

Published in final edited form as:

Biochemistry. 2010 February 16; 49(6): 1127. doi:10.1021/bi901994d.

Degradation of Soluble and Fibrillar Amyloid β -Protein by Matrix Metalloproteinase (MT1-MMP) *in-Vitro*[†]

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Abstract

The progressive accumulation of β -amyloid ($A\beta$) in senile plaques and in the cerebral vasculature is the hallmark of Alzheimer's disease and related disorders. Degradation of $A\beta$ by specific proteolytic enzymes is an important process that regulates its levels in brain. Matrix metalloproteinase 2 (MMP2) was shown to be expressed in reactive astrocytes surrounding amyloid plaques and may contribute to $A\beta$ degradation. Membrane type-1 (MT1)-MMP is the physiological activator for the zymogen pro-MMP2. Here, we show that in addition to MMP2, its activator MT1-MMP is also expressed in reactive astrocytes in regions with amyloid deposits in transgenic mice. Using a Cos-1 cell expression system we demonstrated that MT1-MMP can degrade exogenous $A\beta_{40}$ and $A\beta_{42}$. A purified soluble form of MT1-MMP degraded both soluble and fibrillar $A\beta$ peptides in a time dependent manner yielding specific degradation products. Mass spectrometry analysis identified multiple MT1-MMP cleavage sites on soluble $A\beta_{40}$ and $A\beta_{42}$. MT1-MMP-mediated $A\beta$ degradation was inhibited with the general MMP inhibitor GM6001 or the specific MT1-MMP inhibitor tissue inhibitor of metalloproteinases-2. Furthermore, *in situ* experiments showed that purified MT1-MMP degraded parenchymal fibrillar amyloid plaques that form in the brains of $A\beta$ precursor protein transgenic mice. Together, these findings indicate that MT1-MMP possesses $A\beta$ degrading activity *in vitro*.

A key pathological feature of Alzheimer's disease (AD)¹ is the progressive accumulation of β -amyloid ($A\beta$) in senile plaques and the cerebral vasculature. $A\beta$ is derived from amyloidogenic processing of the amyloid precursor protein (A β PP), which involves sequential cleavage by β -secretase and γ -secretase (1–2). The steady-state level of $A\beta$ peptides in the brain is controlled by a balance between production and clearance (3). Impaired clearance of $A\beta$ peptides is likely important in the pathogenesis of AD, especially in the more common sporadic form. Several major pathways for $A\beta$ clearance have been identified including receptor-mediated cellular uptake, blood-brain barrier transport (4–5), and direct proteolytic degradation.

Several proteinases/peptidases which can degrade $A\beta$ have been reported including neprilysin (6–7), insulin-degrading enzyme (8), plasmin (9), endothelin-converting enzyme (10), angiotensin-converting enzyme (11), myelin basic protein (12), matrix metalloproteinase

[†]This work was supported by National Institutes of Health grants HL72553 and NS55118. We thank Dr. Mahiuddin Ahmed and Dr. Steven Smith for performing the TEM analysis of fibrillar $A\beta$. ELISA antibody reagents were generously provided by Eli Lilly Laboratories.

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¹Abbreviations used are: AD, Alzheimer's disease; $A\beta$, amyloid β -protein; MMP, matrix metalloproteinase; MT1-MMP, membrane type 1 matrix metalloproteinase; Th-T, thioflavin T; Th-S, thioflavin S; DMSO, dimethyl sulfoxide; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; TIMP2, tissue inhibitor of metalloproteinase-2, MT1dTM: soluble transmembrane domain-lacking form of MT1-MMP; GFAP, glial fibrillary acidic protein.

(MMP) 2 (13–14), and MMP9 (15). Regarding MMP2, it has been reported to cleave A β peptides at several sites (14). MMP2 expression and activity is induced in cultured human cerebrovascular smooth muscle cells in response to pathogenic A β (16). Also, in astrocytes the activity of MMP2 is increased in the presence of A β (17–20). Reactive astrocytes are found in regions with fibrillar amyloid deposits in brain tissue of human AD subjects and of APPsw (Tg-2576) transgenic mice, and have been shown to participate in the A β degradation in the extracellular space (20–23).

MMP2 is released in a latent form (pro-MMP2) that requires activation by membrane-type 1 (MT1)-MMP (24). MT1-MMP was the first MMP to be identified as an integral membrane protein with a single transmembrane domain and a short cytoplasmic C-terminal tail (25). MT1-MMP is inhibited by the endogenous tissue inhibitor of MMPs 2 (TIMP-2) and recruits pro-MMP2 forming a ternary complex. Then, adjacent uninhibited MT1-MMP cleaves the tethered pro-MMP2 (26). MT1-MMP is expressed in a variety of tissues including brain (27). In addition to activating pro-MMP2, MT1-MMP is involved in the breakdown of various extracellular matrix components including collagens, laminins, fibronectin, and proteoglycans (28). This function enables it to participate in numerous normal biological processes, such as reproduction, embryonic development, wound healing, angiogenesis, and apoptosis (29) or in pathological processes, such as rheumatoid arthritis, cardiovascular disease, tumor invasion and metastasis (30).

Expression of MT1-MMP can be induced in human glioma cells and human cerebrovascular smooth muscle cells in response to A β (23). It was reported that MT-MMPs induce cleavage and shedding of the A β PP ectodomain and that one of these cleavage sites is within the A β peptide region (31). However, any role for MT1-MMP in the degradation of A β peptides and the pathology of AD is unknown. In the present study, we show that, like MMP2, MT1-MMP is expressed in reactive astrocytes in regions with fibrillar microvascular amyloid deposits in a human A β PP transgenic mouse model. Subsequently, we show that MT1-MMP expressed in Cos-1 cells is capable of degrading soluble A β 40 and A β 42 peptides. A purified soluble truncated form of MT1-MMP also degraded soluble and fibrillar A β *in-vitro*. Mass spectrometry analysis identified multiple MT1-MMP cleavage sites on soluble A β 40 and A β 42. Furthermore, *in situ* experiments show that purified soluble MT1-MMP can degrade parenchymal fibrillar amyloid plaques that form in the brains of A β PP transgenic mice. Together, these data indicate that MT1-MMP possesses A β degrading activity.

MATERIALS AND METHODS

Reagents and Chemicals

Synthetic A β 40 and A β 42 peptides were synthesized by solid-phase Fmoc (9-fluorenylmethoxycarbonyl) amino acid chemistry, purified by reverse phase high performance liquid chromatography, and structurally characterized as previously described (32). Thioflavin-S (Th-S), Thioflavin-T (Th-T) and TIMP2 were purchased from Sigma-Aldrich (St. Louis, MO). The general MMP inhibitor GM6001 was purchased from Calbiochem (La Jolla, CA).

A β PP Transgenic Mice

Generation of Tg-SwDI transgenic mice on a pure C57BL/6 background was previously described (33). These mice express low levels of human Swedish/Dutch/Iowa mutant A β PP in neurons under control of the mouse Thy1.2 promoter. Tg-SwDI mice accumulate extensive cerebral microvascular fibrillar amyloid. Brain tissues from homozygous 24 months old Tg-SwDI and similarly aged control non-transgenic mice were used in this study. In other experiments brain tissues from 18 months old Tg2576 mice, a model of AD-like parenchymal fibrillar amyloid plaques, were used (44).

Immunofluorescent Labeling

Immunofluorescent stainings were performed on paraffin sections as recently described (33). The following primary antibodies were used for immunostaining: monoclonal antibody 66.1 (1:300), which recognizes residues 1 to 5 of human A β (34); rabbit polyclonal antibody to collagen type IV (1:100; Research Diagnostics Inc., Flanders, NJ); mouse monoclonal antibody to glial fibrillary acidic protein (GFAP) for identification of astrocytes (1:300, Chemicon); mouse monoclonal anti-keratan sulfate antibody for the detection of activated microglia (clone: 5D4, 1:200, Seikagaku Corporation, Japan); rabbit polyclonal antibody to MT1-MMP (1:100; Triple Point Biologics Inc., Forest Grove, OR); rabbit polyclonal antibody to MMP2 (1:100; Sigma). Primary antibodies were detected with goat anti-rabbit IgG (Alex 594; 1:2500; Molecular Probes Inc., Eugene, OR) or/and donkey anti-mouse IgG (Alex 488; 1:2500; Molecular Probes, Inc., Eugene, OR). Th-S staining for fibrillar amyloid was performed as described (35).

Gelatin Substrate Zymography

Cos-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Gemini Bio-Products, Woodland, CA). Full-length MT1-MMP and pro-MMP2 in pcDNA3.1 plasmids were the kind gifts of Dr. Jian Cao (Department of Medicine, Stony Brook University, NY, USA). Triplicate near confluent cultures were transfected with plasmids for expression of pro-MMP2, MT1-MMP or both pro-MMP2 and MT1-MMP using FuGENE 6 (Roche, Indianapolis, IN). Transfected cells were incubated in serum-free culture media and 72 h. The conditioned culture media samples were collected and aliquots were electrophoresed on 8% SDS-polyacrylamide gels containing 0.1% gelatin at 100 V for 2 h at 22°C. The gels were removed and incubated in rinse buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl₂, 2.5% Triton-X 100) for 3 h with several changes, washed 3 \times 10 min with ddH₂O, then incubated in assay buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl₂) overnight at 37°C, washed 3 \times 10 min with ddH₂O, stained with 0.25% Coomassie Brilliant Blue R-250 and then destained. Gelatinolytic MMP activity was observed as clear zones of lysis in the gels.

A β Degradation in Cos-1 Cells Expressing Human MT1-MMP

Triplicate near confluent cultures were transfected with purified empty pcDNA3.1 plasmid DNA or full-length MT1-MMP in pcDNA3.1 DNA by FuGENE 6 treatment (Roche, Indianapolis, IN), followed by addition of 2 μ g/ml of A β 40 or A β 42 in serum-free media for 48 h. The culture media samples were collected and cell lysates were prepared. A β in the cell culture media samples was quantitatively analyzed by immunoblotting and sandwich ELISA analysis as described above.

Quantitative Immunoblotting

Samples containing MT1-MMP or A β were added directly into SDS-PAGE sample buffer, and stored at -70°C. Aliquots were loaded onto 12% or 10–20% polyacrylamide gels, electrophoresed and transferred onto Hybond-ECL nitrocellulose membranes (Amersham, Arlington Heights, IL) at 100 V for 1.5 hour at RT. Membranes were blocked in 5% milk/PBS/0.05% Tween20 (PBS-T) for 1 h at RT. Primary antibodies were added (RP1-MMP14 for MT1-MMP; mAb20.1 for A β) for 1 h at RT, washed 3 \times min with PBS-T. Horseradish peroxidase-conjugated mouse sheep anti-rabbit or anti-mouse IgG (1:5000 Amersham-Pharmacia, Piscataway, NJ), and washed 3 \times 5 min with PBS-T. Bands were visualized using the ECL detection method (Amersham-Pharmacia, Piscataway, NJ). Quantitation of MT1-MMP or A β bands was performed using a VersaDoc Imaging System (BioRad, Hercules, CA) and the manufacturer's Quantity One software.

A β ELISA Analysis

The levels of soluble A β 40 and A β 42 peptides were measured using a quantitative sandwich ELISA as previously described (33).

Purification of Soluble MT1dTM Protein

The cDNA for a soluble, truncated form of MT1-MMP encoding residues Met¹-Gly⁵³⁵ that lack the carboxyl-terminal transmembrane and cytosolic domains of full length MT1-MMP (MT1dTM) in pSG5 expression vector was the kind gift of Dr. Jian Cao (Department of Medicine, Stony Brook University, NY, USA). Two hundred ml of serum-free conditioned media from Cos-1 cells overexpressing soluble MT1dTM were passed through gelatin-agarose (Sigma-Aldrich, St. Louis, MO) to remove any gelatinases and then concentrated using an Amicon ultrafiltration unit (NMWL 5000 membrane) (Millipore, Bedford, MA). The enzymatic activity of purified MT1dTM was determined using the specific substrate- Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg (Bachem, California, CA).

In-vitro Soluble A β Degradation

Synthetic A β 40 or A β 42 were first dissolved in DMSO to a concentration of 1 mg/ml. 40 nM of purified MT1dTM were incubated with 1 μ M of synthetic A β 40 or A β 42 in zymogen buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl₂) at 37 °C for specific lengths of time. The A β levels were measured in the samples by SDS-PAGE on 10–20% polyacrylamide Tris-Tricine gels and subsequent quantitative immunoblotting (as described above). In some experiments the selective MMP inhibitors GM6001 (10 μ M; Calbiochem) or TIMP2 (40 nM; Sigma) were added.

To visualize MT1-MMP generated A β cleavage products N-terminal biotin labeled A β 40 or A β 42 were incubated with purified MT1dTM for 24 h at 37 C. The samples were diluted in the sample buffer containing 9M urea/5% acetic acid and methyl green. For analysis an acid/urea 22% polyacrylamide gel was prepared and pre run anode to cathode at 250 V for 30 min at 4°C in 5% glacial acetic acid running buffer (36). Following the pre run, the samples were loaded on the gel and electrophoresed at 4 ° from anode to cathode with increasing the voltage every 15 min as follows: 25, 50, 100, 200 volts and then 275 volts for 15 h until the end of the run. Prior to transfer, the acid/urea gel was neutralized in a glass tray by washing 5x with Tris-HCl/glycine transfer buffer on a rocking platform for 15 min. Then the gel was transferred to PVDF membranes by electroblotting for 2.5 hr (80V) at 4°C. After transfer, the membrane was boiled in PBS for 5 min in a glass dish and was cooled down in PBS. The membrane was blocked in 5% milk/PBS/0.05% Tween20 (PBS-T) for 1 h at RT. The membrane was incubated with streptavidin-horseradish peroxidase (1:5000 dilution) for 1 h at RT, and washed 3 \times 5 min with PBS-T. Bands were visualized using the ECL detection method as described above.

Mass Spectrometry

40 nM purified MT1dTM was incubated with 1 μ M synthetic A β 40 or A β 42 in zymogen buffer (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 5 mM CaCl₂) at 37°C for 2 days. After incubation, the samples were dried in a rotary evaporator (Savant, Farmingdale NY), suspended in 20 μ l of 0.1% TFA, ZipTipped using μ C18 tips (Millipore, Milford, MA) and then eluted to the target. The addition of 1 μ l of matrix consisting of acetonitrile/0.1% trifluoroacetic acid containing ω -cyano-4-hydroxy cinammic acid (CHCA, 5 mg/ml) was dried on the sample plate. Samples were run on a *Voyager-DE STR* (Applied Biosystems, Framingham, MA) using a matrix assisted laser desorption ionization - time of flight (MALDI-TOF) mass spectrometer system operated in the reflector mode unless otherwise indicated. The mass scale (m/z 500–5000) was calibrated with a mixture of peptides or internal calibration was performed using a matrix ion at m/z 568.1330 and A β 42 peptide amino acid 1–13 m/z 1561.6672. For samples

acquired in the linear mode, 1 μ l was dissolved in 10 μ l of a 50% solution of acetonitrile/0.3% trifluoroacetic acid containing sinapinic acid (10 mg/ml) and dried on the sample plate. The mass scale (m/z 1000–25000) was calibrated with myoglobin (400 fM/ μ l).

In-vitro Fibril A β Degradation

To prepare amyloid fibrils, 5 mM A β 42 in DMSO was diluted in PBS to 100 μ M, vortexed for 30 sec, and incubated at 37 °C for 5 days (37). Triplicate samples of 10 μ M of aged fibrillar A β was then incubated with 100 nM of purified MT1dTM in absence or presence of 100 μ M GM6001 at 37 °C for 5 days. After digestion, the remaining fibrillar A β was quantitated using a Th-T fluorescence binding assay. Briefly, 5 μ l of 100 μ M Th-T was added to 100 μ l of sample, mixed and incubated at RT in the dark for 10 min. Th-T fluorescence, an indicator of fibril A β binding, was measured at λ_{ex} of 446 nm and λ_{em} of 490 nm.

Transmission Electron Microscopy

Sample aliquots were deposited onto carbon-coated copper mesh grids. Sample grids were allowed to stand for 60 sec, and excess solution was wicked away. Sample grids were then negatively stained with 2% (w/v) uranyl acetate and allowed to dry. The samples were viewed with an FEI Tecnai 12 BioTwin transmission electron microscope at 80 kV, and digital images were taken with an Advanced Microscopy Techniques camera.

In-Situ Fibrillar Amyloid Plaque Degradation

For this analysis the well-characterized Tg2576 (APP^{sw}) mouse model of AD that develops abundant fibrillar amyloid pathology (38) was used. Brains were removed from anesthetized 18 months old Tg2576 mice after perfusion with cold saline and snap-frozen on dry ice. Five- μ m cryostat sections will be collected on slides. Every other section was flipped 180° so that identical faces of adjacent sections were exposed (37). Paired adjacent sections (one incubated with zymogen buffer, the other with 100 nM purified MT1dTM in absence or presence of 100 μ M GM6001) in triplicate were incubated at 37°C for 5 days, stained with thioflavin-S (ThS), and then imaged with fluorescence microscopy. The parenchymal plaque amyloid area of ThS fluorescence was determined using image analysis software (Image J). Fractional area was compared between paired sections.

Statistical Analysis

Data were analyzed by Student's t-test at the 0.05 significance level.

RESULTS

MT1-MMP and MMP2 are expressed in brain regions with prominent cerebral microvascular fibrillar A β deposits in Tg-SwDI mice

Previously, MMP2 was found increased in reactive astrocytes adjacent to parenchymal amyloid plaques in aged A β PP transgenic mouse brain (39). MMP2 expressed by reactive astrocytes is implicated in extracellular A β catabolism (39). We have generated the Tg-SwDI mouse model, which develops early-onset and progressive accumulation of regional cerebral microvascular fibrillar amyloid deposition (33). Th-S staining of brain sections of aged Tg-SwDI mice revealed extensive fibrillar A β accumulation in the microvessels of the thalamus, but not in the cortex (Fig. 1A,B). MMP2 is also expressed in cells in the thalamus where fibrillar microvascular A β accumulates, but not the cortex (Fig. 1C,D). MMP2 is expressed as an inactive zymogen (pro-MMP2) requiring proteolytic activation by MT1-MMP. Labeling for MT1-MMP also showed strong expression by cells in the thalamus where fibrillar microvascular A β accumulates, but not the cortex (Fig. 1E,F).

MT1-MMP and MMP2 are selectively expressed in reactive astrocytes in brain regions with microvascular fibrillar amyloid deposits in aged Tg-SwDI mice

To identify the cell type that expresses MMP2 and MT1-MMP near microvascular amyloid deposits the Tg-SwDI mouse brain sections were double immunolabeled for GFAP to detect reactive astrocytes and either MMP2 or MT1-MMP (Fig. 2). Immunolabeling for MMP2 and its activator MT1-MMP strongly co-localized with GFAP-positive cells. Immunolabeling for activated microglia in these tissue sections failed to show co-localization with MMP2 or MT1-MMP (data not shown). These data show that like MMP2, its activator MT1-MMP is selectively expressed in reactive astrocytes near cerebral microvascular fibrillar amyloid deposits in aged Tg-SwDI mouse brain.

Exogenous A β 40 and A β 42 degradation in Cos-1 cells expressing human MT1-MMP

We next determined if MT1-MMP, like MMP2, could play a role in A β degradation using a cell culture expression system. Cos-1 cells were chosen since they do not normally express either MT1-MMP or MMP2. Therefore, Cos-1 cells were transfected to express pro-MMP2 alone, MT1-MMP alone or both pro-MMP2 and MT1-MMP. Post transfection, the cells were incubated with serum-free media for an additional 48 h. The cell lysates were collected and analyzed by immunoblotting using an anti-MT1-MMP antibody demonstrating protein expression in the cells transfected with the MT1-MMP plasmid (Fig. 3A). The culture media samples were collected and subjected to gelatin zymography to assay for MMP2 activities (Fig. 3B). The zymography assayed showed that pro-MMP2 was only expressed in the Cos-1 cells transfected with the pro-MMP2 plasmid. Whereas pro-MMP2 alone migrated at ≈ 72 kDa the co-transfection with pro-MMP2 and MT1-MMP exhibited activated MMP2 which migrated as a doublet at ≈ 66 kDa. These experiments demonstrated that MT1-MMP expressed in Cos-1 cells was enzymatically active.

To determine if MT1-MMP expressed in Cos-1 cells could degrade A β , the cells were transfected with either empty plasmid vector (pcDNA3.1) or the MT1-MMP plasmid vector. Post transfection; the cells were incubated with 2 μ g/ml of freshly prepared soluble A β 40 or A β 42 in serum-free media for an additional 48 h. The cell lysates were collected and analyzed by immunoblotting using the anti-MT1-MMP antibody confirming MT1-MMP protein expression in the transfected cells (Fig. 4A). Although small amounts of A β peptides were found associated with the cells present in the cell lysates there was no difference in the amounts between control and MT1-MMP expressing Cos-1 cells (data not shown). The media samples were collected and analyzed for A β 40 and A β 42 peptide levels by immunoblotting using monoclonal anti-A β (Fig. 4B,D, respectively) and by quantitative ELISA measurements (Fig. 4C,E, respectively). These results indicate that both A β 40 and A β 42 were strongly reduced by about 50% and 70%, respectively, in MT1-MMP transfected Cos-1 cells.

***In-vitro* A β 40 and A β 42 degradation by purified soluble MT1-MMP**

MT1-MMP is normally expressed as a membrane bound enzyme. However, a soluble transmembrane domain-lacking form of MT1-MMP (MT1dTM) can be used to study the proteolytic function of the enzyme in solution. Therefore, we used purified MT1dTM protein to investigate if A β peptides could be degraded *in-vitro*. A β 40 or A β 42 (1 μ M) was incubated at 37°C in the presence or absence of purified MT1dTM (40 nM) up to 24 h. At designated time points, samples were collected and analyzed for A β levels by quantitative immunoblotting using the anti-A β mAb. As shown in Fig. 5, A β 40 and A β 42 were degraded by purified MT1dTM *in-vitro* in a time-dependent manner with $\approx 40\%$ reduction in the levels of both peptides in 24 h.

To confirm that the enzymatic activity of purified MT1dTM was required for the A β degradation *in-vitro* we used the general MMP inhibitor GM6001 and specific MT1-MMP

inhibitor TIMP2, A β 40 was incubated with purified MT1dTM at 37°C in the presence or absence of GM6001 or TIMP2 for 24 h. The immunoblotting data presented in Fig. 6 shows that MT1dTM mediated A β 40 degradation was blocked by GM6001 and TIMP2 indicating that the proteolytic activity of MT1dTM was responsible for the observed A β degradation.

The data above demonstrates that MT1-MMP exhibits proteolytic activity towards A β 40 or A β 42 *in-vitro* or in Cos-1 cells expressing MT1-MMP. However, these analyses only show loss of intact A β peptides based on immunoblotting or ELISA analysis. To identify MT1-MMP mediated A β cleavage products we performed acid/urea gel analysis, a technique that can resolve low molecular mass peptides. For this analysis soluble amino-terminal, biotinylated A β 40 or A β 42 peptides were incubated with purified MT1dTM for 48 h. Following incubation, the samples were electrophoresed on a 22% polyacrylamide acid/urea gels, transferred to membranes, and analyzed for biotin-labeled intact A β and amino-terminal fragments using a streptavidin-horseradish peroxidase conjugate. As shown in Fig. 7, the levels of intact biotin-labeled A β 40 and A β 42 were markedly reduced by digestion with MT1dTM and several biotin-labeled amino-terminal fragments of each A β peptide were observed. These data further confirm that MT1dTM degrades soluble A β *in-vitro*.

To identify specific cleavage products, synthetic A β 40 or A β 42 was digested with purified MT1dTM and analyzed by MALDI-TOF mass spectrometry (Fig. 8). The major fragments generated from proteolytic cleavage of A β 40 were similar to those generated from A β 42. Several cleavage sites were identified mainly around V12 through L17, generating major fragments of 1–14 to 1–17, which were consistent with the amino terminal major cleavage products shown in the acid/urea gels (Fig. 7).

***In-vitro* fibrillar A β degradation by purified soluble MT1-MMP**

A β peptides largely accumulate in the AD brain in the form of fibrillar amyloid deposits. To determine whether MT1-MMP could degrade fibrillar A β , we prepared aged fibrillar A β 42, subsequently incubated it with purified MT1dTM at 37° for 5 days and measured the remaining fibrillar A β using a Th-T fluorescence binding assay. Fig. 9A shows a >50% reduction in the Th-T fluorescence signal in the fibrillar A β sample treated with MT1dTM. Importantly, the MMP inhibitor GM6001 largely blocked MT1dTM mediated fibrillar A β degradation indicating the loss of fibrillar A β was dependent on the enzymatic activity of MT1dTM. To further confirm this finding at the ultrastructural level fibrillar A β was incubated in the absence or presence of purified MT1dTM for 5 days and then TEM analysis was performed to visualize the extent fibrillar A β structure (Fig. 9B). Fibrillar A β incubated with MT1dTM showed a marked reduction in the number and length of amyloid fibrils. Together, these data indicate that MT1dTM is also capable of degrading the assembled fibrillar form of A β .

MT1dTM degrades parenchymal fibrillar A β plaques *in situ*

The above data showed that purified MT1dTM was capable of degrading soluble and fibrillar synthetic A β peptides *in vitro*. We next determined if purified MT1dTM could degrade actual amyloid deposits that form in the brains of APP transgenic mice. To do this, adjacent brain slices of aged Tg2576 mice, which contain abundant amyloid plaques, were incubated at 37° C for 5 days with buffer alone or purified MT1dTM in the presence or absence of the MMP inhibitor GM6001. After incubation the sections were stained with Th-S and the area of fluorescence between matching fibrillar plaque deposits from adjacent sections was measured. The area of Th-S fluorescence of adjacent brain sections did not show a difference when incubated with buffer alone, while the area of parenchymal amyloid plaque deposits was significantly decreased ($p < 0.001$) in the brain sections incubated with purified MT1dTM (Fig. 10). Importantly, amyloid plaque degradation by purified MT1dTM was effectively blocked

with the MMP inhibitor GM6001. These results suggest that purified MT1dTM is capable of degrading fibrillar amyloid plaques in brain tissue.

DISCUSSION

In the present study we show that MT1-MMP, the physiological activator of pro-MMP2, can degrade soluble and fibrillar forms of A β *in vitro*. The MMP superfamily consists of secreted and membrane types of metalloproteinases largely involved in degradation and remodeling of the extracellular matrix. It was previously shown that MMP2 and MMP9 are produced by reactive astrocytes surrounding amyloid plaques in aged human A β PP transgenic mice (17–20,39). Similarly, we found that MMP2 and its activator MT1-MMP are expressed in reactive astrocytes in brain regions with microvascular amyloid deposits in aged Tg-SwDI mice (Figs. 1 and 2). Previously, we reported that pathogenic A β stimulates the expression and activation of MT1-MMP and MMP2 in the cultured human cerebrovascular smooth muscle cells (16, 40). Consistent with these earlier *in vitro* findings, in aged Tg-SwDI mice we also found MMP2 and MT1-MMP expression in the smooth muscle cell medial layer of meningeal vessels that occasionally contained fibrillar A β deposits (data not shown).

MT1-MMP was shown to degrade A β peptides in both its natural transmembrane form expressed in Cos-1 cells and as a purified soluble form lacking the carboxyl-terminal transmembrane region (MT1dTM). However, more robust degradation of A β peptides was observed when MT1-MMP was expressed in Cos-1 cells compared to using the soluble MT1dTM form *in vitro*. This disparity may reflect different levels of MT1-MMP present in each type of experiment or, more likely, is a consequence of the soluble MT1dTM exhibiting less enzymatic activity than its natural transmembrane counterpart (41). In any case, soluble MT1dTM provided a useful tool to demonstrate degradation of soluble and fibrillar A β in *in vitro* and *in situ* experiments.

Most of the well-known A β -degrading enzymes such as endothelin converting enzyme, insulin-degrading enzyme, and neprilysin largely show degradative activity towards soluble forms of A β , but not fibrillar A β . However, plasmin and MMP9 are two A β -degrading enzymes shown to be capable of degrading fibrillar A β *in-vitro* (37,42). In the initial experiments of the present study we mainly focused on the degradation of the monomer form of soluble A β in the *in-vitro* assay or in Cos-1 cells. However, in Fig. 4 above the prominent A β monomer a faint A β dimer band was observed which was also degraded in Cos-1 cells expressing MT1-MMP. Although we did not investigate the specific degradation of other soluble forms of A β such as trimers, tetramers, or higher order oligomers our experiments showed that fibrillar A β was degraded by soluble MT1dTM. Based on this latter finding we predict that other soluble oligomeric forms of A β are likely degraded by MT1-MMP although this will need to be confirmed.

Several structural models of amyloid fibrils have been proposed (43). The common feature is the β -pleated sheet structure perpendicular to the fibril axis with a hairpin loop at the C-terminus. The conversion of soluble A β to fibrillar amyloid is accompanied by an increased resistance to proteolytic degradation (44). In this regard it is noteworthy that purified soluble MT1dTM can similarly degrade fibrillar A β *in-vitro* and fibrillar amyloid deposits in brain tissue sections of human A β PP transgenic mice. MMP9, like MT1-MMP, cleaves between residues A³⁰-I³¹ (37). This site is exposed on the surface of A β fibrils allowing access for cleavage by MMP9 and MT1-MMP (37). In contrast, A β fibrils were observed to be more resistant to degradation by MMP2. It was proposed that the major MMP2 cleavage site of Leu34-Met35 within the hydrophobic domain of A β would be inaccessible within an amyloid structure (44). Collectively, these findings suggest that the various A β -degrading enzymes likely work at different sites in the brain for A β catabolism. For example, secreted A β -

degrading enzymes such as IDE, MMP2, and MMP9 may effectively target soluble forms of A β in interstitial fluid whereas membrane-bound A β -degrading enzymes such as neprilysin and MT1-MMP are better suited for deposited fibrillar A β or A β associated with cell surfaces.

MT1-MMP appears to be highly expressed in brain regions exhibiting amyloid pathology and neuroinflammation (Figs. 1 and 2). On the other hand, in normal brain or in the absence of amyloid pathology little, if any, expression of MT1-MMP is observed. This suggests that under normal conditions MT1-MMP likely has little involvement in regulating basal brain A β levels compared with other A β -degrading enzymes that are constitutively expressed. However, when amyloid deposition and neuroinflammation occur, as in AD, reactive astrocytes and vascular smooth muscle cells markedly increase their expression of MT1-MMP which may then play a significant role degrading soluble and deposited A β peptides. This increased expression in response to amyloid deposition implies that MT1-MMP may be an opportunistic A β -degrading enzyme. Future experimentation will be needed to determine if MT1-MMP does indeed contribute to A β degradation *in vivo* under pathological conditions when it is likely expressed.

Various members of the MMP superfamily may play some role regulating the levels of A β in the CNS. For example, MMP2, MMP9, and MT1-MMP possess A β -degrading activity (15, 45). MMP2, MMP3, MMP9 and MT1-MMP exhibit increased expression in response to A β (16,23,46). However, the protein levels and activity of MMP2, MMP3, and MMP9 showed no difference in the frontal cortex of AD patients compared with control patients (47). This may reflect a very limited, focal expression in specific cells that was not discerned in this study. It was reported that MT1-MMP, MT3-MMP and MT5-MMP have α secretase-like shedding activity on A β PP which would preclude A β formation (31). More specifically, recombinant MT3-MMP showed multiple cleavage sites on A β PP within the A β domain. Since the shedding pattern for MT1-MMP and MT3-MMP are very similar, MT1-MMP may also cleave A β PP within the same sites. Here, our mass spectrometry data showed an MT1-MMP cleavage site at the H¹⁴-Q¹⁵, which is the same as an MT3-MMP shedding site on A β PP (30). However, A β peptide was not degraded by recombinant MT3-MMP or by cells expressing MT3-MMP. Therefore, regarding MT-MMPs the A β degradation activity appears specific to MT1-MMP.

In conclusion, we have demonstrated that MT1-MMP is selectively expressed in reactive astrocytes near fibrillar amyloid deposits in human A β PP transgenic mouse brain. MT1-MMP was found to degrade soluble A β 40 and A β 42 as well as fibrillar amyloid. Together, our data suggest MT1-MMP could function as an opportunistic A β degrading enzyme when expressed by reactive astrocytes adjacent to fibrillar amyloid deposits. Future *in vivo* studies are needed to determine its role in relation to other identified A β degrading enzymes in regulating A β levels in brain.

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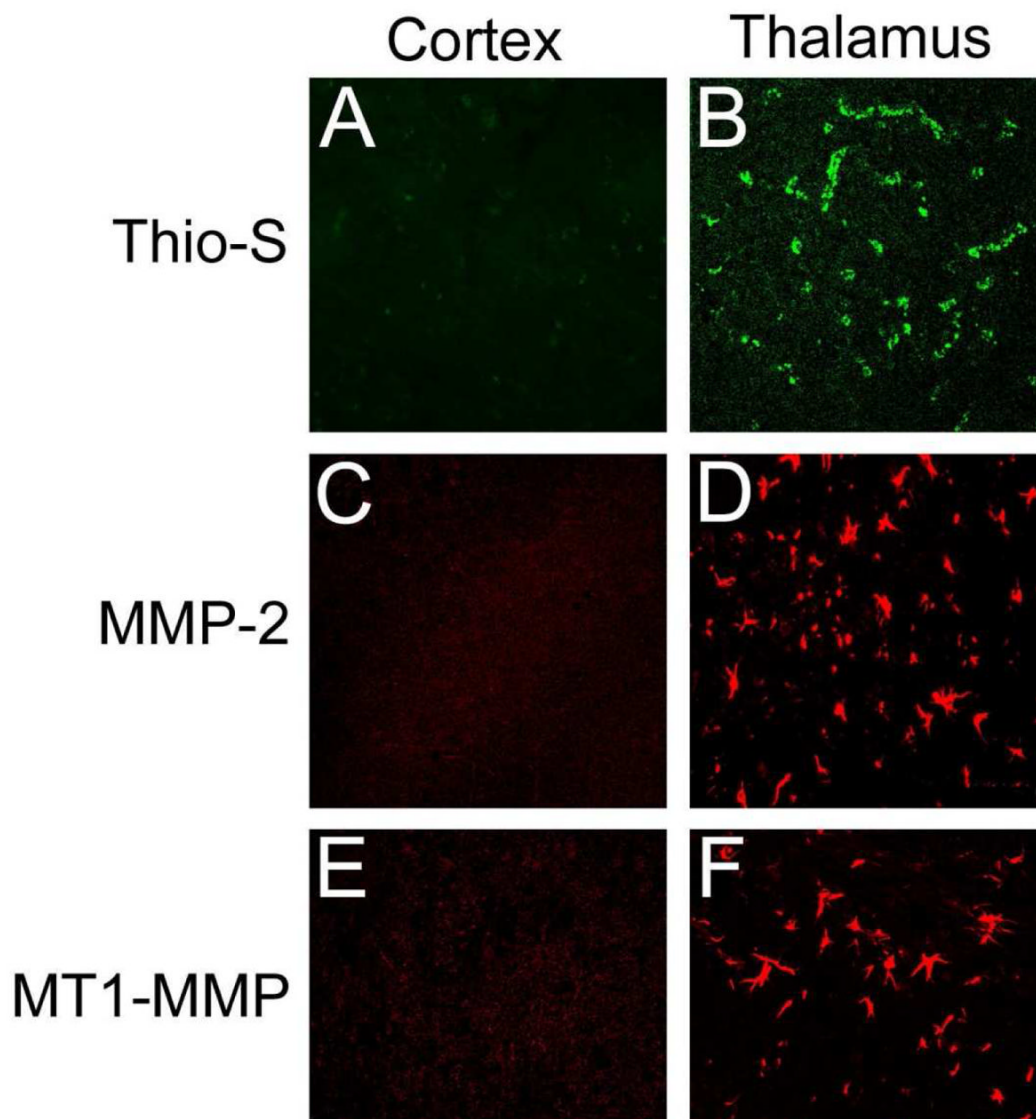


Fig. 1. MT1-MMP and MMP2 are expressed in regions of fibrillar A β accumulation in Tg-SwDI mouse brain

Brain sections from twenty four month old Tg-SwDI mice were labeled for fibrillar A β using Thio-S (green) showing that the cortex (A) lacks appreciable fibrillar amyloid whereas the thalamic region (B) contains extensive microvascular amyloid accumulations.

Immunolabeling for MMP2 or MT1-MMP (red) in adjacent brains sections shows weak expression in the cortex (C and E, respectively) but strong expression in the thalamic region (D and F, respectively) containing abundant microvascular amyloid. Scale bars = 50 μ m.

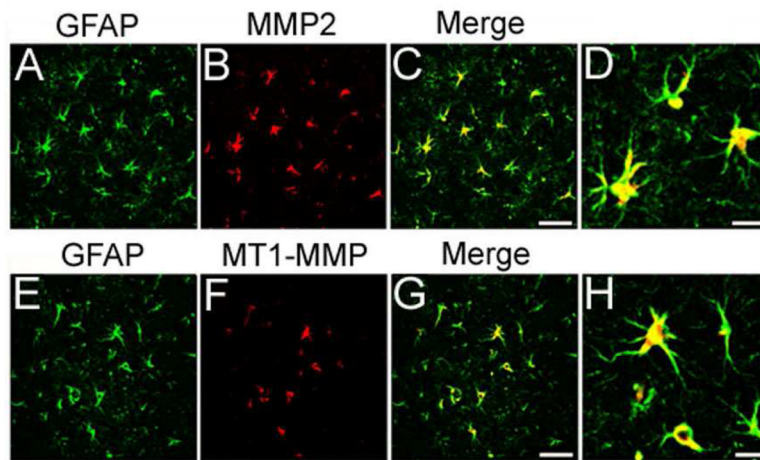


Fig. 2. MT1-MMP and MMP2 are expressed in reactive astrocytes near fibrillar microvascular amyloid deposits in Tg-SwDI mouse brain

Brain sections from twenty four month old Tg-SwDI mice were double immunolabeled for GFAP to identify astrocytes (green) and MMP2 or MT1-MMP (red). The thalamic region, which contains extensive microvascular fibrillar amyloid, is shown. Numerous reactive astrocytes were observed (A and E) as well as strong immunolabeling for MMP2 (B) and MT1-MMP (F). Merging of the images showed strong co-localization of GFAP and MMP2 (C) or MT1-MMP (G). Scale bars = 50 μ m. Higher magnifications of the merged images are shown in (D) and (H), respectively. Scale bars = 10 μ m.

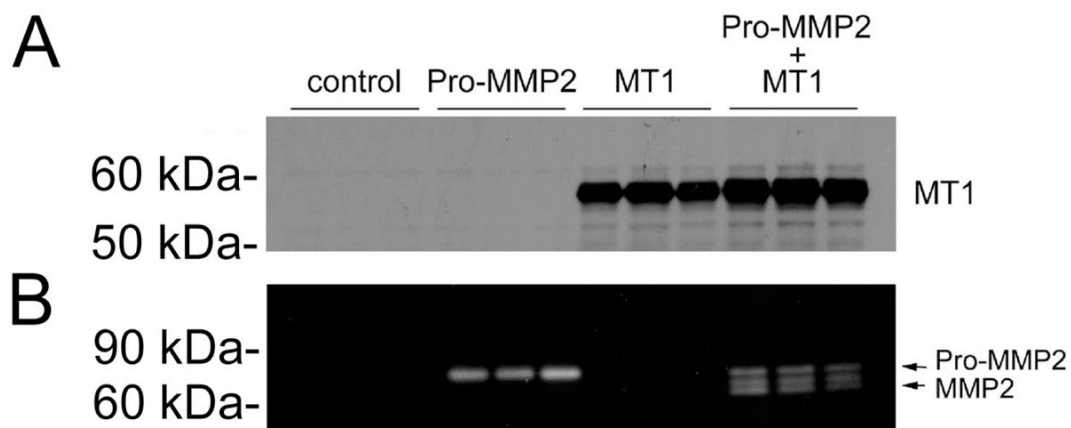


Fig. 3. Activation of pro-MMP2 by MT1-MMP expressed in Cos-1 cells

Triplicate cultures of Cos-1 cells were transfected with empty plasmid vector (pcDNA3.1), Pro-MMP2 vector alone, MT1-MMP vector alone, or both Pro-MMP2 vector and MT1-MMP vector. Twenty four hours post transfection, the cells were incubated in serum-free medium for an additional 48 h. **(A)** The cell lysates were collected and analyzed by immunoblotting by using anti-MT1-MMP. **(B)** The culture media samples were collected and analyzed by gelatin zymography. Co-expression of Pro-MMP2 and MT1-MMP led to conversion of pro-MMP2 to MMP2 demonstrating the MT1-MMP was proteolytically active.

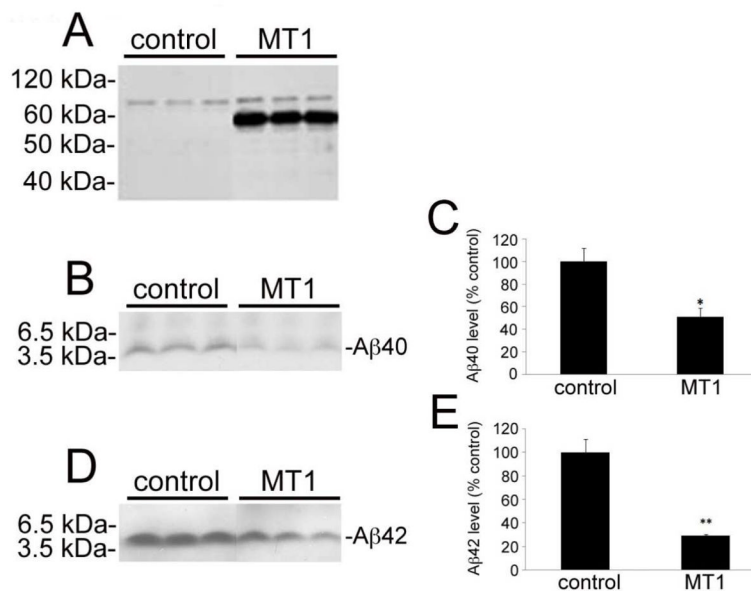


Fig. 4. Aβ40 and Aβ42 are degraded by MT1-MMP expressed in Cos-1 cells

Triplicate cultures of Cos-1 cells were transfected with empty plasmid vector (pcDNA3.1) or MT1-MMP vector. Twenty four hours post transfection, the cells were incubated with 2 μg/ml of freshly solublized Aβ40 or Aβ42 in serum-free media for an additional 48 h. (A) The cell lysates were collected and analyzed by immunoblotting using anti-MT1-MMP. The culture media samples were collected and analyzed for Aβ40 and Aβ42 peptides levels by immunoblotting using anti-Aβ (B and D, respectively) and by ELISA (C and E, respectively). The data shown are the mean ± S.D. (n=3). *, p < 0.05; **, p < 0.01.

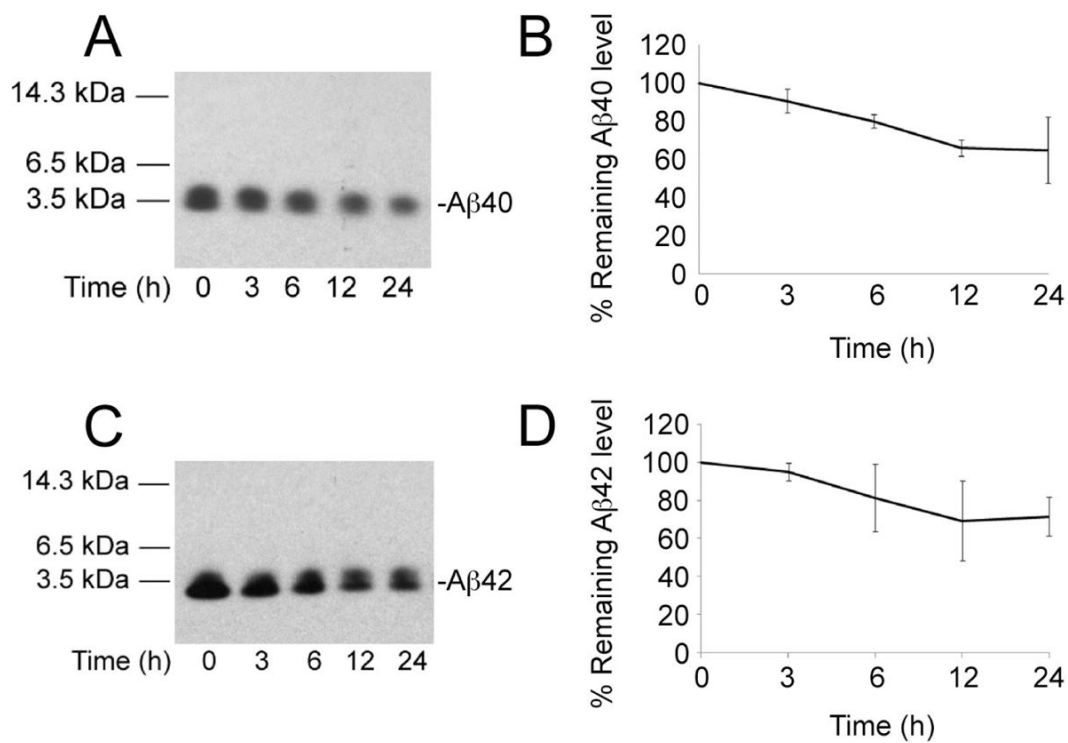


Fig. 5. A β 40 and A β 42 degradation by soluble MT1dTM
A β 40 (**A** and **B**) or A β 42 (**C** and **D**) was incubated at 37°C in the presence or absence of purified 40 nM of MT1dTM. At each time point, samples were collected and analyzed for A β level by quantitative immunoblotting using anti-A β mAb. The data shown are the mean \pm SD of three separate determinations.

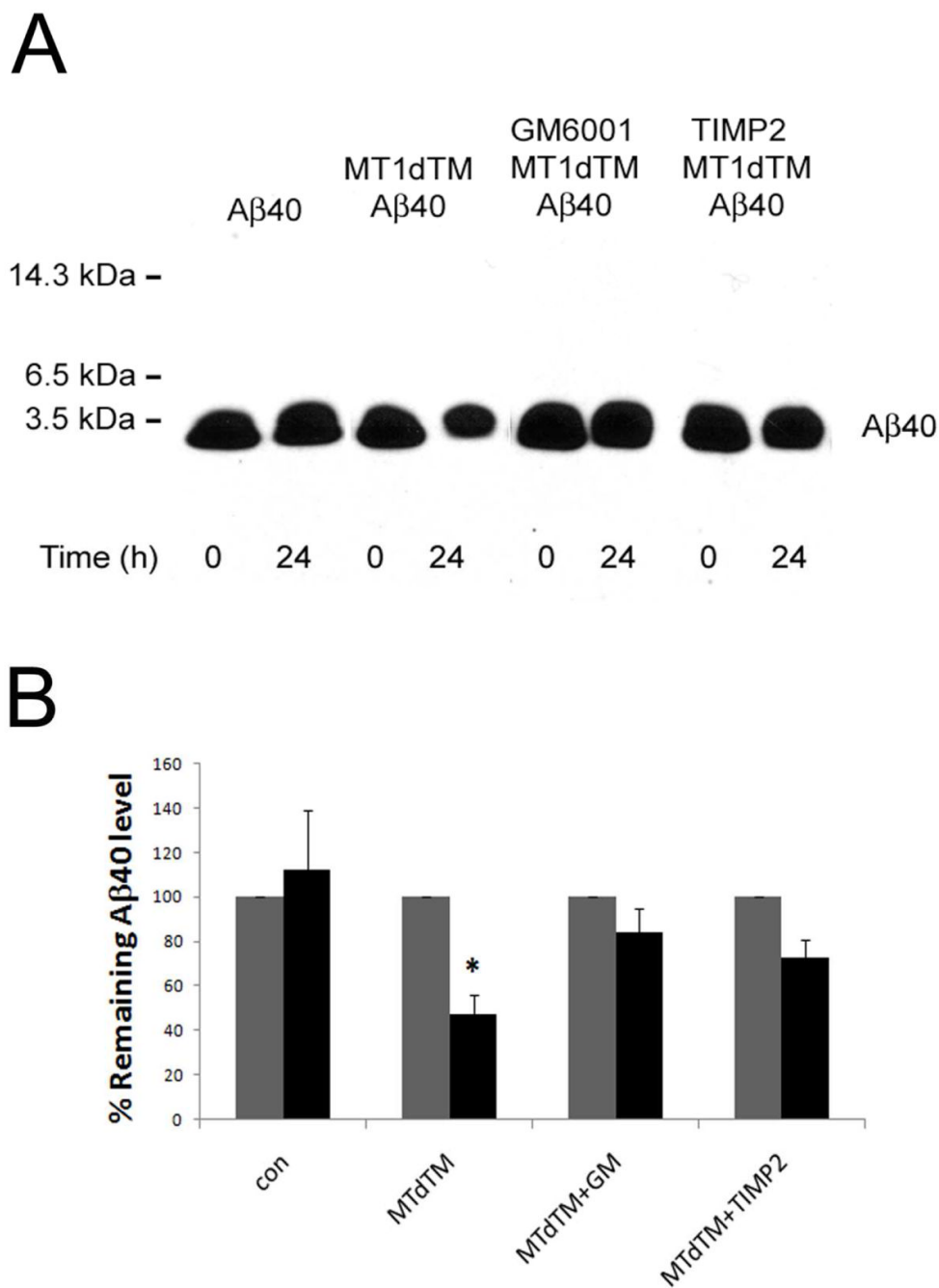


Fig. 6. Aβ40 degradation by soluble MT1dTM is inhibited by GM6001 and TIMP2

Aβ40 was incubated with purified soluble MTdTM at 37°C in the presence or absence of the general MMP inhibitor GM6001 (10 μM) or the specific MT1-MMP inhibitor TIMP2 (40 nM) for 24 h. Following incubation, the samples were collected and analyzed for Aβ levels by quantitative immunoblotting using anti-Aβ (A). The data shown are the mean ± SD of three separate determinations (B). *, $p < 0.05$, paired t test.

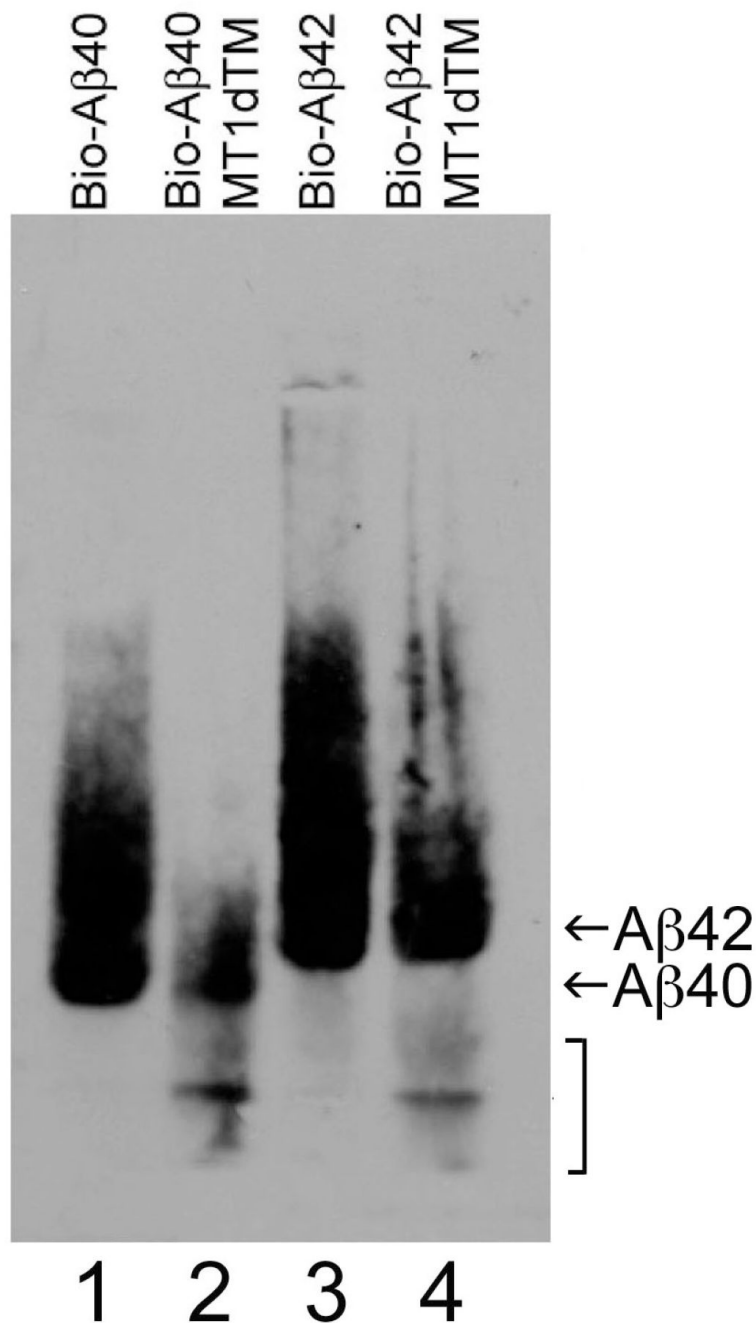


Fig. 7. Analysis of MT1dTM mediated Aβ cleavage fragments on acid/urea gels

Soluble amino terminal, biotinylated Aβ40 or Aβ42 were incubated with purified soluble MT1dTM for 48 h. Following incubation, samples were separated on 22% polyacrylamide acid/urea gels, transferred to membranes, and analyzed for Aβ products by using a streptavidin-horseradish peroxidase conjugate to detect biotinylated peptides and fragments. Lane 1, biotinylated-Aβ40; lane 2, biotinylated-Aβ40 + MT1dTM; lane 3, biotinylated-Aβ42; and lane 4, biotinylated-Aβ42 + MT1dTM. The brackets denote amino terminal cleavage products common to Aβ40 and Aβ42.

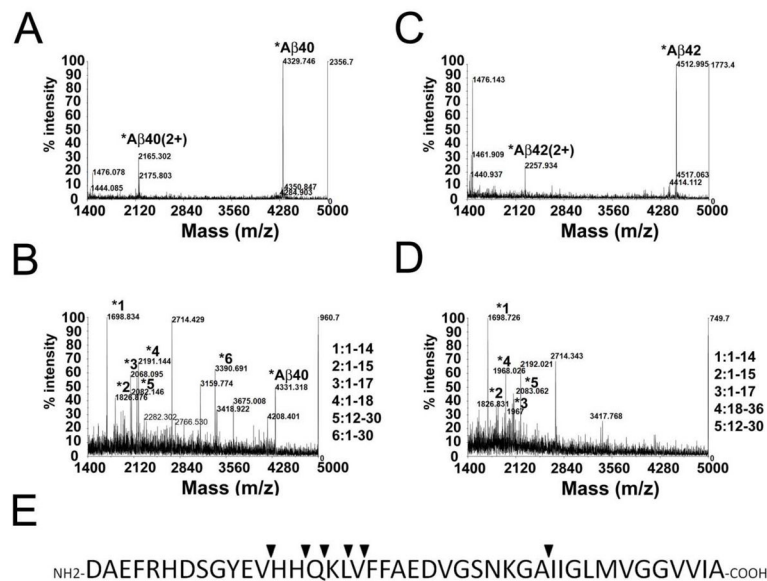


Fig. 8. MALDI-TOF MS analysis of Aβ fragments released from by purified MT1dTM. Synthetic Aβ40 alone (A), Aβ42 alone (C), Aβ40 and purified MT1dTM (B) or Aβ42 and purified MTdTM (D) were incubated with 37°C for 2 days. After incubation the samples were analyzed by MALDI-TOF mass spectrometry. Comparing with Aβ40 or Aβ42 alone, several specific peaks were identified as Aβ fragments (reflector mode). (E) Summary of the MT1dTM cleavage sites on Aβ (▼).

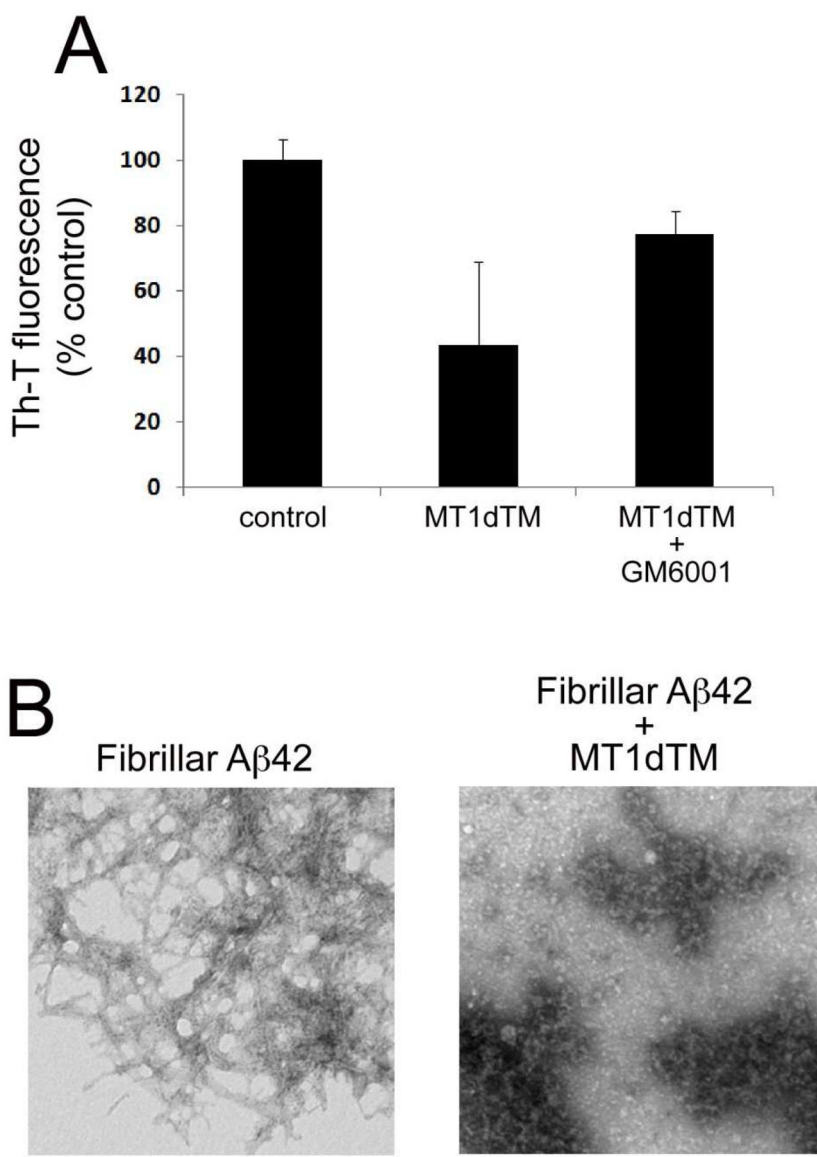


Fig. 9. Fibrillar A β degradation by soluble MT1dTM

(**A**) Fibrillar A β 42 was incubated alone or with MT1dTM in the presence or absence of the MMP inhibitor GM6001 for 37°C for 5 days. The remaining fibrillar A β was quantitated using a Th-T binding fluorescence assay. The data shown are the mean \pm SD of three separate determinations. (**B**) Fibrillar A β 42 was incubated alone or with purified MT1dTM at 37°C for 5 days. The samples were collected and analyzed by TEM. Scale bars = 100 nm.

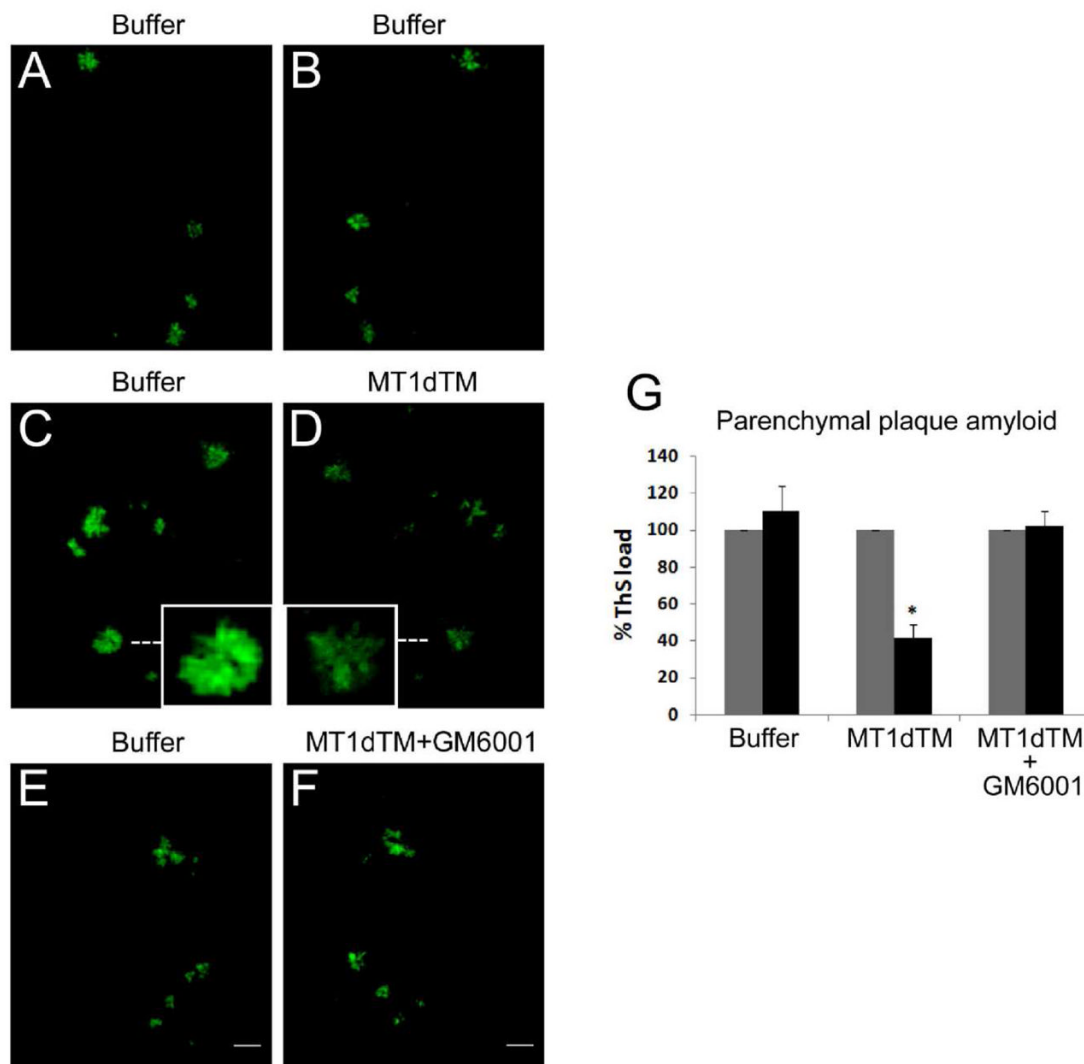


Fig. 10. *In Situ* brain fibrillar amyloid plaque degradation by soluble MT1dTM

Adjacent 5 μ m fresh frozen brain sections from 18 months old Tg2576 mice were incubated alone (A,B,C,E) or with purified MT1dTM (D) or GM6001-treated MT1dTM (F) at 37°C for 5 days. The sections were then fixed, and stained with Th-S. Insets show parallel representative plaques enlarged. Scale bars = 50 μ m. (G) The parallel cortical fibrillar amyloid plaque areas were quantified in the treated and untreated sections and expressed as percent remaining Th-S area. The data presented are the mean \pm S.D. of n = 25 plaques (buffer alone); n = 37 plaques (incubated with MT1dTM); n = 27 plaques (incubated with GM6001-treated MT1dTM). *, p < 0.001, paired *t* test.