Expression of Rat Cathepsin S in Phagocytic Cells*

Suzana Petanceska, Peter Canoll, and Lakshmi A. Devi§

From the Department of Pharmacology, New York University Medical Center, New York, New York 10016

Cysteine lysosomal proteases are essential for turnover of intracellular and extracellular proteins. These enzymes are strongly implicated in normal and pathological processes involving tissue remodeling. Among the cysteine proteases, cathepsin S seems to be best suited for such a process since it retains most of its enzymatic activity at neutral pH. In situ hybridization analyses of the adult rat brain, spleen, and lung reveal that cathepsin S mRNA is preferentially expressed in cells of mononuclear-phagocytic origin. After entorhinal cortex lesion of adult rat brain (a paradigm for neuronal degeneration and reactive synaptogenesis), cathepsin S mRNA is dramatically increased in activated microglia in the deafferented dentate gyrus and in macrophages at the wound site, suggesting a role in lesion-induced tissue remodeling. This possibility is further supported by the finding that cathepsin S degrades a number of extracellular matrix molecules at neutral pH and by the finding that inflammatory mediators stimulate its secretion from the microglia and macrophages. These data suggest that cathepsin S is an important player in degenerative disorders associated with the cells of the mononuclear phagocytic system.

Cysteine lysosomal proteases are essential for the turnover of intracellular proteins, as well as extracellular proteins internalized by endocytosis (1), and therefore they are implicated in a number of pathological conditions that involve tissue destruction such as tumor metastasis, arthritis, multiple sclerosis, and Alzheimer’s disease (2–5). Members of this family of enzymes are strongly implicated in normal and pathological processes involving tissue remodeling. Among the cysteine lysosomal proteases suggest that some members of the family subserve a housekeeping role, while others perform a more specialized and/or tissue-specific function. For example, the mRNAs for cathepsin B and L are fairly evenly distributed throughout the body (12). However, the transcripts for cathepsin S show variation in levels among tissues and exhibit a restricted expression pattern (12–16).

Our previous work showed that in rat brain the expression pattern of cathepsin S mRNA is profoundly different from the expression pattern of the transcripts for cathepsin B and cathepsin L (14). Namely, cathepsin S mRNA was found in cells that are fairly homogeneously distributed in gray and white matter; it could also be seen in some perivascular cells, as well as in a number of cells in the choroid plexus and the leptomeninges (14). The distribution as well as the morphology of these cells suggested that cathepsin S mRNA was expressed in different populations of brain macrophages: microglia in the parenchyma and perivascular and leptomeningeal macrophages.

By in situ hybridization analyses of rat spleen and lungs, combined with immunostaining of the same tissue with OX42 (an antibody that recognizes the C3bi receptor, common to various subpopulations of macrophages), and Northern analyses of a number of cell lines and different primary glial cultures, we show that in rat brain and in peripheral tissues cathepsin S mRNA is preferentially expressed in cells from the mononuclear phagocytic system. This is consistent with previous reports showing that the highest cathepsin S activity is in the bovine spleen (10) and that within the human lung immunoreactive cathepsin S is in the macrophages (9).

Cathepsin S is expressed largely in cells of mononuclear phagocytic lineage; this raises the possibility that cathepsin S may be involved in macrophage-mediated tissue destruction. To address this possibility, we examined the expression and distribution of cathepsin S after entorhinal cortex lesion (ECL) of adult rat brain and compared it to the expression and distribution of cathepsin B and L by in situ hybridization histochemistry. Entorhinal cortex lesion is not only a classical paradigm for studying neuronal degeneration and reactive synaptogenesis, but it is also a lesion with a well characterized microglial response (17), which enabled us to further confirm the cell-specific expression of cathepsin S mRNA. In response to unilateral ECL, cathepsin S mRNA expression was dramatically increased around the wound site and in the outer molecular layer of the deafferented dentate gyrus, areas of infiltration of blood borne macrophages and microglial hyper proliferation, respectively.

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§To whom correspondence should be addressed: Dept. of Pharmacology, New York University Medical Center, 550 First Ave., New York, NY 10016. Tel.: 212-263-7119; Fax: 212-263-7133; E-mail: Lakshmi.Devi@Med.NYU.edu.
†Current address: Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, 1230 York Ave., New York, NY 10021.
‡Current address: Laboratory of Molecular and Cellular Neuroscience, The Rockefeller University, 1230 York Ave., New York, NY 10021.

The abbreviations used are: ECM, extracellular matrix; ECL, entorhinal cortex lesion; PBS, phosphate-buffered saline; LPS, lipopolysaccharide; DTT, dithiothreitol; BSA, bovine serum albumin; CSPG, chondroitin sulfate proteoglycan; HSPG, heparan sulfate proteoglycan.
We also show that classical inflammatory mediators (bacterial endotoxin and IFN-γ) stimulate the secretion of cathepsin S activity from resident and thioglycolate-elicited peritoneal macrophages and from a microglial cell line. Finally, we show that cathepsin S efficiently degrades various ECM molecules (laminin, fibronectin, and brain chondroitin sulfate proteoglycans) at neutral pH in vitro.

Taken together, our results strongly support an important role for cathepsin S in the macrophage/microglia-mediated tissue destruction and remodeling in the brain and in peripheral tissues.

**MATERIALS AND METHODS**

**Tissue Preparation and in Situ Hybridization**—Spleen or lung were quickly removed from 10-week-old male rats (Sprague-Dawley) and frozen in isopentane at −30°C. 15-micron-thick cryostat sections were mounted on poly-L-lysine-coated slides and processed for in situ hybridization as described previously (14). The sections were then hybridized with [35S]UTP labeled antisense riboprobes, diluted in hybridization buffer (75% formamide, 10% dextran sulfate, 30 mM sodium phosphate, pH 7.4, 1 × Denhardt's, 10 mM DTT, 10 μM mgml yeast RNA) to give a final concentration of 1 × 10⁶ cpm/section. Portions of rat cathepsins B, L, and S cDNAs (570, 495, and 502 base pairs, respectively) which span the distance between the conserved domains that contain the active site Cys²⁵ and Asn¹⁷⁵ residues, were chosen to generate cRNA probes as reported previously (14). Adjacent sections were hybridized with riboprobes for cathepsins B, L, or S, with similar specific activities. After overnight hybridization at 55°C, the excess of unhybridized probe was removed from 10-week-old male rats (Sprague-Dawley) and washed in 0.1 × SSC at 65°C for 1 h. After being dehydrated through graded alcohols and air dried, the sections were exposed to XAR-5 (Eastman Kodak) x-ray film. To evaluate the signal at the cellular level, the slides were dipped in L4 or K5 photographic emulsion (Polysciences, Warrington, PA) and stored at 4°C for 1–3 weeks before developing. The developed, emulsion-dipped sections were counterstained with 0.05% Cresyl violet (pH 4.0) and analyzed on a bright field/dark field Leitz microscope.

Two types of controls were used to ensure specificity of the hybridization signal: a sense strand control and an RNase pretreatment control. No signal higher than background was seen on any of the sections when the sense probes were used or when the tissue was treated with RNase A before hybridization to the antisense probes.

**Stereotoxic lesioning of the entorhinal cortex of 10-week-old adult male rats (Sprague-Dawley) was performed as described (18).** Lesioned brains were quickly removed, frozen in isopentane, sectioned, and processed for in situ hybridization as described above.

**Stereotactic lesioning of the entorhinal cortex of 10-week-old adult male rats** was performed as described (18). Lesioned brains were quickly removed, frozen in isopentane, sectioned, and processes for in situ hybridization as described above.

**Culturing of Primary Glial Cells and Cell Lines**—For culturing primary glial cells, adult rat brains were removed from 10-week-old male rats (Sprague-Dawley) and dissociated by gentle trituration into a single cell suspension. The cell suspension was plated onto polylysine-coated dishes and allowed to adhere over-night. After washing with PBS, the cell suspension was treated with 5 mM leucine methylester for 15 min at room temperature and then washed with fresh medium containing the appropriate culture conditions. The purity of the culture was determined by immunocytochemical studies.

**Immunocytochemistry**—For diagnostic immunofluorescence, primary mixed glial cells or cells treated with RNase A before hybridization to the antisense probes. Mock digestions were performed for 15 or 150 min at 37°C. The antibody specificity was confirmed by competition in the presence of 5 μg/ml of lipopolysaccharide (LPS, J5 strain) alone, 30 μg/ml of both IFN-γ and LPS (100 units/ml) or LPS (100 units/ml) in 300 μM of medium. Following a 5-h incubation, the cells were washed and replaced with fresh medium containing the appropriate treatments. The secreted medium was collected for 1 h, and stored frozen at −20°C.

**Cathepsin S Activity Determination**—The spent medium was subjected to a neutral pH incubation step prior to cathepsin S activity determination; this step inactivates the other lysosomal proteases with substrate specificity similar to that of cathepsin S while retaining activity of cathepsin S. For this, an aliquot of spent medium was incubated with 10 volumes of 150 mM Tris-Cl buffer, pH 7.5, containing 2 mM EDTA, 2 mM DTT, 0.01% Triton X-100 for 45 min at 37°C. The protease activity was measured using 6.5 units/ml of BAPNA (4-Methylumbelliferyl β-D-galactoside) (15). Briefly, the enzyme was incubated in 100 mM sodium acetate buffer, pH 5.0, containing 2 mM EDTA, 2 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 5 mM pepstatin A, and 20 μM aminobenzoyl-Phe-Arg-aminomethyl coumarin as the substrate. The product formed was detected at excitation 383 nm and emission 460 nm. Parallel incubation with 5 μM trans-epoxysuccinyl-L-leucylamide (4-guanidino)butane (also known as E-64, Sigma) were carried out as controls. E-64 is a highly selective active-site-directed inhibitor that inhibits majority of the lysosomal cysteine hydrolases with high potency (10). To ensure the specificity of the assay, we took advantage of the neutral pH inactivation of the other lysosomal hydrolases; we also included inhibitors of serine, aspartyl, and metallopeptases in the assay buffer.

**Immunocytochemical Analysis of ECM Molecules Involved in Degradation**—For measuring the degradation of ECM molecules, purified cathepsin S was used. Recombinant human cathepsin S expressed in yeast was purified using 80% ammonium sulfate precipitation followed by affinity chromatography of the resuspended precipitate on thiopropyl-agarose (11). Approximately 300 or 30 ng of purified cathepsin S was incubated with 10 μg of bovine fibronectin, laminin, collagen I and IV, or basement membrane heparan sulfate proteoglycan in 100 mM sodium phosphate, pH 6, containing 2 mM EDTA, 2 mM DTT, and 0.01% Triton X-100 or in 100 mM Tris-Cl, pH 7.5, containing 2 mM EDTA, 2 mM DTT, and 0.01% Triton X-100. The digestions were performed for 15 or 150 min at 37°C. Mock digestions without the enzyme and digestions in the presence of E-64 (5 μM) were used as negative controls. The reaction products were resolved on 4–20% gradient SDS-polyacrylamide gels and visualized by silver staining (21).

**Chondroitinase-Treated Native or Radioiodinated Neuron and Phascan (purified from rat brain) were subjected to proteolysis by purified cathepsin S at acidic and neutral pH in buffers as described above.** Chondroitinase ABC (purified from P. chondroitinum or P. chondrophytes, both 1 unit/mg) was treated with 3 mg/ml of native or 5–10 ng of proteoglycans) were used for each digestion. The proteoglycan preparations contained a 1000-fold excess of BSA (24 μg of BSA in the case of neurcan and 10 μg of BSA in the case of phosphacan) as a radiation scavenger. Each reaction mixture contained 300 or 30 ng of active cathepsin S. The reaction products were resolved on 4–20% SDS-polyacrylamide gels. The gels were dried and exposed to XAR-5 (Eastman Kodak Co.) x-ray film. Digestion of native proteoglycans was carried out essentially as described above except that after being digested with cathepsin S the reaction mixtures were treated with...
Our previous in situ hybridization study in adult rat brain showed that cathepsin S mRNA is expressed in cells that have the morphology typical for different populations of brain macrophages/microglia (14). The results of in situ hybridization analyses of cathepsin S mRNA expression in rat spleen and lung tissue support the notion that this enzyme is preferentially expressed in cells from the mononuclear-phagocytic system throughout the body. In rat spleen, cathepsin S mRNA could be seen in cells from the marginal zone, an area that contains both macrophages and dendritic cells (Fig. 1A); in lungs cathepsin S mRNA is expressed in a small number of large cells in the alveolar wall, most likely representing alveolar macrophages (Fig. 1C). This is supported by the finding that a similar pattern was obtained by immunostaining of the serial sections of rat spleen and lung with OX42 (panels B and D, respectively). OX42 is a monoclonal antibody that recognizes resident tissue macrophages, alveolar macrophages, dendritic cells, Kupffer cells, and microglia.

The expression of cathepsin S mRNA in cells of the mononuclear-phagocytic system is consistent with the results from Northern blot analysis of a number of mouse and rat cell lines from different embryonic origin. Cathepsin S mRNA was detected only in the Wehi 3B monocytic leukemia cell line and the N-13, microglia-like cell line (Table I). We did not detect this transcript either in various cell lines of neuronal (PC12, N2A, N1e-115) or glial (C6) origin, pituitary-derived cells (AtT20, GH4C1), hepatocytes (BRL3A), or in fibroblasts (RAT1, NIH-3T3, CHO) (Table I). In contrast with cathepsin S, cathepsin B and cathepsin L mRNAs were expressed in high levels in all of the cell lines tested (Table I).

Intrigued by the very selective expression of cathepsin S in rat tissues and cell lines of different embryonic origin, and particularly by the possibility that it is confined to the sole immunoeffectector cell type in rat brain, we compared the expression of cathepsin S, B, and L mRNAs after unilateral ECL of adult rat brain, a classical paradigm for neuronal degeneration and reactive synaptogenesis with a very pronounced microglial response (17, 23).

We observed a significant up-regulation of cathepsin S mRNA in the outer molecular layer of the deafferented dentate gyrus and a dramatic increase at the wound site 8 days after lesion (Figs. 2F, 3, and 4). In contrast to this, there was only a slight increase in cathepsin B and cathepsin L mRNA expression in the outer molecular layer and a moderate increase at...
the wound site (Fig. 2, panels B and D). Analyses of emulsion-dipped sections showed that the increased signal for cathepsin S in the dentate gyrus and around the wound was a result of its expression in a greater number of cells and also due to an increased number of transcripts in individual cells (Figs. 3 and Fig. 4).

Staining of an adjacent section with OX42, an antibody that recognizes different populations of tissue macrophages and microglia, by virtue of binding to their C3bi complement receptor, gave a pattern similar to the signal obtained with cathepsin S cRNA probe in the deafferented dentate gyrus and around the wound (not shown), suggesting that the cathepsin S positive cells in these areas are likely to be activated microglia and blood-born macrophages, respectively.

To further address the question of cellular specificity of cathepsin S expression, we generated primary mixed glial cultures from rat neonatal forebrains. Northern analysis of total RNA from mixed glial cultures showed significant levels of mRNAs for cathepsin S, cathepsin B, and cathepsin L (not shown). However, upon subculturing to virtually pure microglia, type 1 astrocytes, and O2A progenitors, cathepsin S mRNA could be detected only in microglia. Cathepsin B and cathepsin L transcripts were present in all three cell types (Fig. 5).

In the light of these findings and the finding that the levels of cathepsin S mRNA are dramatically increased in what appears to be activated microglia/macrophages in vivo, it was interesting to see if cathepsin S activity was secreted from activated macrophages or microglial cells. It is well established that one of the early responses of macrophages to activation by inflammatory mediators is an increase in the levels of lysosomal hydrolases and their secretion (24). We examined the ability of two classical mediators of macrophage activation, bacterial endotoxin (LPS) and IFNγ, to stimulate the secretion of cathepsin S activity from resident and thioglycolate-elicited peritoneal mouse macrophages and from a microglial cell line, N-13. We observed a significant increase in the secretion of cathepsin S activity following a 5-h treatment with LPS (Table II). Treatment with IFNγ for 5 h had no effect on this secretion. Also, treatment with both LPS and IFNγ did not affect the LPS-induced secretion (Table II). The thioglycolate-elicited macrophages were more responsive to LPS than the resident macrophages and the microglial cells. In all cases, there was a substantial increase in cathepsin S activity secreted in response to LPS; these results support the hypothesis that upon activation, macrophages/microglia secrete enzymatically active cathepsin S.

It has been argued that the lysosomal hydrolases and metalloproteases that are secreted from activated macrophages/microglia are involved in the clearance of degenerative debris and in the ECM remodeling (24, 25). Based on the findings that cathepsin S can retain activity at neutral pH and can be actively secreted, and based on the observation that its expression is dramatically increased in activated microglia/macrophages after ECL, we argued that this enzyme takes part in the processes of ECM dissolution. Therefore, we tested the capacity of cathepsin S to degrade various ECM components (fibronectin, laminin, collagen, chondroitin sulfate proteoglycans and heparan-sulfate proteoglycans) in vitro. Purified, recombinant,
human cathepsin S was incubated with various ECM components at acidic or neutral conditions, and the reaction products were analyzed by silver staining after SDS-polyacrylamide gel electrophoresis. Cathepsin S efficiently cleaves laminin, fibronectin (Fig. 6, A and B) and collagen I and IV (data not shown). Fibronectin is a better substrate than laminin at acidic pH since it is more efficiently cleaved by cathepsin S at acidic pH as compared with neutral pH (compare lane 1 in Fig. 6A with lane 3 in Fig. 6B, fibronectin panel). In contrast, laminin is more efficiently cleaved at neutral pH since the concentration of cathepsin S that leads to complete proteolysis at neutral pH results in only a partial proteolysis at acidic pH (compare lane 1 in Fig. 6A with lane 3 in 6B, laminin panel).

Next, we examined the ability of cathepsin S to degrade chondroitin sulfate and heparan sulfate proteoglycans (CSPGs and HSPGs). The CSPGs, two brain proteoglycans, neurocan and phosphacan, were radiiodinated and used. Neurocan is a developmentally regulated, neuronal CSPG with extracellular localization during brain development, while in adult brain it is localized mostly intracellularly (26). Phosphacan is a secreted form of the receptor tyrosine phosphates β1, preferentially expressed in glial cells (27, 28). Immunohistochemical data show an increase in its expression in the outer molecular layer of the dentate gyrus after ECL.2 Upon treatment with cathepsin S, the precursor bands of neurocan were converted to a prominent ~45-kDa intermediate within 15 min (Fig. 7A, left panel). In the case of phosphacan, a series of smaller molecular mass products (between 50 and 90 kDa) was observed (Fig. 7A, right panel). Prolonged incubation of chondroitinase-treated neurocan or phosphacan with cathepsin S resulted in complete degradation of both proteoglycans at both acidic and neutral pH (not shown).

When native proteoglycans (not treated with chondroitinase) were exposed to cathepsin S, the enzyme was capable of generating virtually the same products with similar efficiency at both acidic and neutral pH (Fig. 7B). It is important to note that although the reaction mixtures contained more than a 1000-fold excess of BSA (used as a radiation scavenger), only the proteoglycans were efficiently degraded. It is interesting to note that the incubation of neurocan with a three times higher concentration of cathepsin B, under analogous conditions, did not lead to significant degradation of the CSPGs (not shown). Taken together, these results show preferential degradation of these proteoglycans by cathepsin S at both acidic and neutral conditions.

**DISCUSSION**

Cathepsin S mRNA exhibits a restricted pattern of expression. This is evident from the results of the in situ hybridization analysis of rat spleen and lung tissue (Fig. 1, A and B) as well as the screening for cathepsin S mRNA expression in different primary glial cultures and various cell lines (Fig. 5 and Table I), which demonstrate that, in rat, cathepsin S mRNA is preferentially expressed in cells of mononuclear-phagocytic origin. This is consistent with the results of our previous study that showed that in rat brain, cathepsin S is expressed in cell types that have distribution pattern and morphology of microglia/macrophages (14).

The human cathepsin S has been reported to be expressed in primary fibroblasts (13); we did not detect rat cathepsin S mRNA using a rat-specific cDNA probe in rat, mouse, or hamster fibroblasts (Table I). This difference might be a result of

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**TABLE II**

Secretion of cathepsin S from macrophages/microglia

| Cells                  | Cathepsin S activity | % control |
|------------------------|----------------------|-----------|
| Resident macrophages   | 100 ± 4              | 100 ± 4   |
| Thio-macrophages       | 100 ± 12             | 98 ± 14   |
| Microglial cell line    | 100 ± 3              | 103 ± 2   |

**Fig. 6. Proteolysis of fibronectin and laminin by cathepsin S.** 10 μg of bovine fibronectin or laminin were incubated with 300 or 30 ng of recombinant human cathepsin S for 150 min; lanes 2, same as lanes 1 except 5 μM E-64 was included in the mixture; lanes 3, undigested fibronectin or laminin. B, proteolysis at acidic pH. Lanes 1, 10 μg of fibronectin or laminin incubated with 300 ng of recombinant human cathepsin S for 150 min; lanes 2, same as lanes 1 except 5 μM E-64 was included; lanes 3, undigested fibronectin or laminin.

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2 S. Petanceska, P. Canoll, and L. A. Devi, unpublished observations.
neutral pH; cathepsin L mRNAs in these areas (Fig. 2, panel F) compared to the slight increase in cathepsin B and panel F dentate gyrus and around the wound site after ECL (Fig. 2, panel E). In the deafferented species difference in the regulation of the expression of this enzyme due to differences in promoter structure and/or cellular factors.

The expression of cathepsin S is subject to profoundly tight and differential regulation. This is clear from the overwhelming increase in cathepsin S mRNA levels in the deafferented dentate gyrus and around the wound site after ECL (Fig. 2, panel F) compared to the slight increase in cathepsin B and cathepsin L mRNAs in these areas (Fig. 2, panels B and D). In this respect, it is noteworthy that while the structure of the promoter for human cathepsin B has all the features of a promoter for a genuine “housekeeping” protein (80% GC-rich, with 15 SP1 sites), the promoter for human cathepsin S has low GC content and contains 18 potential AP1 sites, which is a characteristic of promoters for highly regulatable proteins (16, 29).

Microglia are the first cell type to be recruited in response to entorhinal cortex lesion. Hyperproliferation and activation of microglia in the outer molecular layer of the deafferented dentate gyrus occurs within 24 h after lesioning; the activated microglia exhibit a highly phagocytic phenotype (17). There is also a major infiltration of blood-born macrophages at the wound site. We observed increased OX42 immunostaining in these regions (not shown), similar to the pattern of expression of cathepsin S mRNA (Figs. 3 and 4), suggesting that the cells expressing cathepsin S mRNA in the ipsilateral dentate gyrus and around the wound are likely to be activated microglia and blood-born macrophages. It has been established that the microglial response is maximal during the first week and that it declines within the second week when it is being replaced by an astroglial response (17). Therefore, it is possible that cathepsin S mRNA is also expressed in reactive astrocytes that start to appear in the outer molecular layer of the deafferented dentate gyrus at that time (8 days post-lesion). This seems unlikely since we did not detect cathepsin S mRNA in primary cultures of type 1 astrocytes purified from rat brain (Fig. 5). Also, cathepsin S mRNA was not induced in cultures of type 1 astrocytes grown in the presence of dBcAMP (not shown); dBcAMP-treated type 1 astrocytes have been postulated to be in vitro homologs of reactive astrocytes (30).

One of the major functions of microglia/macrophages is phagocytic removal of dead cells or cell remnants during brain development and after injury in adult brain (31). They are also engaged in remodeling of the ECM by phagocytosis as well as by active secretion of neutral proteases such as elastase and plasminogen activator/plasminogen (32) and acid lysosomal hydrolases. In light of this, it is interesting to note that the secreted acid hydrolases have been shown to be solely responsible for the potent elastinolytic activity attributed to the activated macrophages; this has lead to the implication that these enzymes are involved in the pathophysiological remodeling of the extracellular matrix (33). The involvement of cathepsin S in such a physiological function is further supported by our finding that the treatment of macrophages or microglia with LPS leads to 2-5-fold increase in secretion of cathepsin S activity and that cathepsin S cleaves various ECM components at neutral pH conditions, causing their complete and/or partial degradation (Fig. 7).

Cathepsin S efficiently cleaves fibronectin and laminin in vitro and might be involved in their metabolism in vivo, especially during development and after injury in adult brain when there is an increased production of these molecules (34–36). We have also shown that cathepsin S can degrade neurocan and phosphacan, two brain CSPGs, at acidic and neutral pH. We have also found that cathepsin S is capable of degrading HSPGs at neutral pH (data not shown). In AD brains, CSPG and HSPG are components of the senile plaques (37, 38). Interestingly, it has also been shown that CSPGs and HSPGs can protect the potentially neurotoxic amyloid β peptide from proteolysis in vitro (39) and that the protein moiety of the proteoglycans is critical for amyloid β fibril formation and persistence (40). It is therefore possible that cathepsin S plays a modulatory role in the formation and persistence of amyloid fibrils in the senile plaques, particularly since it has been detected by immunocytochemistry around senile plaques in Alzheimer’s disease brains (41).

The fact that the expression of cathepsin S is tightly regulated and that it is capable of degrading extracellular matrix components under neutral pH suggests an important role for cathepsin S in the context of the mononuclear-phagocytic system, regarding processes of normal growth and development as well as during pathological conditions.
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