Abnormal Expression and Distribution of MMP2 at Initial Stages of Alzheimer’s Disease-Related Pathology

Beatrice Ternia, Isidro Ferrera

Institute of Neuropathology, Bellvitge University Hospital, Hospitalet de Llobregat, Spain

Laboratory of Neurobiology, Bellvitge Biomedical Research Institute (IDIBELL), Hospitalet de Llobregat, Spain

University of Barcelona, Hospitalet de Llobregat, Spain

CIBERNED, Institute Carlos III, Ministry of Health, Spain

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Abstract. Previous studies have shown that metalloproteinases (MMPs) participate in the clearance of amyloid-β (Aβ) in Alzheimer’s disease (AD). MMP2 and MMP3 cleave soluble Aβ, and both MMP9 and MT1-MMP are able to degrade soluble and fibrillar forms of Aβ. The present study shows increased expression levels of active MMP2 in the entorhinal cortex at early stages of AD-related pathology (Braak and Braak stages I/II-0 and III/IV-A) as revealed by western blotting and gelatin zymography. Confocal microscopy discloses co-localization of MMP2 and phospho-tau in neurofibrillary tangles and dystrophic neurites. MMP2 has the capacity to cleave recombinant tau in vitro in a dose-dependent manner, consistent with a physiological function of MMP2 in normal tau proteolysis. However, MMP2 does not cleave hyperphosphorylated and dephosphorylated tau from enriched paired helical filament fractions. These observations raise the possibility that accumulation of MMP2 in neurofibrillary tangles and concomitant loss of proteolytic capacity on tau protein is a response geared to eliminating production of toxic truncated tau species in AD brains.

Keywords: Alzheimer’s disease, metalloproteinases, MMP2, neurofibrillary tangles, tau

INTRODUCTION

Alzheimer’s disease (AD) is a progressive neurodegenerative disease causing loss of neurons and neuronal connectivity in several brain regions. In addition, multiple metabolic pathways are altered in the course of the degenerative process [1, 2]. AD is a long-lasting biological process in which neurofibrillary tangles (NFTs) progress from the entorhinal and transentorhinal cortex (stages I and II), hippocampus and limbic system (stages III and IV), and the whole neocortex (V and VI), whereas the progress of amyloid-β (Aβ) plaques follows a different regional pattern with increasing Aβ plaque burden in the whole brain [3].

The metabolism of Aβ production and clearance, and hyperphosphorylation of tau, are very complex and involve several mechanisms, some leading to the formation of oligomeric and fibrillar species and truncated products which are toxic to nerve cells [4, 5].

A growing body of evidence indicates that matrix metalloproteinases (MMPs) may play an important role in the pathogenesis of AD and other neurodegenerative pathologies. MMPs, collectively called matrix metalloproteinases (MMPs), may play an important role in the pathogenesis of AD and other neurodegenerative pathologies.
The distribution of the MMPs in the central nervous system is widespread; they are secreted by neurons, oligodendrocytes, microglia, and endothelial cells [7]. MMPs participate in the clearance of Aβ in AD [8-11]. In particular, growing data point to MMP2 (gelatinase A) and MMP9 (gelatinase B) as important players in the metabolism of Aβ. Uregulation of MMP2 and MMP9 is induced by Aβ in mixed primary culture [12, 13]. AβPP/PS1 transgenic mice have increased levels of MMP2 and MMP9 in astrocytes around Aβ plaques; and treatment with the MMP inhibitor GM-6001 increases Aβ levels in brain interstitial fluid in these transgenic mice [14]. Although most evidence on the role of MMPs in AD points to advanced cases of the disease, some data indicate that deregulation of metalloproteinases may also occur at early stages of AD [15]. Recent studies clearly demonstrated the upregulation of MMP2 and MMP9 in the hippocampus of the 5xP3A mouse model of AD during the asymptomatic and initial stage of the pathology [16].

We hypothesized that MMP2 and MMP9 may participate in the normal metabolism of other proteins involved in AD, in addition to Aβ, and particularly in tau metabolism. Recently, it has been shown that limited cleavage by MMP9 enhances the formation of tau oligomers [17]. Furthermore, we speculated that the effects of MMP deregulation at early stages of AD-related pathology may have implications in tau proteolysis. Therefore, in this study, we started investigating modifications in the expression and localization of MMP2 in the entorhinal cortex in normal middle-aged individuals with no AD-related pathology and in cases with early stages of AD-related pathology mainly consistent with the presence of NFTs. Functional studies assessing the capacity of MMP2 to cleave recombinant tau, along with hyperphosphorylated and dephosphorylated tau aggregates extracted from cortex of AD, were carried out to increase understanding of the contribution of MMP2 in tau metabolism.

MATERIALS AND METHODS

Tissue samples

The selection of cases examined in the present study corresponded to a consecutive series of brain donations of patients dying from unrelated causes at a general hospital. The postmortem delay between death and processing was between 4 and 6.30 h. Cases with neurological symptoms and signs, hepatic or renal function impairment, hypoxia, infection, autoimmune disorders, disseminated neoplasia, or prolonged agonic state were excluded. The clinical records in all cases with AD-related pathology were re-assessed by phone calls or interviews with relatives asking about any neurological or cognitive change. Therefore, all cases with AD-related pathology were considered possible pre-clinical stages. At autopsy, half of each brain was fixed in formalin, while the other half was cut in coronal sections 1 cm thick, and selected brain regions were dissected, collected, and numbered in plastic bags, frozen on dry ice, and stored at ~80°C until use. For diagnostic morphological studies, the brains were fixed by immersion in 4% buffered formalin for 2 or 3 weeks. The tissue was embedded in paraffin, and de-waxed sections, 5 μm thick, were processed for neuropathological study. Staging of AD-related pathology was carried out following Braak and Braak criteria [3], adapted to paraffin sections [18]. Cases with additional pathology (either tau, i.e., grains; α-synuclein; Lewy bodies in other brain regions; TDP-43 pathology; or vascular in any area) were excluded. Special care was also taken to minimize the amount of Aβ plaques in the present series to increase the weight of tau pathology. Following these conditions, the following cases were used in the present study: middle-aged subjects with no neuropathological lesions (MA): 9 (8 men, 1 woman; age: 53.6 ± 9.3), AD-related pathology stages III/IV-A: 8 (4 men, 4 women; age: 66 ± 7.5), and AD-related pathology stages III/IV-A: 8 (4 men, 4 women; age: 79 ± 5.3). Studies were focused on the entorhinal cortex.

Preparation of samples

80 mg of entorhinal cortex from MA, ADIII/IV-0, and ADIII/IV-A cases was homogenized in 800 μl of RIPA buffer (50 mM Tris HCl pH 8; 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate; 0.1% SDS) plus 1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitor tablets (Roche Molecular Systems, Barcelona, Spain). The samples were then centrifuged at 10,000 x g for 10 min and the total protein of the supernatant was quantified with the BCA (bicinchoninic acid) assay (Biorad, CA, USA).

Western blot

20 μg of total protein was run in SDS-PAGE using 8% acrylamide gel and then transferred to a nitrocellulose membrane for 2 h at 200 mA. The membrane was then blocked with 5% skimmed milk for 1 h prior to overnight incubation at 4°C with anti-MMP2 monoclonal antibody (Millipore-Merck, Barcelona, Spain) at a dilution of 1:1,000. The blots
were then incubated for 2 h at room temperature with anti-mouse horseradish conjugated secondary antibody (Dako, Barcelona, Spain) and visualized with the ECL chemiluminescence method (Amersham, Barcelona, Spain). The monoclonal anti-β-actin antibody (Sigma-Aldrich, Madrid, Spain) was used as a control of protein loading. Protein expression levels were determined by densitometry of the bands using Image J software. Measurements were expressed as arbitrary units and the results were normalized for β-actin. The numerical data were statistically analyzed using ANOVA test.

In gel zymography

Gelatinolytic activity of MMP2 was measured with in gel zymography assay following a protocol adapted from one previously described [14]. Briefly, 60 mg of entorhinal cortex from MA, ADIII/0, and ADIII/A cases was homogenized in Lysis buffer (50 mM Tris-HCl pH 7.6, 150 mM NaCl, 5 mM CaCl2, 0.5% Triton, 0.05% Brij-35) along with 1 mM PMSF and protease inhibitors, and left for 30 min in agitation at 4 °C. The supernatant was collected by centrifugation at 10,000 × g for 10 min and the total amount of protein was quantified with BCA method. The same amount of protein from each case was incubated with 50 μl of gelatin-Sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) for 1 h with constant shaking at 4 °C. After centrifugation, the gelatin sepharose pellet was washed twice with lysis buffer and then incubated with Elution buffer (lysis buffer plus 10% DMSO) for 30 min in agitation at 4 °C. 30 μl of the eluted material containing gelatinases was mixed with an equal volume of 2X sample buffer pH 6.8 and run at 30 mA in a 10% SDS-PAGE gel containing 0.1% gelatin. After running, the gel was washed twice with 2.5% Triton for 20 min, equilibrated for 15 min in the activity buffer (50 mM Tris-HCl pH 7.5, 10 mM CaCl2, 0.025 NaCl), and finally incubated in the same buffer for 2 h. The gel was then stained for 1 h with 0.5% Coomassie brilliant blue in 40% methanol and 10% acetic acid, and destained until bands clearly appeared. The bands corresponding to the proteolytic activity of MMP2 were quantified using the Image J software and the data corresponding to MA and AD and cases were statistically analyzed using ANOVA test.

Double-labeling immunofluorescence and confocal microscopy

5 μm thick sections from entorhinal cortex were first de-waxed and then stained with a saturated solution of Sudan black for 10 min to block the autofluorescence of lipofuscin. After one wash in 70% ethanol and several in distilled water, the sections were blocked for 1 h with 10% fetal bovine serum (FBS) in PBS and incubated overnight at 4 °C with 1% PBS/PBS solution containing a combination of mouse anti-MMP2 (Abcam, Clone 4D3, Cambridge, UK) and rabbit anti-Tau-Thr181 (Calbiochem, Barcelona, Spain) primary antibodies used at 1:50 and 1:100 dilutions, respectively. After washing with PBS, the sections were incubated for 45 min in the dark at room temperature with a cocktail of Alexa488 anti-mouse and Alexa555 anti-rabbit secondary antibodies at a dilution of 1:400 in the same vehicle solution of primary antibodies. Nuclei were stained with DRAQ5 (Molecular Probes, Oregon, USA) diluted 1:2,000 for 10 min. Some sections were incubated only with the secondary antibodies. In addition, some samples were pre-incubated with recombinant MMP2 previously boiled to expose the antibody epitopes. The antigen-antibody mixture was made at 10:1 ratio in PBS and left overnight at 4 °C with gentle shaking. Sections from the same individual were then incubated with either MMP2 antibody alone or pre-absorbed MMP2 antibody in a parallel experiment. After washing in PBS, the sections were finally mounted in Immuno-Fluore Mounting medium (ICN Biomedicals), sealed, and dried overnight. Sections were examined with a Leica TCS-SL confocal microscope. Sections pre-incubated with the antigenic peptide and sections incubated only with the secondary antibodies were negative.

Cleavage of recombinant tau by MMP2

Full-length MMP2 (Sigma) was diluted to a final concentration of 100 μg/ml and activated through its incubation with 1 mM APMA (4-aminophenylmercuric acetate) (Sigma) for 1 h at 37 °C. Afterwards, 2 μg of the longest form of recombinant tau, which includes exons 2 and 3, and four microtubules-binding repeats (T40) (r-peptide) were incubated with 50 ng, 200 ng, and 500 ng of activated MMP2 in a buffer containing 50 mM Tris-HCl pH 7.5, 10 mM CaCl2, and 150 mM NaCl. The reaction was left at 37 °C for 3 h and then stopped by the addition of Laemmli SDS sample buffer and followed by boiling. The samples were subjected to 12% SDS-PAGE and the tau proteolytic profile was visualized first on silver staining (Amersham) and then by immunoblotting with both N-terminal tau (Millipore) and Tau 46 C-terminal (Abcam) antibodies diluted 1:1,000.
PHF cleavage by MMP2

Paired helical filaments (PHFs) made of hyperphosphorylated tau were extracted as previously described [19]. The samples were incubated with or without E. coli alkaline phosphatase (18 Units/ml, Sigma) at 68°C for 3 h. After this treatment, half of the sample was incubated with 500 ng of activated MMP2 at 37°C for 3 h. The reaction was stopped by adding Laemmli SDS sample buffer followed by boiling. The other half was immediately mixed with Laemmli SDS sample buffer and boiled. The samples were subjected to 12% SDS-PAGE and immunoblotted with N-terminal tau and Tau46 C-terminal antibodies.

RESULTS

Levels of MMP2 are increased in entorhinal cortex at early stages of AD-related pathology

Total homogenates from entorhinal cortex from MA, ADI/II-0, and ADIII/IV-A cases were run in SDS-PAGE gels and immunoblotted with antibodies recognizing full-length MMP2 (gelatinase A). The monoclonal antibody for MMP2 revealed an increment of this protein in ADI/II-0 and ADIII/IV-A with respect to MA cases (Fig. 1A, B; Supplementary Fig. 1). Values of MMP2 were normalized to β-actin; ANOVA test, used to compare the AD groups with MA group, produced p-values for ADI/II-0 and ADIII/IV-A of 0.021 and 0.0027, respectively.

To further investigate the possible alteration of this metalloprotein at early stages of AD-related pathology, MMP2 was isolated from entorhinal cortex. The gelatin zymography assay showed one typical band of 72 kDa corresponding to MMP2 (Fig. 2). A significant increase in MMP2 gelatinolytic activity was observed in ADI/II-0 (p = 0.007) and ADIII/IV-A (p = 0.008) groups with respect to MA cases with no AD-related pathology (Fig. 2A, B), in agreement with the difference in MMP2 expression levels shown by western blot.

Immu-co-localization of MMP2 with tau lesions in the entorhinal cortex at early stages of AD-related pathology

MMP2 is weakly expressed in neurons in the normal brain; however, MMP2 appears to accumulate in neurons containing NFTs in AD. Sections of entorhinal cortex of AD and MA cases were double stained with anti-MMP2 and anti-phosphoTau-Thr181 antibodies.
in order to study the distribution of the metalloproteinase with respect to the tau lesions (Fig. 3). MMP2 co-localizes with Tau-Thr181 in some NFTs and dystrophic neurites in cases with AD-related pathology.

**MMP2 cleaves recombinant tau at C-terminal region**

Full-length tau 4R (Tau40) was incubated with increasing concentrations of recombinant active MMP2 for 3 h at 37°C. The pattern of tau cleavage was analyzed in 12% SDS-PAGE and the proteolysis bands were visualized with silver staining (Fig. 4A). A gradual increase in MMP2 concentrations from 50 ng to 500 ng reduced the intensity of the 55 kDa band, corresponding to full-length tau (lane 1), while low molecular weight bands, resulting from tau cleavage, appeared in a dose-dependent manner (lanes 2–4). At the lowest concentration assayed (50 ng MMP2), three major bands of 35, 30, and 25 kDa were observed, but the band at 30 kDa become much more intense and new low molecular weight bands ranging from 25 to 10 kDa appeared at higher concentrations of MMP2.

In order to validate tau cleavage by MMP2, the proteolytic bands were visualized with immunoblotting using N-terminal or C-terminal tau antibodies (Fig. 4B). The N-terminal antibody raised against the first 16 amino acids of tau showed a band of 55 kDa corresponding to full-length tau and several proteolytic bands between 40 and 30 kDa corresponding to truncated tau (Fig. 4B, left, lane 1). Incubation with 200 ng and 500 ng of MMP2 reduced the expression of the upper bands and resulted in the appearance of intermediate bands of about 35 kDa and below 20 kDa (Fig. 4B, left, lanes 3, 4). Additionally, immunoblotting with the C-terminal tau antibody showed some bands, ranging between 37 and 25 kDa, which were present in the baseline samples and after incubation with 50 ng MMP2. Incubation with higher doses of MMP2 abolished C-terminal immunoreactivity thus indicating that major cleavage of tau occurs at the C-terminal domain. (Fig. 4B, right, comparing lanes 1, 2 with lanes 3 and 4).

**Lack of proteolysis of PHF tau by MMP2**

To further investigate whether MMP2 had an effect on tau aggregates in vivo, we analyzed MMP2 degradation of PHF-tau extracted from AD brains. Samples were analyzed with western blot using phospho-tau AT8 antibody and N-terminal and C-terminal (Tau46) antibodies (Fig. 5A, B, C, respectively). PHF-tau was treated either with or without *E. coli* alkaline phosphatase to obtain dephosphorylated and phosphorylated PHF-tau samples. Typical bands of 68 kDa, 64 kDa, and 60 kDa [19] together with a higher band of 73 kDa and several bands of low molecular weight, corresponding to truncated tau...
species, were found in non-treated samples (not subjected to dephosphorylation Fig. 5A, in all panels). No signal of phospho-tau protein was seen in the dephosphorylated sample using the AT8 antibody (Fig. 5A; lane B).

Several bands ranging between 70 and 50 kDa were observed instead following tau dephosphorylation (Fig. 5B, C; lanes B). Samples of PHF-tau treated either with or without *E. coli* phosphatase were then incubated with MMP2. Immunoblots with N-terminal and C-terminal (Tau46) antibodies did not show any difference in the degradation pattern between MMP2-non-incubated and MMP2-incubated samples independently of the phosphorylation state of tau (Fig. 5B, C, lanes C, D). No additional PHF cleavage by MMP2 was observed in phosphorylated or dephosphorylated samples even at high exposition time of the membrane (Fig. 5B, C).

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**Fig. 3.** Immunofluorescence and confocal microscopy of entorhinal cortex (layer II) of middle aged (MA) (A-C), ADIII/0 (D-F), and ADIII/IV-A (G-I) cases show MMP2 immunoreactivity in neurons with NFTs (arrows) and some dystrophic neurites (arrowhead) in ADIII/0 and ADIII/IV-A. A, D, G (green): MMP2 antibody; B, E, H (red): anti Tau-Thr181 antibody; C, F, I: merge. L-O: Detail of MMP2-Tau co-localization in tangles of cases of ADIII/0 (L, M) and ADIII/IV-A (N, O).
Fig. 4. A) MMP2 cleaves recombinant tau in a dose-dependent manner. MMP2 concentration from 50 ng to 200 ng and 500 ng (lanes 2, 3, and 4, respectively) reduces the intensity of the 55 kDa band corresponding to full-length tau. Low molecular weight bands appear following tau incubation with increasing concentrations of MMP2; silver staining. B) Immunoblotting with antibodies against N-terminal tau and C-terminal tau (Tau46) shows that incubation of recombinant tau with MMP2 results in reduced levels of full-length tau and in the appearance of bands of lower molecular weight revealed with N-terminal tau antibody. In contrast, no lower bands appear with C-terminal tau antibody following incubation of recombinant tau with 200 ng and 500 ng of MMP2, respectively.

Fig. 5. PHF-tau was extracted from AD cases and half of the sample was treated with E. coli alkaline phosphatase (lanes B and D) while the other half was not subjected to phosphatase treatment (lanes A and C). Samples were analyzed with western blot using phospho-tau AT8 antibody, N-terminal and C-terminal (Tau46) antibodies (A, B, and C, respectively). Samples without alkaline phosphatase pre-treatment showed the typical AD tau pattern of 68 kDa, 64 kDa, and 60 kDa bands together with several bands of lower molecular weight corresponding to truncated tau. This pattern is best visualized with short incubation time as seen with AT8 antibody (A, lane A). Longer incubation time, as employed with anti-N-terminal and C-terminal antibodies, results in a blurred band due to overexposure (B and C, lanes A, asterisk). Yet overexposure is needed to better visualize the effects of MMP2 incubation (lanes C and D). Hyperphosphorylated tau band pattern is modified following pre-treatment with alkaline phosphatase: no bands are seen using AT8 antibody (A, lane B), whereas several bands ranging between 70 and 50 kDa are identified with N-terminal and C-terminal antibodies (B and C, lanes B, double asterisk). Immunoblots with N-terminal and C-terminal (Tau46) antibody show no modifications of the profile of tau bands, following incubation with MMP2 (B and C, lanes C, D).

DISCUSSION

The present study, using western blotting and gelatin zymography, shows increased expression levels of MMP2 at early (Braak and Braak III/IV-0) and middle (Braak and Braak III/IV-A) stages of AD-related pathology compared with middle-aged cases with no AD-related pathology. Since the mean age in MA cases was lower than in cases with AD-related pathology, it can be suggested that increased MMP2 expression...
levels might be the effect of aging rather than the effect of AD pathology. However, MMP2 was found in some neurons with NFTs and in dystrophic neurites, co-localizing with hyperphosphorylated tau, as visualized with double-labeling immunofluorescence and confocal microscopy, thus indicating a close relationship between MMP2 and hyper-phosphorylated tau in AD.

Proteolysis of tau contributes to tangle formation [20–22]. An important fraction of tau in fibrils is truncated at positions Glu-391 and Asp-421, and tau is cleaved at Asp-421 by caspases [23]. Removal of the C-terminal domain of tau enhances nucleation and fibrillization reactions in vitro [24]. Moreover, time-course analysis and antibody labeling of tau inclusions demonstrates that tau oligomers appear early in the AD pathogenesis and are the toxic form of tau, as they are able to inhibit axonal transport in neurons and are involved in synapse and neuron loss [25–27].

So far, MMPs have always been considered to be only extracellular degrading enzymes, and their substates have always been identified in proteins of the extracellular matrix. However, MMP2 has intracellular proteolytic activity in variegated pathological conditions such as tumor growth and heart and brain ischemia [28]. MMP9 is able to cleave tau at N- and C-terminal regions, thus liberating the hexapeptide domain of the protein and facilitating tau aggregation [17]. The present study demonstrates that MMP2 is able to cleave tau in vitro after co-incubation of recombinant full-length tau with MMP2; western blotting further shows that tau fragments are likely generated at the C-terminal region of the protein. Based on these evidences, overexpression of MMP2 in ADIII and ADIV at neurofibrillary lesions, may suggest a role of MMP2 in the promotion of tau fibrillization.

It may also be suggested that MMP2 has physiological functions in normal tau proteolysis whatever tau degradation occurs in the extracellular space or in the cytoplasm of neurons. Thrombin and pro-thrombin are found in NFTs, dystrophic neurites, and reactive microglia [29], thus suggesting that they may be involved in tau proteolysis. However, increased tau phosphorylation inhibits this process, and PHFs are more resistant to thrombin cleavage [30] in the same line as we can here observed for MMP2. We can then conclude that upregulation of MMP2 at early and middle stages of AD-related pathology may be an additional mechanism which promotes tau fibrillization. Moreover, the incapacity of MMP2 to degrade hyperphosphorylated tau in paired helical filaments may further contribute to the accumulation of tau aggregates.

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SUPPLEMENTARY MATERIAL

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