this study to identify whether TIMP would be localized in the lesions.
In the Alzheimer’s disease and aged non-demented controls, areas of brain studied included ependyma, choroid plexus, frontal cerebral cortex, hippocampus, nucleus basalis of Meynert, caudate, putamen, globus pallidus, midbrain, pons, medulla and cerebellum. The patients with Alzheimer’s disease were all clinically demented and clinically classified as probable Alzheimer’s disease. They all satisfied neuropathological criteria for the diagnosis of Alzheimer’s disease (31).

The six other pathological entities included lesions of active multiple sclerosis (two cases), HIV encephalitis (one case), HIV-associated toxoplasmosis (one case), HIV-associated progressive multifocal leukoencephalopathy (PML) (one case), HIV-associated cytomegalovirus encephalitis (one case) and HIV vacular myelopathy (one case). All cases had autopsy intervals of less than 21 hours (b) (range 5–20 h, mean 13 h).

HT1080 fibrosarcoma, a cell line which makes TIMP, gelatine A, and gelatine B in culture (32), was used to develop the immunohistochemical protocol for the rabbit anti-TIMP antibody. Cell pellets were prepared for immunohistochemistry using four methods of fixation and processing which included: (1) immediate quick freezing of tissue samples in liquid nitrogen-chilled isopentane, followed by cryostat sectioning; (2) 24 h fixation in freshly prepared 4% paraformaldehyde with subsequent cryoprotection by incubation in 20% sucrose, followed by quick freezing, as above, and subsequent thick section frozen sectioning, 16 microns, to produce flat sections; (3) 24 h fixation in 10% neutral buffered formalin with subsequent dehydration in graded alcohols and standard embedment in paraffin; and (4) 1 h fixation in the B-5 mercuric chloride-formalin mixture followed by fixation in 10% neutral buffered formalin with processing as above for the formalin-fixed samples. We also evaluated whether preincubation microwaving, at 1.4 kW in two 5 min cycles in citrate buffer, pH 6.0 (33, 34), enhanced subsequent TIMP immunoreactivity of the fixed, embedded samples.

The histochromic protocol consisted of preincubation of sections with 0.1% H2O2 for 15 min, followed by 30–60 min incubation in diluting buffer (phosphate-buffered saline (PBS) with 10% normal goat serum and 0.3% Triton X-100) followed by overnight incubation at 4°C with the primary antibody in diluting buffer. Sections were then washed with PBS and incubated for 1 h in 1:250 biotinylated goat anti-rabbit IgG in diluting buffer followed by a PBS wash and 1 h incubation with the Vectastain ABC reagent (Vector Labs, Burlingame, CA). The horseradish peroxidase substrate was visualized with a 50 mg/100 ml solution of 3,3′-diaminobenzidine tetrahydrochloride chromogen (Polysciences, Inc., Warrington, PA) in the presence of 0.03% H2O2. Controls included sections subjected to incubation in the secondary serum (goat) in lieu of primary antibody and sections incubated with primary antibody that had been preabsorbed with purified antigen (recombinant human TIMP) at a 10:1 ratio of antigen recombinant protein to antibody protein.

In addition, microwaved and non-microwaved sections were also immunoreacted with SP40 and SP18. Microwave antigen capture markedly enhanced staining with these antibodies, which was similar to that described by others, in 4% paraformaldehyde-fixed samples (35). It was the method employed for these antibodies. No microwaving was employed with the tau, HAM 56 and GFAP antibodies. The tau and HAM 56 antibod-
Fig. 2. This figure presents staining of hippocampal cortex with a panel of antibodies which includes TIMP, tau, SP18 and SP40 antibodies. TIMP antibody is depicted with and without antigen preabsorption. Sections a and b show TIMP immunoreactivity at lower and higher magnifications. Reactivity can be seen to be localized to neuronal neurofibrillary tangles and to senile plaques. Senile plaque staining is characterized by a dystrophic neuritic pattern of oblong and rod-like positive images. Central compact plaque amyloid does not stain. In sections c and d, lower and higher magnifications of antigen-preabsorbed TIMP antibody, it can be seen that preabsorption eliminated plaque and tangle staining. Sections e and f depict the neuritic pattern of senile plaque staining which was also observed with antibodies to tau (e) and to SP18 (f); the latter was raised to a synthetic
ies are mouse monoclonal antibodies. For these antibodies, goat anti-mouse antibody was employed in lieu of goat anti-rabbit antibody in our immunohistochemical protocol (see above).

RESULTS

TIMP positivity was present in the quick frozen formalin- and B5-fixed HT1080 cell pellets. Fixation in 4% paraformaldehyde prior to freezing was detrimental to subsequent immunoreactivity. Pre-incubation of TIMP antisera, with recombinant TIMP at a 10:1 ratio of antigen protein to antibody protein, eliminated subsequent HT1080 TIMP immunoreactivity. Figure 1 depicts the immunoreactivity of formalin-fixed, paraffin-embedded HT1080 cell pellets incubated with a 1:20 dilution of rabbit anti-TIMP antibody with and without antigen preabsorption. This dilution of the primary rabbit polyclonal antibody also yielded clearly positive immunoreactivity with little background in microwaved samples of archival formalin-fixed, paraffin-embedded brain samples. Preincubation microwaving markedly enhanced TIMP positivity and reduced background staining of tissue samples which were fixed and embedded. These were the conditions employed for this study.

In Alzheimer's disease hippocampal and cerebral cortex, positive TIMP immunoreactivity was seen in relationship to neuritic senile plaques and neuronal neurofibrillary tangles. Under these conditions, normal neurons and glia were not immunoreactive to the TIMP antibody. TIMP-positive senile plaques exhibited staining of discrete linear or rod-like profiles and oblong or ball-shaped masses of variable size, some in apparent continuity with the linear profiles. Central compact plaque core amyloid was TIMP-negative. The pattern of TIMP immunoreactivity in plaques was similar to that produced by tau and SP18 antibodies. Tau antibodies also strongly labeled neuronal neurofibrillary tangles. SP40 antibodies, in contrast to TIMP, SP18, and tau antibodies, strongly labeled central plaque cores and fine fibrillar plaque material. Anti-HAM 56, the macrophage marker, stained microglial/macrophage cell somas and a fine haze of their processes associated with plaques, and GFAP antibody immunoreacted with stellate-shaped astrocytes. Preincubation of the TIMP antibody with recombinant TIMP antigen completely inhibited subsequent cortical neuronal neurofibrillary and senile plaque immunoreactivity in these Alzheimer's disease brains. Figure 2 depicts the typical staining of cortical neuronal neurofibrillary tangles and senile plaques which was produced by this panel of antibodies.

Careful search of subcortical nuclear regions of the cerebral hemispheres and brainstem including cortical projection nuclei, such as the nucleus basalis of Meynert, failed to reveal a deep neuronal source of TIMP. As in the cerebral cortex, the only cell body TIMP positivity found was within neurons containing neurofibrillary tangles. Figure 3 depicts findings in the nucleus basalis of Meynert and medullary tegmentum.

In contrast, strong TIMP positivity was seen in the soma of cerebellar Purkinje cells and their dendrites in control and Alzheimer's disease brains. When Purkinje cells had been lost, immunoreactivity was seen in axons which embraced empty Purkinje cell spaces. Preabsorption of TIMP antibody with synthetic immunogen markedly reduced subsequent Purkinje cell immunoreactivity. These cerebellar findings are depicted in Figure 4.

No TIMP-reactive cells were seen in any of the pathological lesions studied. These contained inflammatory processes characterized by macrophages, microglia, multinucleated giant cells, lymphocytes and activated astrocytes.

DISCUSSION

Miyazaki et al (19) demonstrated that highly activated gelatinase A (41 kDa species) has an APP secretase-like activity which hydrolyses the Lys16-Leu 17 bond in the β-amyloid protein sequence; this is the site of normal cleavage of APP in cells which produces the ~100 kDa secretory form and a residual 9 kDa membrane-bound fragment. Based on the localization of a form of gelatinase A in the plasma membrane of cells and the demonstration that this proenzyme is activated to the 41 kDa form by the plasma membrane, Miyazaki et al (19) proposed a physiologic role for the 41 kDa enzyme in producing secretory APP on the plasma membrane and degrading soluble β-amyloid protein in the extracellular matrix. Both actions would serve to prevent β-amyloid protein accumulation in the extracellular matrix. Walsh et al (36), however, demonstrated that the addition of TIMP to cultured cells failed to interfere with cell secretion of soluble forms of APP, which suggests that alphasecretase is not a soluble MMP capable of generating APP; this observation does not exclude a membrane-bound or cytosolic form of gelatinase A in this mechanism. Of relevance, Miyazaki et al (19) also isolated a 28 kDa fragment of APP (in the C-terminal secretory form of APP) that functions as a metalloproteinase inhibitor (19). In spite of a similar molecular weight, this gelatinase inhibitor shares no sequence homology with TIMP, the dom-

peptide containing the amino terminal residues 45–62 of the amyloid precursor protein (APP). In contrast, antibody SP40 (g) raised to the entire 40 residue beta protein sequence immunoreacted with the amyloid core of the center of plaques and with fine peripheral plaque material. a, c 200X; b, d, e, g 800X; and f 400X.
inant inhibitor of MMP, which is secreted by many types of neuronal cells (23). They proposed that overproduction of APP under certain conditions may inhibit the activity of 41 kDa gelatinase A, favoring the extracellular deposition of β-amyloid protein.

In the current study, we have identified TIMP in a distribution which corresponds to the distribution of paired helical filaments (PHF) in Alzheimer's disease brains, namely in swollen neurites within senile plaques and in neuronal soma occupied by neurofibrillary tangles. TIMP senile plaque staining was similar to that produced by antibodies to tau and SP18 antibodies. The tau antibody recognizes biochemically abnormal tau forms in PHF in Alzheimer's disease and SP18 has been shown to have a similar pattern of staining (29, 34, 37–39). We could not locate a source of TIMP in cell bodies which project to cortex, or in any cerebral hemispheric neuronal or glial cell bodies. The only TIMP-immunoreactive population found were the Purkinje cells of the cerebellum. Since this cell population has been shown to take up proteins from the subarachnoid space and TIMP is present in relatively high concentrations in cerebrospinal fluid (10–100 ng/ml) (Zucker, Gijbels, unpublished), we do not know whether Purkinje cell reactivity represents synthesis or uptake (40, 41).

Our failure to detect TIMP immunoreactivity in other cells of the nervous system and in inflammatory lesions may be due to rapid secretion of TIMP by cells and low intracellular pools rather than to an absence of protein production. TIMP has been previously detected by bioassay in a variety of cells including cells of the CNS (26).

We propose that the colocalization of TIMP and APP in dystrophic plaque neurites supports the hypothesis that TIMP may participate in the process of β-amyloid protein production. One potential explanation is that a MMP might be excessively produced or activated in selected locations of β-amyloid deposition and, based on the high binding affinity of TIMP for activated MMP, the TIMP localizes to these sites. TIMP also forms a complex with latent gelatinase B (92 kDa) and thus might colocalize with this MMP; complexed latent gelatinase B is able to be activated but in a more controlled fashion (42).

Finally, the codistribution of TIMP and the distribution of abnormal biochemical forms of the tau protein suggest that an imbalance of proteases and their inhibitors may produce accumulation of post-translational modified forms of tau which characterize the PHF-enriched pathologies, neuritic plaques and neurofibrillary tangles (36–38).
Fig. 4. This figure depicts TIMP immunoreactivity in the cerebellar cortex without and with antigen preabsorption of TIMP antibody. Sections a and b, at lower and higher magnifications, depict strongly labeled TIMP-immunoreactive Purkinje cell somas and dendrites. Under these conditions, the cells of the molecular and granular layers are not immunoreactive. In sections c and d, the effects of antigen preabsorption of antibody are shown. Purkinje cell staining is markedly reduced. Section e is taken from a cerebellum in which the Purkinje cell population was depleted by a non-Alzheimer-related secondary pathological process. TIMP-positive axonal processes are seen in the Purkinje cell layer. This positivity is virtually eliminated by preabsorption of TIMP antibody with recombinant antigen as shown in f. a, c 200X; b, e, f 800X; and d 600X.

REFERENCES


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