Degradation of Amyloid β-Protein by a Metalloprotease Secreted by Microglia and Other Neural and Non-neural Cells*

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Wei Qiao Qiu, Zhen Ye, Dora Kholodenko‡, Peter Seubert‡, and Dennis J. Selkoe§

From the Center for Neurologic Diseases, Harvard Medical School, Brigham and Women’s Hospital, Boston, Massachusetts 02115 and Athena Neurosciences, Inc., South San Francisco, California 94080

Amyloid β-protein (Aβ) is the major component of neuritic (amyloid) plaques in Alzheimer’s disease, and its deposition is an early and constant event in the complex pathogenic cascade of the disease. Although many studies have focused on the biosynthetic processing of the β-amyloid precursor protein and on the production and polymerization of Aβ, understanding the degradation and clearance of Aβ has received very little attention. By incubating the conditioned medium of metabolically labeled Aβ-secreting cells with media of various cultured cell lines, we observed a time-dependent decrease in the amount of Aβ in the mixed media. This factor principally responsible for this decrease was a secreted metalloprotease released by both neural and non-neural cells. Among the cells examined, the microglial cell line, BV-2, produced the most Aβ-degrading activity. The protease was completely blocked by the metalloprotease inhibitor, 1,10-phenanthroline, and partially inhibited by EDTA, whereas inhibitors of other protease classes produced little or no inhibition. Substrate analysis suggests that the enzyme was a non-matrix metalloprotease. The protease cleaved both Aβ1–40 and Aβ1–42 peptides secreted by β-amyloid precursor protein-transfected cells but failed to degrade low molecular weight oligomers of Aβ that form in the culture medium. Lipopolysaccharide, a stimulator of macrophages/microglia, activated BV-2 cells to increase their Aβ-degrading metalloprotease activity. We conclude that secreted Aβ1–40 and Aβ1–42 peptides are constitutively degraded by a metalloprotease released by microglia and other neural cells, providing a potential mechanism for the clearance of Aβ in brain tissue.

The defining pathological features of Alzheimer’s disease (AD) are extracellular deposits of amyloid β-proteins (Aβ) that form senile plaques and amyloid angiopathy and intraneuronal deposits of modified tau proteins that form neurofibrillary tangles. Aβ are 40–43-amino acid proteolytic fragments generated by unidentified proteases from the transmembrane glycoprotein, β-amyloid precursor protein (βAPP) (1, 2). The gene encoding βAPP is on human chromosome 21q, and missense mutations in and around the Aβ coding region of this gene are a rare cause of familial AD (FAD). Moreover, trisomy 21 (Down’s syndrome) is characterized by overexpression of βAPP due to increased gene dosage, resulting in very early Aβ deposition followed by the gradual development of the classical neuropathological lesions of AD (3, 4). Recently, mutations in the presenilin 1 and 2 genes, which cause severe early onset FAD (5–7), have been shown to selectively increase the production and cerebral deposition of the highly amyloidogenic 42-residue form of Aβ (8, 9). These and other findings provide strong evidence that disordered βAPP metabolism can increase the production of Aβ peptides, particularly the Aβ1–42 peptide, thereby initiating amyloid plaque formation and the pathological cascade of AD. In support of this model, transgenic mice overexpressing the V717F mutation or K670N/M671L mutation of βAPP progressively develop Aβ deposits and plaque-associated neuritic, microglial, and astrocytic pathology with age and may even show concomitant memory impairment (10–12).

Much attention has been focused on the secretory and endocytic processing of βAPP by cells in order to understand how Aβ is generated normally and in AD. In contrast, little is known about how Aβ, once secreted, is degraded and cleared from tissues. At present, only the relatively rare forms of FAD linked to the βAPP or presenilin genes are thought to involve overproduction of Aβ. The excessive cerebral accumulation of Aβ occurs in all other cases of the disease could be explained in part by a decrease in the ability of the brain to degrade and clear Aβ. If specific Aβ-degrading proteases can be shown to be released by neural cells, changes in the structure or activity of such proteases could be sought in as yet unexplained forms of FAD, and their up-regulation could represent a therapeutic approach to AD in general.

Here, we have screened certain neural and non-neural cell lines to ascertain whether they constitutively release proteases capable of degrading the Aβ peptides naturally secreted by cells. We have identified a secreted metalloprotease activity that efficiently degrades endogenous Aβ into fragments under cell culture conditions. Among the neural cell lines tested, a microglial line, BV-2, secretes the active protease robustly. Substrate analysis suggests that it is a non-matrix metalloprotease. Its production or activity is upregulated by activating BV-2 cells. Interestingly, the metalloprotease is far more effective in degrading secreted Aβ140 and Aβ142 monomers than Aβ oligomers formed in cell culture. These findings have implications for the normal and pathological clearance of Aβ in brain.

**EXPERIMENTAL PROCEDURES**

Cell Culture—Chinese hamster ovary (CHO) cells stably transfected with βAPP770 cDNA containing the Val → Phe mutation at residue 717 (7PA2 cells) (13) were routinely cultured in Dulbecco’s modified Eagle’s
medium, 10% fetal bovine serum (FBS) with G418 (200 µg/mL). Untransfected CHO cells, monkey kidney COS cells, and the human neuroblastoma cell lines, M17 and SY5Y, were grown in Dulbecco’s modified Eagle’s medium, 10% FBS. All these cells were passaged by adding 3 ml of 50 mM EDTA, immediately aspirating it off, and incubating the cells at 37°C for 2 min followed by washing with Hank’s balanced salt solution (Life Technologies, Inc.). The cells were passaged at 1:10 dilution. Mouse microglial cells, BV-2, were cultured in RPMI 1640, 10% FBS (14). Because they were semisuspended, BV-2 cells were shaken to be lifted during passage.

**Assays of Aβ-degrading Activity**—To obtain conditioned media (CM) containing Aβ-degrading activity, different cultured cells were washed 3 times with serum-free N2 medium (N2 supplement (Life Technologies, Inc.), 1% ovalbumin, 1 mM pyruvate in MEM) (N2), N2 was added, and cells were conditioned at 37°C for various times. CM were collected and centrifuged at 3,000 × g for 30 min to remove cells. Some CM were concentrated 10 times in a Centricon 30 (Amicon) filter. To characterize Aβ-degrading activity, confluent monolayers of 7PA2 βAPP-transfected CHO cells in 10-cm dishes were preincubated for 30 min in methionine-free medium and labeled for 4 h with 300 µCi of [35S]methionine. The labeled media were collected, combined, and centrifuged at 3,000 × g for 30 min. An amount of labeled medium (3 ml) was mixed with an equal amount of cell conditioned medium from the various cell lines tested or with unconditioned N2 medium as a control, and the mixtures were incubated at 37°C for 16 h. The amount of labeled Aβ remaining in each sample was assessed by immunoprecipitation with the high-affinity Aβ antibody, R1282 (4). This antibody was generated to synthetic Aβ1–40 peptide and characterized similarly to our Aβ antibody, R1280, used in our previous study (15).

Aβ degradation in cultures was also quantified by incubating 7 µl of conditioned or unconditioned medium with 125I-labeled Aβ1–40 (3.3 × 10^10 cpm) (kind gift of Dr. John Maggio (15) in 14 µl of reaction buffer (187 mM NaCl, 0.02 µM NaH2PO4, 10 µM Tris-HCl, pH 7.5) at 37°C for 16 h. After quenching the reaction with 7 µl of glacial acetic acid and 5 µl of 0.1% pyronin Y, the sample was analyzed by acid-urea-PAGE (15) and autoradiography.

Protease inhibition assays were conducted by adding different protease inhibitors at the indicated concentrations to the mixtures before incubating at 37°C for 16 h. All inhibitors shown in Table I were purchased from Sigma.

**ELISA**—Cold 4-h conditioned medium of 7PA2 cells was mixed with plain N2 or with CM of CHO or BV-2 cells. After incubating at 37°C for 16 h, total Aβ or specific Aβ1–42 remaining in each sample was quantified by highly specific sandwich ELISA assays described previously (16). Two different ELISA assays were performed using distinct capture antibodies: 266 (raised against residues 13–28 of Aβ), for total Aβ or 21F12 (raised against residues 33–42 of Aβ), for Aβ peptides ending specifically at residue 42. The reporter antibody was 3D6 (to residues 1–5 of Aβ) in both assays. The ratio of Aβ1–42 to total Aβ in each sample was calculated.

**LPS Treatment**—Bacterial lipopolysaccharide (LPS) (Escherichia coli serotype O111:B4) was purchased from Calbiochem. Cells were either left unstimulated or stimulated with LPS (10 µg/mL) in culture medium containing 10% FBS at 37°C for various times. Conditioned media were collected and centrifuged before assaying Aβ-degrading activity as described above.

**RESULTS**

Decrease of Aβ Is Mediated by the Conditioned Media of Non-neural and Neural Cells—We examined several non-neural and neural cell lines for the secretion of an Aβ-degrading protease activity: COS monkey kidney cells; Chinese hamster ovary (CHO) cells; the human neuroblastoma cell lines, M17 and SY5Y; and the murine microglial line, BV-2. All cultures were passaged by brief incubation with EDTA rather than trypsin, in order to avoid the formation of a serine protease/α2-macroglobulin complex in the medium, which we recently showed can occur during trypsinic passage of cells and degrade secreted Aβ (15). To search for Aβ-degrading activity, confluent cultures were washed and changed to the serum-free medium, N2, for further cultivation. After conditioning for 24 h, the media were collected and centrifuged to remove floating cells. CM from certain cell lines (CHO, COS, M17, and SY5Y) were concentrated 10-fold by Centricon filtration before assaying for proteolytic activity. To detect degradation of endogenously secreted Aβ, CHO cells stably transfected with βAPP770 cDNA containing the V717F mutation (7PA2; Ref. 13) were labeled with [35S]methionine for 4 h in serum-free medium. Equal amounts of the labeled 7PA2 CHO CM were incubated with unconditioned medium (lane 1), CHO CM (lane 2), or BV-2 CM (lane 3) at 37°C for 16 h, followed by characterization as in A.

**Decrease of secreted Aβ is mediated by the conditioned media of various cell lines.** A, CHO cells stably transfected with an APPV717F gene containing V717F mutation (7PA2) were washed and labeled with [35S]methionine for 4 h in serum-free medium. Equal amounts of the labeled supernatant were incubated with the 10× concentrated unconditioned N2 medium (lane 1) or 10× concentrated CM of CHO (lane 2), or COS (lane 3) cells at 37°C for 16 h. The incubated media were immunoprecipitated with the Aβ antibody, R1282, and electrophoresed on Tris-Tricine SDS-PAGE gels, followed by autoradiography. Molecular size markers (kDa) are indicated. B, aliquots of the labeled 7PA2 supernatant were incubated 1:1 with 10× concentrated unconditioned N2 medium (lane 1) or 10× concentrated CM of CHO, COS M17, and SY5Y cells at 37°C for 16 h. The incubated media were immunoprecipitated as described in A. C, equal amounts of the labeled 7PA2 CHO CM were incubated with unconditioned medium (lane 1), CHO CM (lane 2), or BV-2 CM (lane 3) at 37°C for 16 h, followed by characterization as in A.
characterized.

**Aβ Degradation Is Mediated by a Secreted Metalloprotease in Both CHO and BV-2 Conditioned Media**—To determine whether the loss of Aβ in CM was caused by degradation by secreted protease(s), we examined the effects of several different protease inhibitors with an assay similar to that described above. Using concentrations known to cause maximal inhibition of other secreted proteases (18), various well-characterized inhibitors were incubated for 24 h at 37°C with either N2 medium or CHO CM in the presence of synthetic Aβ1–42 peptide (40 ng/ml) as a substrate, followed by a sandwich ELISA to detect remaining intact Aβ. The inhibitor profile clearly demonstrated an Aβ-degrading metalloprotease in the CHO medium (Table I). The degradation of cell-secreted Aβ and p3 by both CHO and BV-2 media was essentially abolished by 1,10-phenanthroline, whereas EDTA generally produced less inhibition (Fig. 3). The different degrees of inhibition of the degradation of endogenous (Fig. 3) versus synthetic (Table I) Aβ by EDTA are unexplained, but may be due to different conformations of these two substrates, and we are conducting experiments to address this issue. Although phosphoramidon is also a metalloprotease inhibitor, it did not inhibit the degradation of either synthetic or endogenous Aβ in this system (Fig. 3). These results indicate that the major Aβ-degrading activity present in both CHO and BV-2 CM is a secreted metalloprotease. Another protease inhibitor, 4-[(2-aminoethyl)-benzenesulfonfonyl fluoride (Pefabloc), produced slight inhibition of Aβ degradation (Fig. 3), suggesting that a serine protease may also make a small contribution to Aβ proteolysis by CHO and BV-2 media.

To detect the products of the Aβ degradation, acid-urea-PAGE (15) was used to analyze 125I-labeled synthetic Aβ1–40 after incubation in CHO or BV-2 CM for 24 h (Fig. 4). The amount of intact Aβ decreased markedly, and 3 peptide bands appeared that migrated differently from intact Aβ and were not produced by incubation in N2 medium alone. The production of these fragments was completely inhibited by 1,10-phenanthroline, confirming that Aβ is degraded by a secreted metalloprotease in both CHO and BV-2 CM and generates similar peptide products.

**Degradation of Aβ1–40, Aβ1–42 and Aβ Oligomers by the Metalloprotease**—Because Aβ peptides ending at residue 42 (Aβ1–42) are the initially deposited species in AD and normal aged brain tissue, we examined the substrate specificity of the metalloprotease on endogenous Aβ1–40 and Aβ1–42. Confluent 7PA2 cells were washed and cultured in serum-free N2 medium for 4 h. Equal amounts of this CM were incubated with plain N2, CHO CM, or BV-2 CM for 24 h, followed by sandwich ELISA measuring either total Aβ (including Aβ1–40 and Aβ1–42) or Aβ1–40 alone. The amounts of total Aβ and Aβ1–42 remaining in CHO and BV-2 media were decreased approximately 75% compared to those in N2 (Fig. 5, A and B). As a result, the ratios of Aβ1–40 to total Aβ showed only a slight and insignificant increase (Fig. 5C).

We also characterized the specificity of the metalloprotease in the 24-h conditioned media of CHO and BV-2 cells for Aβ-related species found in the culture medium of our βAPP-
expressing 7PA2 cells. Aβ was substantially decreased by 4 h of incubation, whereas p3 showed relative stability for the first 8 h of incubation (Fig. 6). Importantly, the endogenous 6–12-kDa oligomeric Aβ species that we previously documented in the CM of these V717F βAPP-transfected CHO cells (13) were completely resistant to proteolysis (Fig. 6).

Regulation of the Secreted Metalloprotease—During the course of these studies, we noticed that high Aβ-degrading activity in CM (>90% decrease of Aβ compared to that in N2) occurred when cells were conditioned in the absence of serum. In contrast, only a 10–15% decrease in Aβ occurred when 10% FBS was present during conditioning (data not shown). To clarify the role of serum in the production and activity of the Aβ-degrading protease, we conditioned CHO cells in N2 medium containing increasing concentrations of FBS for 24 h. These CM were then incubated with labeled CHO 7PA2 medium at 37 °C for 16 h, followed by the Aβ immunoprecipitation assay described above. As a control, 10% FBS was added to a sample of medium conditioned without serum just prior to the incubation. CHO CM containing 1% FBS degraded Aβ as much as CHO CM without FBS, whereas the amount of Aβ in CHO CM containing 10% FBS appeared virtually unchanged from that in plain N2 (Fig. 7). Increasing the serum concentration between 1% and 10% led to a graded decrease in the amount of Aβ-degrading activity (Fig. 7). Using the ELISA assay, we were similarly able to detect only a 10–20% decrease in Aβ levels in medium conditioned in 10% FBS (data not shown). These results indicate that increasing the percentage of serum during conditioning results in a corresponding decrease in Aβ proteolysis. On the other hand, adding 10% FBS solely during the incubation period did not decrease the strong Aβ-degrading activity generated by CHO cells conditioned without serum. Serum also decreased the metalloprotease activity released from BV-2 cells but to a lesser degree than CHO cells (Fig. 8B). Thus, serum appears to decrease the cellular production of the Aβ-degrading metalloprotease or increase the secretion of a metalloprotease inhibitor rather than directly inhibiting the protease.

To further characterize the regulation of the metalloprotease in the microglial line, E. coli LPS, a general stimulator of macrophage and microglial cells (14), was applied to the cells...
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During conditioning in the presence of 10% FBS, the latter generally decreased the amount of Aβ-degrading activity. LPS stimulated the BV-2 cells to release more Aβ-degrading protease, but failed to do so as expected in the CHO fibroblast line (Fig. 8A). As the conditioning time in LPS was prolonged, the stimulation of Aβ-degrading activity in BV-2 CM rose. The released protease was specifically blocked by 1,10-phenanthroline (Fig. 8B), suggesting that the protease activity stimulated by LPS in the presence of serum represented the same metalloprotease observed constitutively in the absence of serum.

DISCUSSION

The degree of Aβ deposition seen in AD and aged normal brains is determined by the rates of both Aβ production and Aβ removal. Therefore, it is important to understand the contribution of Aβ removal under normal and pathological circumstances, identify its mechanisms, and search for methods to enhance clearance. In this report, we describe a metalloprotease secreted by both non-neural and neural cells that is capable of efficiently cleaving Aβ and p3 but not oligomeric Aβ species found endogenously in the medium of βAPP-expressing cells.

Our studies using [35S]methionine-labeled CM of βAPP-transfected CHO cells as a source of Aβ and related species and the CM of different cell lines as a source of proteases show that several non-neural and neural cell lines secrete Aβ-degrading proteases, although the levels of activity vary substantially among cell types. Because the two culture media were incubated together in the absence of cells, our paradigm excludes the possibilities of cell-mediated internalization of Aβ (19, 20) or the degradation by a cell surface protease, if such exists. By using EDTA to lift the cells during passage, we also excluded the possibility that the serine protease/α2-macroglobulin complex we previously described in vitro (15) was responsible for Aβ degradation. Two different methods, immunoprecipitation and ELISA, were employed to assess the amounts of Aβ remaining in the media after incubation and to confirm unequivocally that it was Aβ itself that was the substrate. We consistently observed the degradation of Aβ by proteases secreted by two non-neural cell lines, CHO and COS, and three neural cell lines, M17 and SY5Y neuroblastoma and BV-2 microglial cells (Fig. 1). Among them, BV-2 and CHO showed the strongest activity, followed in decreasing order by COS, M17, and SY5Y. BV-2, a murine microglial cell line, released the most Aβ-degrading activity per cell (Fig. 2), suggesting that microglial cells could play an important role in the clearance of Aβ from the extracellular space of brain. Furthermore, unlike the CHO fibroblast cells, which released little Aβ-degrading activity into medium in the first 6 h of conditioning, BV-2 cells immediately and continually secreted proteolytic activity after being placed in serum-free medium (Fig. 3).

The metalloprotease inhibitors 1,10-phenanthroline and EDTA substantially blocked the degradation of endogenous Aβ by the CM of both CHO and BV-2 cells (Fig. 3). Phosphoramidon, another metalloprotease inhibitor, which can only block certain metalloproteases (18), lacked the ability to inhibit the protease we describe. Selected inhibitors of the cysteine and aspartyl classes likewise produced little or no detectable inhibition of Aβ degradation under the conditions of our experiments. However, Pefabloc, a broad spectrum serine protease inhibitor, modestly inhibited the catabolism of Aβ by the conditioned media we examined (Fig. 3), suggesting that a serine protease may contribute to Aβ degradation in our cultures, although it plays a minor role. Naidu et al. (22) have reported that a mixture of inhibitors of all four major protease classes (leupeptin, pepstatin, EDTA, and phosphoramidon) inhibited Aβ degradation by CHO cells. In the cell lines we used, a metalloprotease is responsible for the major Aβ degradation, as confirmed when the specific Aβ-derived proteolytic products seen in acid-urea gels were completely abolished by 1,10-phenanthroline (Fig. 4). This inhibitor was not tested by Naidu et al.

Because the major class of secreted metalloproteases is the matrix metalloproteases, we have recently characterized both CHO and BV-2 CM by chromatography on gelatin-Sepharose, a...
matrix metalloprotease binding gel (23). The flow-through frac-
tions of this column showed no loss of Aβ-degrading activity.
Moreover, p-aminophenylmercuric acetate, a matrix metallo-
protease activator, did not change the level of Aβ degradation of
CHO and BV-2 CM, whereas it increased the degree of gelatin
degradation by these CM, as shown by gel zymograpy.2 These
results suggest that the protease we have charac-
terized belongs to a class of metalloprotease different from
the principal matrix metalloproteases.

Because accumulating studies have shown that Aβ42 pep-
tides are the initial species involved in the formation of amyloid
deposits in AD and Down’s syndrome (4, 8, 24, 25) despite the
fact that cells secrete much more Aβ40 than Aβ42 (8), we char-
acterized the specificity of the metalloprotease for secreted
peptides ending at Aβ40 and Aβ42. Both CHO and BV-2 CM
decreased the amounts of total Aβ and Aβ42 to similar levels and
left the ratio of Aβ40 to total Aβ largely unchanged (Fig. 5),
suggesting that the metalloprotease has equal avidity for en-
ogenous Aβ1–40 and Aβ1–42 peptides. We found that the p3
peptide (Aβ17–40/42) was more resistant to degradation by the
metalloprotease than Aβ (Fig. 6). Interestingly, the low mole-
cular weight Aβ oligomers that can be detected in CHO medium
(13) and that are composed of both Aβ40 and Aβ42 peptides3
were unchanged after 48 h incubation in conditions that al-
lowed virtually complete degradation of monomeric Aβ and p3.
Therefore, we speculate that the threshold concentration of Aβ
monomer needed to allow aggregation could be reached by
increased production and/or impaired degradation of Aβ. After
Aβ40 and Aβ42 Peptides are oligomerized, they will apparently
be cleared inefficiently by secreted proteases, even those rele-
ased by activated microglial cells in senile plaques. These
oligomers could then accumulate and ultimately form high
molecular weight assemblies, including the potentially neuro-
toxic amyloid fibrils characteristic of AD.

Because the clearance of Aβ should help determine the levels
of extracellular Aβ and thus the rate of fibril formation, meth-
ods to stimulate Aβ degradation represent one approach to
slowing the development of AD neuropathology. During our
experiments, we noticed that the activity of Aβ-degrading met-
alloprotease decreased in parallel with increasing levels of
serum in the cultures (Fig. 7), suggesting that the enzyme is
probably regulated by extracellular factors under physiological
conditions. It is currently unclear whether this loss of activity
is caused by the increased secretion of a protease inhibitor by
the cells or by decreased release of the protease itself. We also
found that LPS, a general stimulator of macrophage/microglia,
activated BV-2 cells to increase Aβ-degrading metalloprotease
activity in their media under the proteolytically adverse con-
dition of culturing in 10% serum (Fig. 8). It is possible that
the activated microglia which are consistently found in mature
(neuritic) plaques but less frequently present in immature (dif-
fuse) plaques (21) release proteases such as that described
here. Our data lead to the hypothesis that at early stages of AD,
microglial cells could play an active role in slowing the de-
velopment of Aβ deposits and amyloidosis; however, at later
stages there could be an adverse effect, because the released
metalloprotease cannot efficiently degrade oligomeric Aβ but
would be available to act upon other cellular and extracellular
substrates, thereby potentially aggravating the inflammatory
cytotoxic events in and around the mature plaque. Full
purification of the metalloprotease should allow the develop-
ment of antibodies and probes useful for examining the role of
this and related proteases in the normal and abnormal biology
of Aβ.

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