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Protective mechanisms by cystatin C in neurodegenerative diseases

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1. ABSTRACT

Neurodegeneration occurs in acute pathological conditions such as stroke, ischemia, and head trauma and in chronic disorders such as Alzheimer's disease, Parkinson's disease, and amyotrophic lateral sclerosis. While the cause of neuronal death is different and not always known in these varied conditions, hindrance of cell death would be beneficial in the prevention of, slowing of, or halting disease progression. Enhanced cystatin C (CysC) expression in these conditions caused a debate as to whether CysC up-regulation facilitates neurodegeneration or it is an endogenous neuroprotective attempt to prevent the progression of the pathology. However, recent *in vitro* and *in vivo* data have demonstrated that CysC plays protective roles via pathways that are dependent on inhibition of cysteine proteases, such as cathepsin B, or by induction of autophagy, induction of proliferation, and inhibition of amyloid-beta aggregation. Here we review the data demonstrating the protective roles of CysC under conditions of neuronal challenge and the protective pathways induced under various conditions. These data suggest that CysC is a therapeutic candidate that can potentially prevent brain damage and neurodegeneration.

2. INTRODUCTION

CysC (1), also known as gamma trace (2), belongs to the cystatin type 2 super family (3). It is a 120 amino acids protein preceded by a 26 amino acids amino-terminal secretory signal and contains two intramolecular disulfide bridges (4, 5). It is ubiquitously expressed by all mammalian tissues and is secreted into all body fluids (1, 6). CysC is a marker of kidney dysfunction (7) and has a broad spectrum of biological roles in numerous cellular systems, ranging from anti-viral and anti-bacterial properties (1), bone resorption (8), tumor metastasis (9), modulation of inflammatory responses (1, 10), and cell proliferation and growth (11, 12). Similar to a variety of activities that have been associated with other protease inhibitors in the brain (13-15), CysC has been implicated in the response of the nervous system to neuronal degeneration. While the involvement of CysC in pathological conditions of the central nervous system has been demonstrated, its role has been a matter of debate [reviewed in (16, 17)]. Changes in the levels of CysC in the cerebral spinal fluid were documented in a number of neurodegenerative diseases and it was suggested to be of diagnostic importance (18-23). Enhanced CysC expression

occurs in specific neuronal cell populations in the brains of human patients with Alzheimer's disease (AD) (24, 25) and in animal models of neurodegenerative conditions caused by facial nerve axotomy (26), noxious input to the sensory spinal cord (27), perforant path transections (28), hypophysectomy (29), transient forebrain ischemia (30, 31), photothrombotic stroke (32), and induction of epilepsy (33-35). Enhanced CysC gene expression and higher CysC protein levels were also shown in dopaminergic-depleted rat striatum following a 6-Hydroxydopamine (6-OHDA)-induced lesion in nigrostriatal neurons, astrocytes, and microglia cells (36). Administration of human CysC into the rat substantia nigra pars compacta partially rescued nigral dopaminergic neurons following a 6-OHDA-induced lesion. An *in vitro* study showed that loss of dopaminergic-neurons in fetal mesencephalic cultures due to exposure to 6-OHDA is partially reversed by treatment with human CysC (36). Augmented CysC expression was also observed *in vitro* in cultured rat brain neurons in response to oxidative stress (37). PC12 cell lines that stably expressed rat CysC showed resistance to high oxygen atmosphere and to glutamate- and 13-L-hydroperoxylinoleic acid (LOOH)-induced cell death (38). A similar up-regulation of CysC was shown in human cerebral microvascular smooth muscle cells and in aortic smooth muscle cells after 48 hours of hypoxia or hypoxia followed by reoxygenation (39).

Several hypotheses can be envisioned to explain the involvement of CysC in the brain: it can be involved in regulation of apoptosis, cell proliferation, mitogenic activity of the cell, promotion of survival, or prevention of cell death. The main focus of this review is to unfold the mechanisms of protection induced in different pathological processes based on observations from cell cultures and animal models of neurological disorders.

3. MECHANISMS OF NEUROPROTECTION BY CYSTATIN C

3.1. Neuroprotection by inhibition of cysteine proteases

CysC is considered an important endogenous inhibitor of cysteine protease activity because of its potent *in vitro* inhibition of cathepsins (Cat) B, H, K, L, and S [reviewed in (6, 40)]. Cathepsins are proteinases required for housekeeping function during protein turnover that differ in structure, substrate-specificity, and biochemical characteristics [reviewed in (41)]. CysC itself is a target of proteolysis (42, 43) and is inactivated by proteolytic degradation by Cat D and elastase (44, 45). Imbalance between endogenous inhibitors and cysteine proteinases has been associated with different diseases such as AD (46), rheumatoid arthritis (47), renal failure (48), multiple sclerosis (49, 50), muscular dystrophy (51), inflammatory periodontal disease (52), inflammatory lung disease (53), inflammation and trauma (54), and various types of cancer (55-58). Enhanced expression of several cathepsins in the brain has been documented in response to injuries, similar to those inducing CysC expression upregulation, such as in transient ischemia (59), and inhibitors of Cat B and Cat L have been found to reduce neuronal damage in the hippocampus after ischemia (60, 61).

There are data placing both cathepsins and CysC in intracellular as well as extracellular locations and establishing the respective subcellular distribution of these proteases and their endogenous inhibitors is fundamental to the understanding of the regulation of lysosomal proteolysis and its disruption in neurodegenerative diseases. The primary structure of CysC is indicative of a secreted protein and accordingly, it was demonstrated that most of the CysC synthesized by mouse neuroblastoma N2a cells (62), human embryonic kidney HEK293 cells (62), or human retinal pigment epithelial cells (63), is targeted extracellularly via the secretory pathway. However, CysC was also found in endocytic cellular compartments and it was shown that it inhibits cathepsin activities within the lysosomal system (64). Moreover, it was demonstrated that CysC added to cell-culture media is internalized (65, 66). Although Cat B and Cat D are typically localized in lysosomes, they are also found at other cellular sites (67) such as trans-Golgi vesicles (68), transport vesicles (69), secretory vesicles (70), clathrin-coated vesicles (71), and endosomes (72). Cathepsins may be released through exocytosis, and activated microglia secrete several proteases including Cat B (73) that can trigger neuronal apoptosis (74). It was suggested that, in degenerating neurons, cathepsins could be released into the cytoplasm and neuropil after disruption of lysosomes (75-78). Release of Cat B to the extracellular fluid can cause proteolytic tissue damage leading to organ failure, and matrix destruction associated with inflammation, tumor invasion, and metastasis [reviewed in (79-80)]. Localization of cathepsins in senile plaques in the brain of AD patients led to the suggestion that degenerating neurons or their processes are a major source of extracellular Cat D and various other lysosomal hydrolases and hydrolase activities within amyloid deposits (81, 82).

Pharmacological inhibition of cathepsins has been shown to reduce neuronal damage after brain ischemia, thus it was suggested that CysC, an endogenous inhibitor of cathepsins, is an endogenous neuroprotectant. *In vivo* studies demonstrated increased Cat B activity in the brain of CysC knockout mice, confirming the inhibitory function of CysC (84). Larger brain infarcts were found in CysC knockout mice after focal ischemia induced by 40 minutes occlusion of the origin of the middle cerebral artery as compared to wild type mice (85). However, brain damage in the CA3 region of the hippocampus, the dentate gyrus, and cortex of CysC knockout mice was diminished after global ischemia induced by 12 minutes occlusion of both common carotid arteries (85). The different responses after focal and global ischemia in CysC knockout mice suggest that the protective role imparted by CysC is differential and restricted to certain brain regions or certain brain insults.

In vivo neuroprotection by CysC, modulated by inhibition of cysteine proteases was recently demonstrated in a mouse model of an inherited neurodegenerative disorder, progressive myoclonic epilepsy (86). Cystatin B (CysB) is an inhibitor of cysteine proteases, including Cat B, H, L, and S (3, 4), a member of the cystatin family 1 of cysteine protease inhibitors. It is mainly localized in

lysosomes (86) and is diffusely distributed in the cytoplasm (86, 87). Loss-of-function mutations in the CysB gene lead to a rare autosomal disorder, Unverricht-Lundborg disease (EPM1) (87-89), the most common form of progressive myoclonus epilepsies (90). EPM1 has an onset of symptoms at 6-15 years of age and progression with age leads to myoclonic and tonic-clonic seizures (91, 92), neurological decline, and severe ataxia (91, 93). A CysB knockout (CysBKO) mouse model develops myoclonic seizures and ataxia, similar to symptoms seen in the human disease (94). Degeneration of cerebellar granule cells (94), hippocampal neurons, and cells within the entorhinal cortex was observed in the developing brains of CysBKO mice (95). These mice also show gliosis and increased expression of apoptotic and glial activation genes (95, 96). The progressive cerebellar atrophy caused by CysB deficiency implicates a required role for CysB expression in the development of the cerebellum and in normal neuronal survival. Increased mRNA, protein, and enzymatic activity levels of the two lysosomal enzymes Cat B and Cat D were demonstrated in the brains of CysBKO mice (86). Data suggest that increased proteolysis by lysosomal cathepsins is responsible for the phenotypic characteristics of EPM1 (87, 97, 98) and deletion of Cat B in CysBKO mice resulted in a reduction in the amount of cerebellar granule cell apoptosis depending on mouse age (87).

An endogenous upregulation of CysC mRNA and protein was observed in the brains of EPM1-mimicking CysBKO mice (86). The increase in CysC expression in CysBKO mice might represent a compensatory intrinsic neuroprotective mechanism to rescue neurons by inhibiting the apoptosis-promoting actions of cathepsins. However, the level of CysC expression in these mice may not be sufficient to counteract the progression of the disease. In order to test the hypothesis that CysC overexpression can rescue the loss-of-function of CysB, CysBKO mice were crossbred with CysC overexpressing transgenic mice. It was demonstrated that clinical symptoms and neuropathologies, including deficient motor coordination, cerebellar atrophy, neuronal loss in the cerebellum and cerebral cortex, and gliosis caused by CysB deficiency, are rescued by CysC overexpression (86). CysC overexpression in CysBKO mice decreased Cat B and Cat D activities in the brain (86). These data show that CysC partially prevents neurodegeneration in CysBKO mice through inhibition of cathepsins activity. These findings demonstrate that CysC could be a therapeutic candidate with a potential of preventing EPM1.

3.2. Neuroprotection by induction of neurogenesis

It has been shown that independent of its effects on Cat activity, CysC regulates cell proliferation (11, 12). In rats undergoing acute hippocampal injury or status epilepticus-induced epileptogenesis, the expression of CysC mRNA and protein are increased in the hippocampus and in the dentate gyrus (33-35). The time of increased CysC expression parallels the time of prominent neurogenesis (99, 100). *In vitro* experiments showed that rat CysC evokes the proliferation of glomerular rat mesangial cells in an autocrine manner (12). It was shown that fibroblast growth factor 2 (FGF-2)-responsive neural stem cell proliferation requires a glycosylated form of rat CysC (101). *In vivo* grafting of adult rat hippocampus-derived neural progenitor cells coexpressing a secreted form of FGF-2 and the glycosylated form of rat CysC to adult rat hippocampus showed a 4-fold increased proliferation of endogenous progenitor cells in the proximal areas to the grafts within the granular layer of the dentate gyrus, compared to cells not expressing both FGF-2 and this form of CysC (101). Moreover, the basal level of neurogenesis in the subgranular layer of dentate gyrus was decreased in CysC knockout mice, supporting a role for CysC in neurogenesis (101, 102). Finding further revealed CysC as a critical factor for differentiating embryonic stem cells into neural stem cells, when recombinant mouse CysC was added to embryonic stem cell cultures in the presence of FGF2 and EGF (103). The proliferation and migration of newborn granule cells in the dentate gyrus are impaired in CysC knockout mice (102).

CysC is also involved in astrocytic differentiation during mouse brain development. *In vitro* studies showed that CysC upregulates glial fibrillary acidic protein (GFAP) promoter activity in an immature astrocyte cell line (104). This regulating role of CysC in glial development has been further confirmed: addition of human CysC into the culture medium of primary brain cells increased the number of GFAP-positive and nestin-positive cells, as well as the number of neurospheres formed from embryonic brain (105). Furthermore, CysC gene expression started earlier than that of GFAP in astrocyte progenitor cells in the ventricular zone of mouse forebrain (104). Thus, stimulation of cell proliferation and promotion of the mitogenic activity of cells may be another avenue for CysC mediated neuroprotection.

3.3. Neuroprotection by induction of autophagy

In vitro studies have demonstrated a concentration dependent protective effect of exogenously applied human CysC on neuronal cell lines and primary cortical neurons against the toxicity induced by nutrition-deprivation, oxidative stress, the microtubule-depolymerizing agent colchicine, or staurosporine, a potent wide spectrum inhibitor of protein kinases (106). Moreover, endogenous CysC overexpression in primary cortical neurons isolated from brains of CysC transgenic mice also protected the cells from spontaneous death induced by culturing and from B27-supplement-deprivation (106). Consistent with a protective role for CysC, cells isolated from CysC knockout mice were more sensitive to *in vitro* toxicity compared to cells isolated from brains of wild type mice (106).

In neuronal cultures exposed to cytotoxic challenges, the neuroprotective action of CysC does not require Cat B inhibition but involves induction of fully functional autophagy (106). Autophagy usually occurs in normal cells to maintain cellular turnover, clearance, and regeneration of new cellular components to restore balance in the system and promote neuronal health, and is greatly increased in cells under pathological conditions that cause cell dysfunction such as trophic stress or nutritional

deprivation, hypoxia, ischemia, endotoxin shock, and metabolic inhibition [reviewed in (107-109)]. The autophagic pathway consists of sequestration and turnover of organelles and cytoplasm in autophagic vacuoles that following maturation fuse with lysosomes, leading to degradation of their content. Autophagy activation reduces the size of cells and thereby decreases their metabolic burden, while generating new substrates for energy and cellular remodeling. Excessive or imbalanced induction of autophagic recycling on the other hand can actively contribute to neuronal atrophy, neurite degeneration, and cell death. Autophagy induction may protect cells from apoptosis by eliminating damaged mitochondria and other organelles that have the potential to trigger apoptosis [reviewed in (110-111)]. However, sustained over-activity or dysfunction of the autophagic pathway in pathologic states mediates a caspase-independent form of cell death that shares certain features with apoptosis (112-115). Multiple methods were used to demonstrate that CysC induces autophagy under nutrition-deprivation conditions above the levels of autophagy observed in stressed cells not treated with CysC. It was demonstrated (a) that the neuroprotective effects of CysC are prevented by inhibiting autophagy with *beclin 1* siRNA or 3-methyladenine; (b) that CysC causes microtubule-associated protein Light Chain 3 (LC3-I) conversion to membrane bound LC3-II; (c) that CysC increases the number of autophagic vacuoles in the cytoplasm of cells with otherwise normal ultrastructural morphology; (d) that it reduces mTOR activity (p70S6 kinase dephosphorylation); (e) and that the rate of long-lived proteins breakdown following metabolic labeling is increased by CysC treatment under nutrition deprivation conditions. These assays demonstrated that the observed increase in the number of autophagosomes after exposure to CysC reflects induction of a fully functional autophagy via the mTOR pathway that includes competent proteolytic clearance of autophagy substrates by lysosomes (106). Thus, enhanced lysosomal turnover can protect against neurodegeneration and CysC serves to modulate the efficiency of the autophagic pathway. Maintaining a balanced level of autophagy, promoting clearance and regeneration of new cellular components, is thought to be necessary for the maintenance and restoration of neuronal health (116). It remains to be demonstrated that CysC induces autophagy *in vivo* as a protective mechanism in brain injury and in neurodegenerative disorders.

3.4. Neuroprotection by inhibition of oligomerization and amyloid fibril formation

A variant form of CysC composes the amyloid deposited in the cerebral vasculature of patients with hereditary cerebral hemorrhage with amyloidosis, Icelandic type (HCHWA-I). HCHWA-I (117, 118), also called hereditary CysC amyloid angiopathy (HCCAA) (119), is an autosomal dominant form of cerebral amyloid angiopathy (CAA). Amyloid deposition in cerebral and spinal arteries and arterioles leads to recurrent hemorrhagic strokes causing serious brain damage and eventually fatal stroke (118). The amyloid deposited is composed mainly of a Leu68Gln variant of CysC (120-124). A heterozygous point mutation, identical to that found in the CysC gene of these patients, was also identified in a Croatian man with CAA and intracerebral hemorrhage (125). Thus, sporadic CAA in some patients may be associated with mutations in the CysC gene. The molecular pathogenesis of variant CysC has been studied intensively [reviewed in (126)]. While the variant form of CysC is amyloidogenic, data show that the soluble form of wild type CysC has anti-amyloidogenic properties (127-129).

AD is a progressive neurodegenerative disorder characterized by profound behavioral disorder, loss of memory and reasoning, and personality changes. Neuropathologic hallmarks of AD are loss of neurons with accelerated atrophy of specific brain areas, decreased synapse number in surviving neurons, formation of amyloid deposits in the brain composed mainly of amyloid-beta, a processing product of a larger amyloid beta protein precursor (APP), and presence of neurofibrillary tangles. Extensive research suggests that amyloid-beta has an important role in the pathogenesis of neuronal dysfunction in AD [for reviews see (130-132)], although the pathologically relevant amyloid-beta conformation remains unclear (133). While it was demonstrated that fibrillar amyloid-beta plays a central role in neurotoxicity in AD brains [for review see (134)], both *in vitro* and *in vivo* reports describe a potent neurotoxic activity for soluble, nonfibrillar, oligomeric assemblies of amyloid-beta [for reviews see (135, 136)].

Investigations of the roles of CysC in AD have revealed its participation in many of the pathologies that characterize the disease. The involvement of CysC in AD was originally suggested by its colocalization with amyloid-beta in amyloid-laden vascular walls, and in senile plaque cores of amyloid in brains of patients with AD, Down's syndrome, hereditary cerebral hemorrhage with amyloidosis, Dutch type (HCHWA-D), and cerebral infarction (25, 137-140). CysC also colocalizes with amyloid-beta amyloid deposits in the brain of non-demented aged individuals (25), aged rhesus and squirrel monkeys (141), and transgenic mice overexpressing human APP (25, 142).

More recently, genetic data were presented demonstrating linkage of the CysC gene (*CST3*), localized on chromosome 20 (143, 144), with B/B homozygosity associated with an increased risk of developing late-onset AD (145, 146). While some studies were unable to replicate these findings (147-149), the linkage was supported by others (150-152). For update on the linkage of the *CST3* polymorphism with AD see the Alzgene Internet site of the Alzheimer Research Forum (153). The polymorphism in *CST3* results in an amino acid exchange, which alters the hydrophobicity profile of the signal sequence (146), resulting in a less efficient cleavage of the signal peptide and thus a reduced secretion of CysC (154, 155), and decreased CysC in cerebrospinal fluid (18). Mutations in the presenilin 2 gene linked to familial AD (PS2 M239I and T122R) alter CysC trafficking in mouse primary neurons causing reduced CysC secretion (156). Reduced levels of CysC may represent the molecular factor responsible for the increased risk of AD and/or increased susceptibility to insult.

Immunohistochemical analyses have shown intensely CysC immunoreactive neurons and activated glia in the cerebral cortex of some aged human cases and of all AD patients (24, 157). Higher neuronal immunostaining of CysC in AD brains is primarily limited to pyramidal neurons in cortical layers III and V (24, 25). The regional distribution of CysC neuronal immunostaining duplicated the pattern of neuronal susceptibility in AD brains: the strongest staining was found in the entorhinal cortex, in the hippocampus, and in the temporal cortex; fewer pyramidal neurons were stained in the frontal, parietal, and occipital lobes (24). Pyramidal neurons in layers III and V in the cortex of AD patients have also displayed a quantitative increase in aspartic protease Cat D immunoreactivity (158). Immunostaining of CysC within neurons showed a punctate distribution, which colocalized with the endosomal/lysosomal protease Cat B (24). Upregulation of cathepsin synthesis in AD neurons and accumulation of hydrolase-laden lysosomes indicate an early activation of the endosomal/lysosomal system in vulnerable neuronal populations, possibly reflecting early regenerative or repair processes (159). Using an end-specific antibody to the carboxyl-terminus of amyloid-beta$_{42}$, intracellular immunoreactivity was observed in the same neuronal subpopulation (25). These data suggest that amyloid-beta$_{42}$ accumulates in a specific population of pyramidal neurons in the brain, the same cell type in which CysC is highly expressed.

The significance of the colocalization of CysC with amyloid-beta in the brain was revealed when it was shown that CysC has an anti-amyloidogenic property. It was demonstrated that CysC binds to the amyloid-beta region within full-length APP and that this association does not affect amyloid-beta generation both *in vitro* (127) and *in vivo* in transgenic mice expressing the human CysC gene (160). The association of CysC with APP was confirmed using a method for the *in vivo* mapping of protein interactions in intact mouse tissue (161). CysC does not bind only to amyloid-beta sequences within APP, but also to the peptide itself (127). Analysis of the association demonstrated that CysC interacts with both amyloid-beta$_{40}$ and amyloid-beta$_{42}$ in a concentration dependent manner at physiological pH and temperature. A specific, saturable, and high affinity binding between CysC and amyloid-beta was observed (127).

The binding between amyloid-beta and CysC was also observed in the human central nervous system. While CysC binding to soluble amyloid-beta was observed in tissues from AD patients and controls, an SDS-resistant CysC/amyloid-beta complex was detected exclusively in brains of neuropathologically normal controls (162). The association of CysC with amyloid-beta in brains of control individuals and in cerebrospinal fluid reveals an interaction of these two polypeptides in their soluble form. Most importantly, *in vitro* studies have demonstrated that CysC association with amyloid-beta inhibits amyloid-beta oligomerization and fibril formation (127, 163, 164). The same role of CysC was demonstrated *in vivo* in amyloid-beta depositing APP transgenic mice overexpressing human CysC. Several lines of transgenic mice, expressing human CysC either under control sequences of the human CysC gene (128), or specifically in cerebral neurons (129), were crossbred with mice overexpressing human APP. CysC bound to the soluble, non-pathological form of amyloid-beta in the brains and plasma of these mice and inhibited the aggregation and deposition of amyloid-beta plaques in the brain (128, 129). However, deletion of CysC in knockout mice resulted in an increase in Cat B activity and an enhanced amyloid-beta degradation (84). Unlike a complete deletion of CysC, reduced or enhanced levels of CysC expression affect the aggregation of amyloid-beta, not amyloid-beta levels (128, 129).

In addition to its anti-amyloidogenic property, CysC directly protects neuronal cells from amyloid-beta toxicity. The extracellular addition of human CysC together with preformed either oligomeric or fibrillar amyloid-beta to cultured primary hippocampal neurons and to a neuronal cell line increased cell survival (164). It was shown that CysC does not dissolve preformed amyloid-beta fibrils or oligomers (127, 163). The data obtained show that subtle modifications in CysC expression levels in the central nervous system, or possibly in the periphery, affect amyloid deposition and protect from the toxicity of aggregated amyloid-beta.

Multiple studies have shown changes in CysC serum concentrations associated with a variety of conditions, such as chronic kidney disease, urinary infection, cancer, hypertension, cardiovascular disease, rheumatoid arthritis, glucocorticoid treatment, thyroid function, and aging [reviewed in (7)]. As described above, in the brain, enhanced CysC expression has been observed in response to different types of injury to the central nervous system, such as ischemia or induction of epilepsy as well as in specific neuronal population in AD compared to normal brains. Altered CysC trafficking and a reduction in its secretion are caused by two PS2 mutations (PS2 M239I and T122R), linked to familial AD. A decreased CysC secretion is associated with a polymorphism found in the CysC gene, revealing a mechanism for the increased-risk of late-onset sporadic AD conferred by this polymorphism and suggesting that a reduced CysC brain concentration is associated with the disease.

Recent studies have shown that proteins associated with neurodegenerative disorders are selectively incorporated into intraluminal vesicles of multivesicular bodies and released within exosomes. Exosomes are 40-100 nm bioactive vesicles of endocytic origin that are secreted by diverse cell types and are found *in vivo* in body fluids such as blood, cerebrospinal fluid, urine, and amniotic fluid. Exosomes can mediate communication between cells, facilitating processes such as antigen presentation and in trans signaling to neighboring cells, in tumor metastasis, and in transmitting infectious agents [for reviews see (165-167)]. However, little is known about the biogenesis and function of exosomes in the brain. It has been suggested that exosomes in the brain are involved in cell-cell signaling and glial-neuronal communication, regulation of neurotransmitter receptor levels at the synapse by targeting certain subunits for degradation, and control the production

and turnover of myelin membranes proteins [for reviews see (166, 167)]. It was also proposed that exosomes have roles in removal of unwanted proteins, and the transfer of pathogens between cells, such as HIV-1. Recent studies have demonstrated that proteins associated with neurodegenerative disorders (AD and prion diseases such as Creutzfeldt-Jakob disease of humans or bovine spongiform encephalopathy of cattle) can be selectively incorporated into intraluminal vesicles of multivesicular bodies and subsequently released into the extracellular environment, enriched within exosomes (168). More recently it was shown that proteins and peptides associated with APP metabolism are released in association with exosomes. These include full-length APP, APP carboxyl-terminal fragments, amyloid-beta, and proteins involved in APP processing such as BACE, PS1, PS2, and ADAM10 (169-171). The identification of amyloid-beta in association with exosomes and of other exosomal proteins, such as alix and flotillin, in plaques deposited in AD brains (169) suggest a role for exosomal release of amyloid-beta in extracellular amyloid deposition in the brain (168-171). However, it is conceivable that exosomes are protective against amyloid aggregation by relieving the cells from toxic accumulation of peptides such as amyloid-beta or by releasing amyloid-beta together with anti-amyloidogenic proteins into the extracellular space.

Given the neuroprotective roles of CysC in neurodegenerative disorders and mainly in AD, determining whether CysC is localized in exosomes, is specifically imperative. The studies have shown that while CysC is constitutively targeted extracellularly via to the classical secretory pathway as a soluble protein, it is also secreted by mouse primary neurons in association with exosomes (172). The presence of CysC in exosomes was demonstrated by immunoelectron microscopy and by immunoproteomic analysis using SELDI TOF MS (172). Moreover, the over-expression of the two familial AD-associated PS2 mutations (PS2 M239I and T122R) that alter CysC trafficking in mouse primary neurons, reducing secretion of its glycosylated form (156), resulted in reduced levels of CysC and of APP metabolites within exosomes (172). The presence of both amyloid-beta and CysC in exosomes suggests an additional location for the anti-amyloidogenic function of CysC. A better understanding of the mechanisms involved in exosomal processing and release and the function of CysC within exosomes will have important implications for the development of therapies against AD and other neurodegenerative diseases.

We hypothesize that endogenous CysC is a carrier of soluble amyloid-beta in body fluids such as cerebral spinal fluid and blood, as well as in the neuropil. It has an ongoing role in inhibition of amyloid-beta oligomerization and amyloidogenesis and protection against neurotoxic insults during an individual's lifetime. Endogenous levels of CysC seem not to be sufficient to prevent amyloid deposition in diseased brain, especially under conditions of reduced CysC concentration. Thus, manipulation of CysC concentration or a CysC peptidomimetic compound that will have enhanced anti-amyloid-beta and neuroprotective properties will be useful for slowing, halting, or reversing AD progression.

Wild type CysC colocalization with amyloid, other than amyloid-beta, was observed in a variety of disorders, such as hereditary gelsolin amyloidosis (familial amyloidosis, Finnish type) (173, 174) and familial cerebral amyloid angiopathy, British type, (175). It remains to be determined whether, similar to beta-amyloidoses, CysC binds other amyloid proteins and prevents their aggregation.

4. PERSPECTIVE

In vitro and *in vivo* data have demonstrated that CysC, an endogenous secreted protein, plays important roles in neuroprotection against various toxic stimuli. The protective effects of CysC are conferred by several mechanisms that may be activated individually or together under specific conditions. The mechanisms include inhibition of cysteine proteases, induction of cell division, induction of autophagy, and anti-amyloidogenesis. Thus, CysC is an attractive candidate for the development of a novel therapeutic strategy for the prevention, attenuation, and/or treatment of brain injury, Alzheimer's disease and other neurodegenerative disorders.

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Abbreviations: Alzheimer’s disease (AD); amyloid beta protein precursor (APP); cathepsin (Cat); cerebral amyloid angiopathy (CAA); cystatin B (CysB); cystatin C (CysC); glial fibrillary acidic protein (GFAP); hereditary cerebral hemorrhage with amyloidosis (HCHWA); presenilin 2 (PS2); Unverricht-Lundborg disease (EPM1)

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