Journal of Alzheimer's Disease 19 (2010) 885–894
DOI 10.3233/JAD-2010-1291
IOS Press

Cystatin C Protects Neuronal Cells from Amyloid-β-induced Toxicity

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Handling Associate Editor: Luciano D'Adamio

Accepted 15 September 2009

Abstract. Multiple studies suggest that cystatin C (CysC) has a role in Alzheimer's disease (AD) and a decrease in CysC secretion is linked to the disease in patients with a polymorphism in the CysC gene. CysC binds amyloid-β (Aβ) and inhibits formation of Aβ fibrils and oligomers both *in vitro* and in mouse models of amyloid deposition. Here we studied the effect of CysC on cultured primary hippocampal neurons and a neuronal cell line exposed to either oligomeric or fibrillar cytotoxic forms of Aβ. The extracellular addition of the secreted human CysC together with preformed either oligomeric or fibrillar Aβ increased cell survival. While CysC inhibits Aβ aggregation, it does not dissolve preformed Aβ fibrils or oligomers. Thus, CysC has multiple protective effects in AD, by preventing the formation of the toxic forms of Aβ and by direct protection of neuronal cells from Aβ toxicity. Therapeutic manipulation of CysC levels, resulting in slightly higher concentrations than physiological could protect neuronal cells from cell death in AD.

Keywords: Alzheimer's disease, amyloid-β, cystatin C, neurodegeneration, neuroprotection

INTRODUCTION

The major neuropathological hallmarks of AD are senile plaques, neurofibrillary tangles, and neuronal loss. Senile plaques comprise an extracellular core of aggregated, fibrillar Aβ accompanied by microglial cells, astrocytes, and dystrophic neuronal processes. Although Aβ is the major constituent of the amyloid deposits, minor components were identified, including CysC that has been reported to co-localize with Aβ in parenchymal and vascular amyloid deposits [1–4]. More recently, multiple studies have shown the genetic linkage of the CysC gene (*CST3*) with late-onset AD (for review see [5]). For an updated record of all published AD-associated studies for CST3 see the Alzgene Internet site of the Alzheimer Research Forum [6]. A synergistic risk association between the *CST3* polymorphism and apolipoprotein E (APOE) ε4 alleles has been reported [7,8]. Furthermore, *in vitro* and *in vivo* studies have shown that CysC binds to Aβ (Aβ_{1-40} and Aβ_{1-42}) and inhibits fibril formation and oligomerization of Aβ [9–12].

In addition to its anti-amyloidogenic role, CysC has a broad spectrum of biological roles in numerous cellular systems, with growth-promoting activity, inflammation down-regulating function, and anti-viral and anti-bacterial properties (for review see [5]). It is involved in numerous and varied processes such as cancer, renal diseases, diabetes, epilepsy, and neurodegenerative diseases. Its function in the brain is unclear but it has been implicated in the processes of neuronal degeneration

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ISSN 1387-2877/10/$27.50

and repair of the nervous system (for review see [5]). *In vitro* studies that have used various cell types exposed to a variety of toxic stimuli have reached conflicting conclusions, as to whether CysC is protective or toxic to the cells.

Extensive research suggests that Aβ has an important role in the pathogenesis of neuronal dysfunction in AD (for reviews see [13–15]), although the pathologically relevant Aβ conformation remains unclear [16]. Aβ spontaneously aggregates into the fibrils that deposit in senile plaques. Synthetic Aβ peptides are toxic to hippocampal and cortical neurons, both *in vitro* and *in vivo* [17–19]. Aβ_{1-42} has been shown to induce protein oxidation and lipid peroxidation both *in vitro* and *in vivo* and thus was suggested to play a central role as a mediator of free radical induced oxidative stress and neurotoxicity in AD brains (for review see [20]). However, there is evidence that fibrillar Aβ may not be the most neurotoxic form of the peptide. The quantity and temporal progression of amyloid plaques do not show a simple relationship to the clinical progression of the disease [21]. Furthermore, extensive cortical plaques, mostly of the diffuse type, are present also in non-demented elderly [22]. Both *in vitro* and *in vivo* reports describe a potent neurotoxic activity for soluble, nonfibrillar, oligomeric assemblies of Aβ (for reviews see [23,24]).

The primary structure of CysC is indicative of a secreted protein and accordingly, it has been demonstrated that most of the CysC is targeted extracellularly via the secretory pathway (for review see [5]). Therefore, we have studied the effect of exogenously applied human CysC on cells of neuronal origin under Aβ-induced neurotoxic stimuli, showing that CysC protects neuronal cells from death induced by both fibrillar and oligomeric Aβ. The data demonstrate that CysC has multiple neuroprotective functions, underscoring the importance of developing CysC-dependent therapy for AD.

MATERIALS AND METHODS

Cell culture

N2a cells (ATCC, Manassas, VA) were grown in Dulbeco's Modified Eagle Medium (DMEM) (Gibco Life Technologies-Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Gemini Bio-products, West Sacramento, CA), 1% penicillin-streptomycin, and 1% glutamine. Cultures were incubated at 37°C in 5% CO_2 atmosphere. Cell cultures were washed twice with warm phosphate-buffered saline (PBS) (20 mM NaH_2PO_4, 140 mM NaCl, pH 7.4) and once with warm serum-free medium prior to incubation in either serum-supplemented or serum-free medium. Either fibrillar or oligomeric preformed Aβ was added into serum-free medium in the absence or presence of human urinary CysC (Calbiochem- EMD Bioscience, San Diego, CA).

Primary cultures of hippocampal neurons

Primary cultures of hippocampal neurons were established from E18 pups of pregnant Sprague-Dawley rats (Charles River Labs, Wilmington, MA). Procedures involving experiments on animal subjects were done in accord with the provisions of the PHS "Guide for the Care and Use of Laboratory Animals" and the "Principles for the Utilization and Care of Vertebrate Animals". Brains were harvested and maintained in sterile PBS, hippocampi were dissected out using a dissection microscope, triturated using a sterile glass Pasteur pipette and maintained in serum-free medium. Viable cells were counted using a hemacytometer. Neurons were plated in Neurobasal medium supplemented with 2% B27 without antioxidants, 0.30% glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin, at a density of 300,000 cells/ml in 24-well plates and 6-well plates coated with poly-D-lysine and incubated at 37°C in 5% CO_2 atmosphere. Neuronal cultures were treated 7 days post-plating.

Aβ preparation

Aβ_{1-42} peptide was purchased from Dr. David Teplow (California, UCLA) and was resuspended in 100% 1,1,1,3,3,3 hexafluoro-2-propanol (HFIP) at a final concentration of 1 mM. For complete solubilization the peptide was homogenized using a Teflon plugged 250 μl Hamilton syringe. HFIP was removed by evaporation in a SpeedVac, Aβ_{1-42} resuspended at a concentration of 5 mM in dimethylsulfoxide (DMSO) and sonicated for 10 minutes. Oligomers were prepared as previously described [25]: Aβ_{1-42} was diluted in PBS to 400 μM and 1/10 volume 2% sodium dodecyl sulfate (SDS) in H_2O added. Aβ was incubated for 24 hours at 37°C and further diluted to 100 μM in PBS followed by 18 hours incubation at 37°C. For generation of Aβ fibrils, the 5 mM DMSO Aβ_{1-42} solution was diluted in 10 mM HCl to make a 100 μM solution, vortexed for 30 seconds and incubated for 24 hours at 37°C as previously described [26].

N2a viability assay using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (*MTS*)

20 μl of the CellTiter 96 Aqueous One Solution Reagent (Promega, Madison, WI) were added into each well of the 96 wells plate containing cells in 100 μl culture medium, incubated with the reagent for 3 hours at 37°C in 5% CO_2 and absorbance at 490 nm was recorded. As a negative control cell-free medium was included. Neuronal survival is expressed as percentage of neuronal survival in control cultures. Mean and SEM are calculated for 4 separate experiments and significance is calculated by Student's t-test.

Neuronal viability assay for hippocampal cultures

The number of viable cells was determined by quantifying the number of intact nuclei as described previously [27,28]. Briefly, culture medium was removed by aspiration and 100 μl of detergent-containing lysis solution was added to the well. This solution dissolves cell membranes providing a suspension of intact nuclei. Intact nuclei were quantified using a hemacytometer. Triplicate wells were scored and are reported as mean $\pm$ SEM and significance is calculated by Student's t-test.

Western blot analysis

The proteins were electrophoretically transferred onto a 0.2 μm nitrocellulose membrane (Whatman) in 2.5 mM Tris/19.2 mM Glycine/20% methanol transfer buffer. The membrane was blocked in Odyssey blocking buffer (Licor, Lincoln, NE) diluted 1:1 in PBS and incubated for 1 hour at room temperature, incubated with a monoclonal anti-caspase-2 antibody (1:1000; BD Bioscience, Franklin Lakes, NJ) overnight at 4°C, and with secondary antibody (IRdye 800; 1:25,000; Rockland Immunochemicals, Gilbertsville, PA) for 2 hours at room temperature. The membrane was washed 3 times in PBST followed by a final wash in PBS. Bands were visualized and quantified using the Odyssey Infrared Imager (Licor).

Dot blot analysis

Samples containing mixtures of Aβ and CysC were incubated in conditions in which Aβ forms oligomers as described above and blotted onto a 0.2 μm nitrocellulose membrane (BioRad, Hercules, CA). The membrane was blocked in 5% milk (BioRad) in 10 mM Tris, 150 mM sodium chloride, pH 7.5, 0.1% Tween-20 (TBST), incubated with either anti-CysC antibody (1:1000; Upstate, Temecula, CA) or A11 antibody (1:1000; Abcam, Cambridge, MA) for 2 hours at room temperature, and with secondary antibody (1:2000) for 1 hour at room temperature. The membrane was incubated in chemiluminiscent fluid (Millipore, Temecula, CA) for 60 seconds prior to exposure. The membrane was imaged using a Fuji LAS-3000 gel documentation unit for 10–60 seconds. Quantification was performed by digital image using the native Fuji software, ImageGauge. Mean and SEM are calculated for 4 separate experiments.

Biotin VADfmk isolation of active caspases

bVADfmk (biotin-valyl-alanyl-aspartyl-fluoromethylketone) assay was used to detect the presence of active caspase-2. Rat hippocampal neurons were pre-treated for 2 hours with 50 μM bVADfmk (MP Biomedical, Solon, OH) at 37°C. Cells were treated for an additional 3 hours with 3 μM oligomeric Aβ and harvested in CHAPS buffer (150 mM KCl; 50 mM HEPES; 0.1% CHAPS; pH 7.4) supplemented with protease inhibitor cocktail tablets (Complete Mini, Roche, Indianapolis, IN). Cells were spun at 15,000 g for 10 minutes and the supernatant was collected and boiled for 5 minutes. Streptavidin agarose beads were added to the boiled supernatant and incubated in a rotor overnight at 4°C. Beads were spun at 7500 g for 5 minutes, washed 5 times with PBS, resuspended in sample buffer (1% SDS, 3% Glycerol, and 20 mM Tris-Hcl, pH 6.8) and boiled 5 minutes. Beads in sample buffer were spun at 15,000 g for 10 minutes, supernatant was collected and supplemented with 5% β-mercaptoethanol and samples loaded onto a 12% acrylamide-bis-acrylamide gel for Western blot analysis.

RESULTS

CysC protects neuronal cells from Aβ-induced cell-death

In vitro studies have demonstrated that CysC inhibits aggregation of Aβ into either fibrils [9] or oligomers [10] when incubated together under conditions that result in Aβ aggregation. Here we show that the addition of soluble CysC together with preaggregated Aβ directly protects N2a neuroblastoma cells from the toxicity induced by either fibrillar (Fig. 1A) or oligomeric (Fig. 1B) Aβ. Soluble CysC

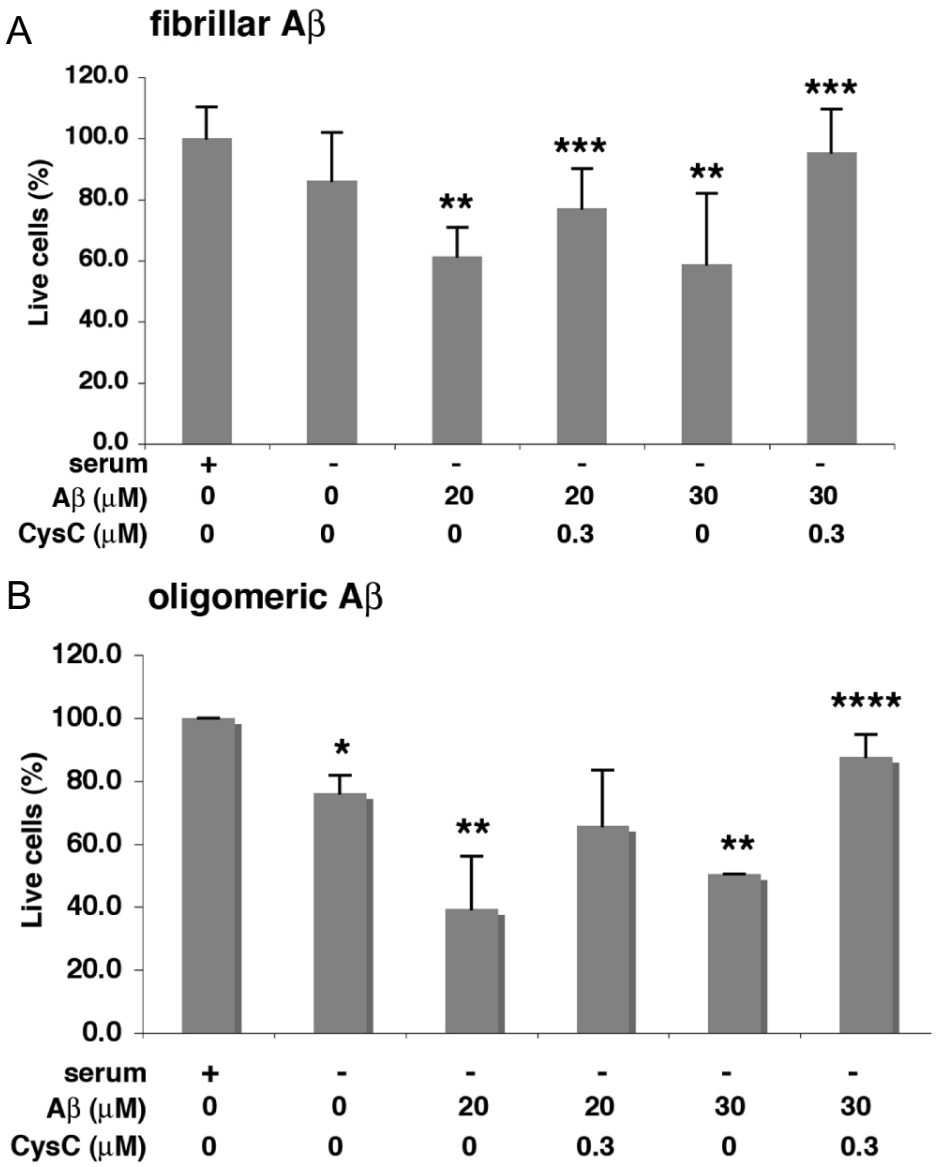

Fig. 1. *CysC protects N2a neuroblastoma cells from Aβ-induced cell-death.* N2a cells were incubated for 40-44 hours in serum-free medium, in the presence or absence of either fibrillar (A) or oligomeric (B) Aβ, in the presence or absence of human CysC. MTS analysis of live cells, presented as mean $\pm$ standard deviation of percent of live cells incubated in serum-containing medium without either Aβ or CysC (mean of 4 experiments; $^{*}p \leqslant 0.02$ and $^{**}p \leqslant 0.003$ for the difference from cells with serum; $^{***}p \leqslant 0.03$ and $^{****}p \leqslant 0.002$ for the difference from cells with Aβ without CysC).

also protects rat primary hippocampal cells from Aβ-induced cell-death when cells were incubated in the presence of either fibrillar (Fig. 2A) or oligomeric (Fig. 2B) Aβ. *In vitro* studies have previously demonstrated that CysC does not dissolve pre-formed Aβ fibers or oligomers [9,10]. Furthermore, we have found that under the incubation conditions used here CysC does not solublize aggregated Aβ. This suggests that CysC directly protects neuronal cells from Aβ-induced toxicity.

In vitro binding of CysC to Aβ inhibits Aβ oligomerization and prevents Aβ oligomers-induced cell death

For analysis of the effect of CysC on Aβ oligomerization, we prepared *in vitro* oligomeric Aβ_{1-42} from structurally homogeneous unaggregated starting mate-

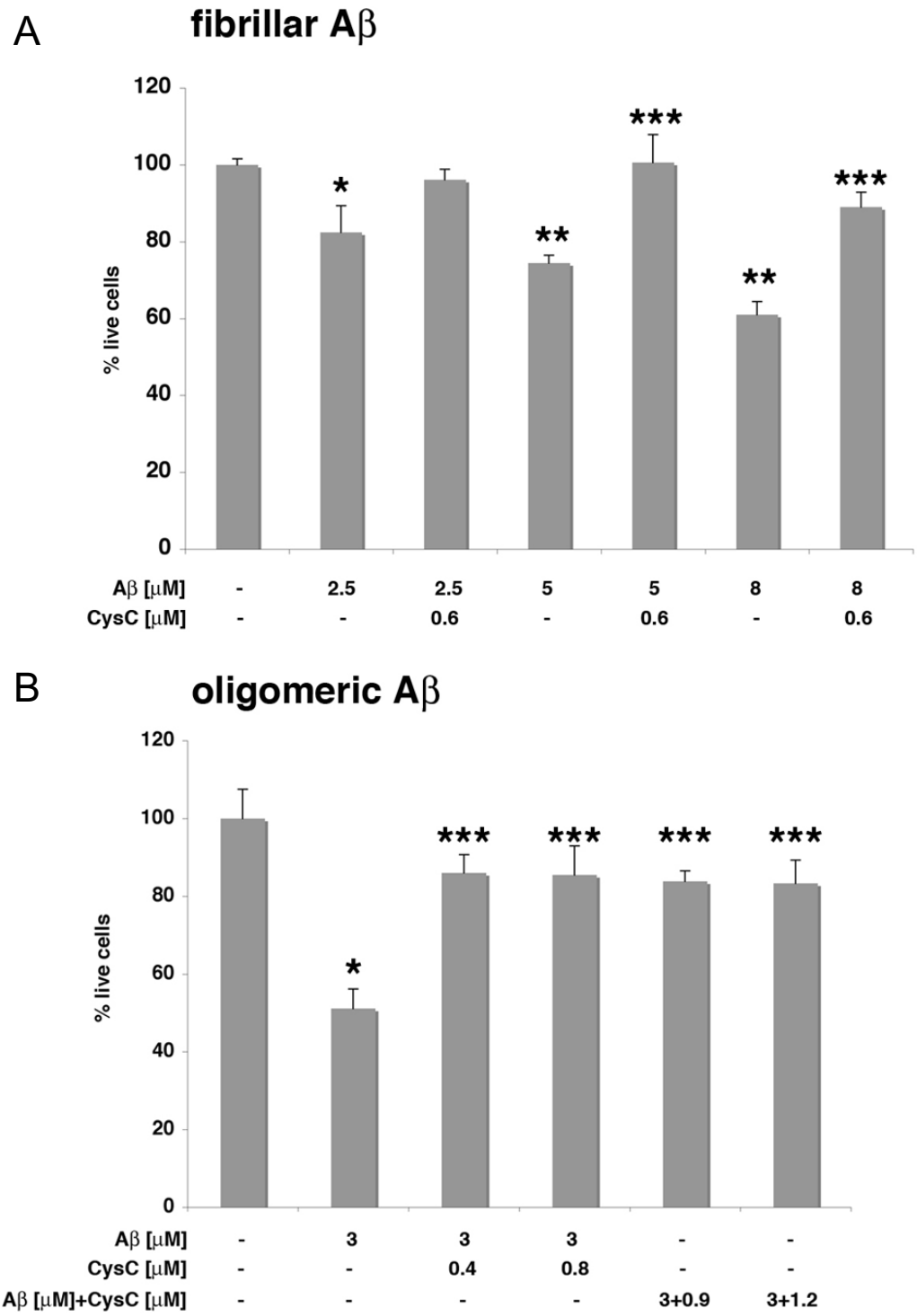

Fig. 2. *CysC protects rat primary hippocampal neurons from Aβ-induced cell-death.* Cells were incubated for 24 hours, in the presence or absence of either fibrillar (A) or oligomeric (B) Aβ, in the presence or absence of human CysC. Additionally, Aβ was preincubated with CysC at conditions in which Aβ forms oligomers and the mix was added to the cells (B). Quantification of live cells, presented as mean $\pm$ standard deviation of percent of live cells incubated in serum-containing medium without either Aβ or CysC (mean of 4 experiments; $^{*}p \leqslant 0.05$ and $^{**}p \leqslant 0.0003$ for the difference from cells without Aβ and CysC; $^{***}p \leqslant 0.003$ for the difference from cells with Aβ without CysC).

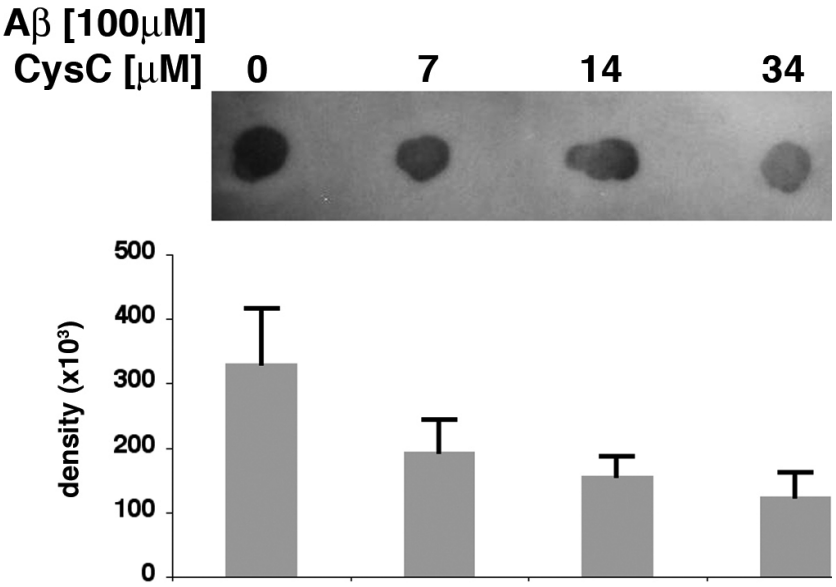

Fig. 3. *CysC inhibits Aβ oligomerization in vitro*. Dot blot analysis with the antibody specific for oligomeric conformation (A11) of $A\beta_{1-42}$ (100 μM) incubated with CysC (0, 7, 14 or 34 μM). Mean $\pm$ standard deviation of quantification of the intensity of the blots from 3 experiments.

rial. Dot blot analysis with the conformation-dependent antibody A11 [29–32] demonstrated the formation of oligomers by $A\beta_{1-42}$ in the absence of CysC (Fig. 3). When soluble $A\beta_{1-42}$ was incubated under the same experimental conditions together with different concentrations of CysC, a CysC-concentration-dependent decrease in the oligomeric form of Aβ was observed (Fig. 3).

In order to test whether *in vitro* prevention of Aβ oligomerization decreases the toxicity of Aβ oligomers, the preincubated mixture of Aβ and CysC was added to rat primary hippocampal neurons. A significantly lower toxicity was observed as compared to the Aβ oligomers that were formed in the absence of CysC (Fig. 2B). Thus, prevention of Aβ oligomerization protects rat hippocampal neurons against Aβ-induced toxicity.

CysC prevents Aβ-induced caspase-2 activation

Rat primary hippocampal neurons were incubated in the presence or absence of oligomeric Aβ, in the presence or absence of CysC. In order to detect active caspase-2, we used an active site affinity ligand, bVADfmk. The biotinylated substrate irreversibly and covalently binds to the active cysteine site of most caspases in the cell trapping them in the activated form. Once bound to bVADfmk, the caspase is no longer active and downstream events are inhibited. Pretreatment with bVADfmk, pull down of active caspases with streptavidin agarose beads followed by Western blot analysis with an antibody to caspase-2 revealed that while oligomeric Aβ induces caspase-2 activity, incubation of the cells with Aβ in the presence of CysC suppresses caspase-2 activity (Fig. 4).

DISCUSSION

CysC is a secreted protein, targeted extracellularly via the secretory pathway and is taken up by cells (for review see [5]). Therefore, we have studied the *in vitro* effect of exogenously applied human CysC on cells of neuronal origin under neurotoxic stimuli. Here we show that CysC protects N2a neuroblastoma cells and rat primary hippocampal neurons from the toxicity induced by either oligomeric or fibrillar Aβ.

Aβ has been implicated in the pathophysiology of AD. The histopathological hallmarks of AD include the formation of Aβ plaques and neurofibrillary tangles and the loss of synapses. While the temporal order in which these events occur and their relationship to one another is not clear, there is evidence of a direct toxic effect of Aβ on neurons [33]. Using different promoters a variety of transgenic models have been engineered to overexpress in the brain mutant forms of amyloid-β protein precursor (AβPP). Several models reproducibly deposit Aβ and develop some of the prominent patho-

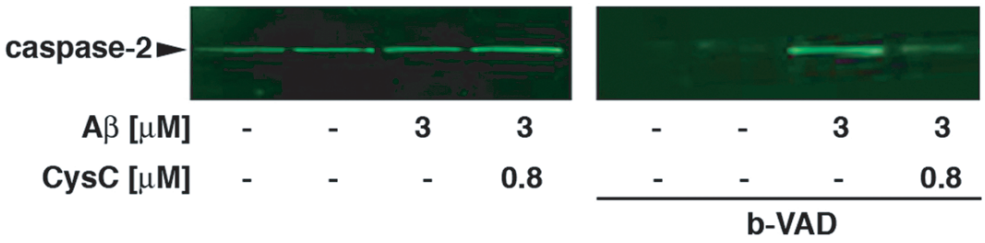

Fig. 4. *CysC prevents Aβ-induced caspase-2 activation.* Rat primary hippocampal neurons were pre-treated for 2 hours with 50 μM bVADfmk. After 2 hours cells were incubated for an additional 3 hours in the presence or absence of 3 μM oligomeric Aβ and in the presence or absence of 0.8 μM CysC. Active caspase-2 was pulled down using streptavidin beads and identified by Western blot analysis with a monoclonal caspase-2 specific antibody. Representative blots are shown; the experiments were replicated 3 times.

logical and behavioral features of AD [34–40]. These findings resulted in focusing research on drugs that reduce the production of Aβ or enhance its degradation, and lowering cortical Aβ levels in some AD patients may be associated with stabilization of memory and cognitive decline [41,42]. There are indications that both fibrillar Aβ and soluble oligomeric Aβ are neurotoxic [43–46]. However, Aβ has a widespread distribution through the brain and body and there is evidence that at physiological concentrations soluble Aβ serves a variety of physiological functions, including modulation of synaptic function, facilitation of neuronal growth and survival, protection against oxidative stress, and surveillance against neuroactive compounds, toxins and pathogens (for review see [47]). These physiological functions should be taken into account when strategies are developed to reduce Aβ load in AD, targeting oligomeric or fibrillar forms of Aβ, leaving monomeric soluble Aβ intact. Our approach does not reduce the level of soluble Aβ, focusing on enhanced binding of soluble Aβ to its carrier CysC, preventing its aggregation and fibril formation and enhancing CysC-mediated direct protection of neuronal cells from the toxicity induced by either form of Aβ.

Caspases are cysteine-dependent aspartate-specific proteases critically involved in apoptosis (for review see [48]). The detection of cleaved caspases and the accumulation of cleaved caspase substrates in brains of AD patients support the hypothesis that apoptosis may play a role in the subsequent neuronal loss found in AD brains [49,50]. Elevated mRNA expression of several caspases was shown in the brain of AD patients compared with controls [51]. Pyramidal neurons from vulnerable regions involved in the disease showed an increase in activated caspases-3 and -6 [52,53]. Synaptosomes prepared from AD brain frontal cortices showed an enrichment in caspase-9 compared with non-demented controls [54]. Studies have shown induction of apoptosis by Aβ in multiple neuronal cell types in culture [55–57]. Aβ-induced cell death was blocked by the broad spectrum caspase inhibitor N-benzyloxycarbonyl-val-ala-asp-fluoromethyl ketone and more specifically by the downregulation of caspase-2 with antisense oligonucleotides. In contrast, downregulation of caspase-1 or caspase-3 did not block Aβ-induced death [28]. Neurons from caspase-2- or 12-knockout mice are resistant to Aβ [28,58]. The results indicate that caspase-2 is necessary for Aβ-induced apoptosis. Here we demonstrate that while Aβ induces activation of caspase-2 in primary hippocampal neurons, CysC inhibits this activation. This suggests that CysC protects neuronal cells from caspase-dependent apoptotic cell death induced by Aβ.

There are several indications that CysC has a role in AD: 1) Genetic studies have linked a *CST3* polymorphism with an increased risk of developing AD (for review see [5,6]). The amino acid exchange from Ala to Thr at the -2 position for signal peptidase cleavage [59], causes a less efficient cleavage of the signal peptide and thus a reduced secretion of CysC [60–62]. 2) Analysis of human cerebrospinal proteins by protein-chip array technology revealed that the combination of five polypeptides, including CysC, could be used for the diagnosis of AD and perhaps the assessment of its severity and progression [63]. 3) Recent findings that low serum CysC levels predict the development of AD in subjects free of dementia at baseline [64], suggest that low serum CysC levels precede clinical sporadic AD. 4) Immunohistochemical studies revealed strong dual staining with antibodies to Aβ and to CysC in a subpopulation of pyramidal neurons in the prefrontal cortex and hippocampus. Co-localization of CysC with Aβ was found predominantly in amyloid-laden vascular walls, and in senile plaque cores of amyloid in the brains of AD, Down syndrome, cerebral amyloid angiopathy, and cerebral infarction patients and non-demented aged individuals (for review see [5]). Co-localization of CysC with Aβ deposits was

also found in brains of transgenic mice overexpressing human AβPP [4,65]. 5) Furthermore, *in vitro* studies have shown that CysC binds to Aβ and inhibits fibril formation and oligomerization of Aβ in a concentration dependent manner [9,10]. Such inhibitory effect was confirmed *in vivo* in Aβ-depositing transgenic AβPP mice overexpressing human CysC. A reduction in Aβ load was observed in the AβPP/CysC double transgenic mice compared to single AβPP transgenic mice [11, 12]. *In vitro* studies have shown that CysC inhibits the formation of high molecular weight Aβ oligomeric assemblies [10]. Moreover, the binding between Aβ and CysC in human CNS was detected in brains and in cerebrospinal of neuropathologically normal controls and in AD cases. The association of CysC with Aβ in brain from control individuals and in cerebrospinal fluid reveals an interaction of these two polypeptides in their soluble form [66]. In addition to these previous data, showing that CysC prevents amyloid fibril formation and oligomerization of Aβ, the demonstration of direct protection of cells from the toxic forms of Aβ highlights the important defensive roles that CysC plays in AD.

Multiple studies have shown changes in CysC serum concentrations in a variety of conditions, including aging (for review see [67]). Enhanced CysC expression occurs in human patients with epilepsy and animal models of neurodegenerative conditions, in response to injury, including facial nerve axotomy, noxious input to the sensory spinal cord, perforant path transections, hypophysectomy, transient forebrain ischemia, and induction of epilepsy (for review see [5]). It has been suggested that this upregulation of CysC expression in response to injury and in various diseased conditions represents an intrinsic neuroprotective mechanism that may counteract progression of the disease. A reduction in CysC secretion is caused by the *CST3* polymorphism in patients with late-onset sporadic AD and by two presenilin 2 mutations (PS2 M239I and T122R) linked to familial AD [68]. We propose that CysC is a carrier of soluble Aβ in body fluids such as cerebrospinal fluid and blood, as well as in the neuropil, where it plays an ongoing role in inhibiting the association of Aβ into insoluble plaques. Furthermore, CysC directly protects neuronal cells from Aβ-induced apoptotic cell death. The inhibition of Aβ aggregation caused by binding of CysC to Aβ and the direct protection of neuronal cells from Aβ-induced death suggest two mechanisms by which a reduced CysC brain concentration is associated with AD. These protective roles of CysC in the pathogenesis of AD suggest that a novel therapeutic approach that involves modulation of CysC levels may have important disease modifying effects.

ACKNOWLEDGMENTS

This research was funded by NIH grants AG017617 (EL) and NS043089 (CMT), and the Alzheimer's Association (IIRG0759699) (EL).

Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=158).

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