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Involvement of cystatin C in pathophysiology of CNS diseases

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1. ABSTRACT

Cystatin C Leu68Gln variant is known to induce amyloid deposition in cerebral arterioles, resulting in Icelandic type cerebral amyloid angiopathy (CAA). Wild-type cystatin C is also observed in solitary CAA involving amyloid β protein (Aβ), and accelerates the amyloidogenicity of Aβ *in vitro*. In neurological inflammatory diseases and leptomeningeal metastasis, low cystatin C levels are accompanied with high activities of cathepsins in the cerebrospinal fluid. Among the cells in CNS, astrocytes appear to secrete cystatin C in response to various proteases and cytokines. Co-localization of Aβ and cystatin C in the brains of Alzheimer's disease (AD) led to the hypothesis that cystatin C is involved in the disease process. We demonstrated that cystatin C microinjection into rat hippocampus induced neuronal cell death in dentate gyrus. Furthermore, apoptotic cell death was observed in neuronal cells treated with cystatin C *in vitro*. Up-regulation of cystatin C was observed in glial cells with neuronal cell death *in vivo*. These findings indicate the involvement of cystatin C in the process of neuronal cell death.

2. CYSTATIN C-TYPE CEREBRAL AMYLOID ANGIOPATHY

The deposition of abnormal fibrillar protein aggregates (so-called amyloid) in the walls of arteries, arterioles, and sometimes capillaries and veins of the central nervous system (CNS) is known as cerebral amyloid angiopathy (CAA). The most prevalent form of CAA is the β-amyloid (Aβ) type that frequently accompanies Alzheimer's disease (AD). In AD, both parenchymal amyloid and vascular deposition are seen. Mutated cystatin C deposition was also observed in hereditary CAA with amyloidosis, Icelandic type (HCHWA-I) (1). The common features in CAA are vasculopathies associated with amyloid infiltration, such as clusters of multiple arteriole lamina, glomerular formation, obliterative intimal changes and double-barreling, especially in cortical arterioles and leptomeningeal vessels (2). CAA often leads to recurrent brain hemorrhage or infarction in cortical and subcortical regions.

Patients with HCHWA-I have been extensively studied by molecular biological methods, and the deposited

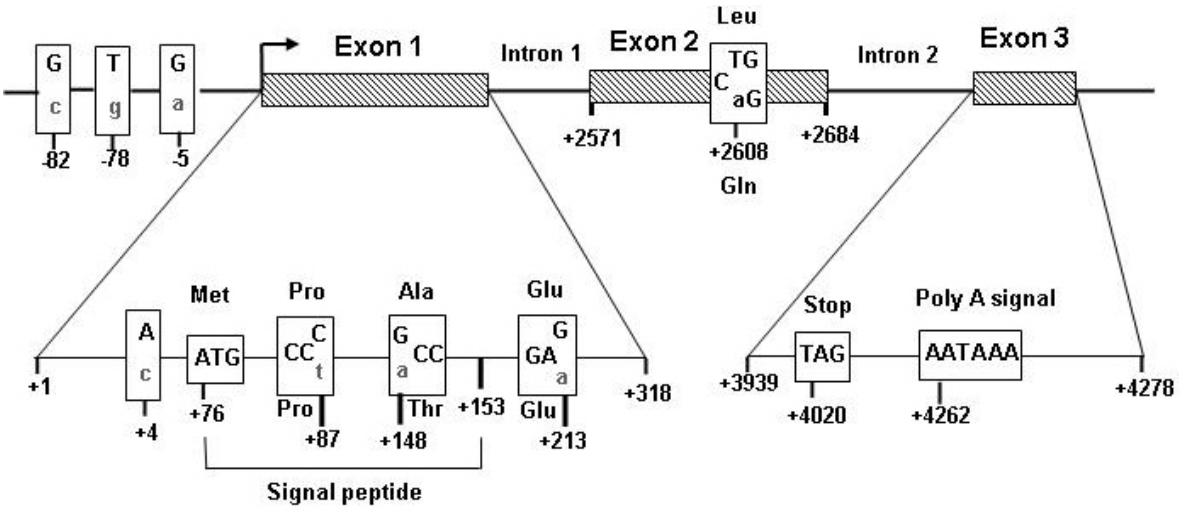

Figure 1. Cystatin C gene structure and sequence variations. The genetic mutation A for T at position 2608 causes the amino acid substitution of Leu (CTG) for Gln (CAG), as is seen in HCHWA-I. Only G/A at position +148 among seven polymorphs causes an amino acid mutation, Ala/Thr, which is at position 73, and may be associated with AD.

amyloid was shown to be composed of the Leu68Gln variant of cystatin C (Figure 1), which is directly associated with recurrent young-onset cortical hemorrhage or subcortical infarction, leading to death. Cystatin C isolated from the leptomeninges was found to be truncated, lacking the first 10 N-terminal amino acids (3, 4). Sporadic CAA with the same mutation of HCHWA-I was also identified in a Croatian patient (5). We also reported a familial CAA case showing deposition of Aβ and cystatin C, but mutation of the cystatin C gene was not identified (6).

On the other hand, wild-type cystatin C has been found even in the sporadic type of CAA, with Aβ deposition, in a ratio of about 1:100 (6). Immunohistochemical studies revealed that cystatin C was also co-localized with Aβ in the outer lamina of amyloid-laden vascular walls in patients with AD, Down's syndrome, hereditary cerebral amyloid angiopathy with amyloidosis, Dutch type (HCHWA-D), and elderly patients (7-10). The co-localization of both proteins in CAA was associated with fatal subcortical hemorrhage (9). A further study analyzing biopsy cases showed that severe cystatin C immunoreactivity was a risk factor for the occurrence and enlargement of cerebral hemorrhage, with loss of vascular smooth muscle (11).

In human and mouse atheroma, increased expression of cysteine and aspartic proteases correlated with decreased cystatin C (12, 13). Decrease of cystatin C in the lesions was closely related to the incidence of collagen and elastic lamina degradation in the vessel walls, leading to aneurysms (14, 15). Thus, it is postulated that cystatin C is an intrinsic factor that influences the stability of CAA and the occurrence of stroke.

3. AMYLOID FIBRIL FORMATION BY CYSTATIN C

As has been clearly shown in the case of transthyretin, many different point mutations can lead to amyloid formation. Therefore, there is a tendency to explain amyloidogenity in terms of reduced stability of the proteins (16, 17). However, an early study suggested that cystatin C and its L68Q variant in HCHWA-I exhibit similar patterns (18).

Amino-terminally truncated cystatin C lacking the first 10 amino-acid residues is deposited as amyloid in CAA in patients with HCHWA-I (4), but full-length cystatin C was detected in patients with non-hereditary CAA (19). The cleavage is thought to be a secondary event in amyloid formation (20). The amino acid substitution does not affect the activity of cystatin C as a cysteine protease inhibitor. However, replacement of Leu68 in the hydrophobic core of cystatin C with Gln may induce conformational changes in cystatin C, leading to dimerization and further amyloid fibril formation (21).

The Leu68Gln mutation causes cystatin C to be more unfolded than the wild-type when exposed to denaturing agents, low pH or high temperature *in vitro*. In fact, cystatin C monomer and dimer were detected in the serum and cerebrospinal fluid (CSF) of HCHWA-I patients, whereas only monomer was detected in control subjects (22). Even wild-type cystatin C has amyloidogenic properties, which might be significant in relation to the deposition of cystatin C with Aβ in CAA or in amyloid plaques in AD patients. Crystal structure analysis revealed that the protein refolds to produce very tight 2-fold symmetric dimers, retaining the secondary structure of the monomeric form (23). The dimerization occurs through 3-dimensional domain swapping, which could lead to infinite linear polymerization and amyloid fibril formation (24).

Not only the HCHWA-I variant, but also the wild-type cystatin C formed dimers in a concentration-dependent manner (25). Analysis of intracellular accumulation of cystatin C revealed that insoluble variant cystatin C existed in the endoplasmic reticulum (ER) in cystatin C-transfected Chinese hamster ovary cells (26). A

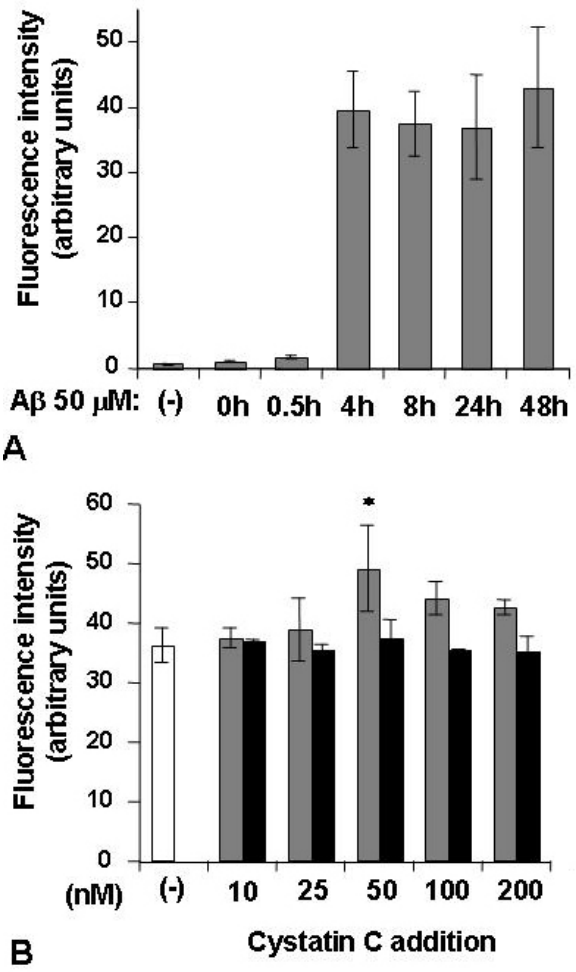

Figure 2. Effect of cystatin C on amyloid β (Aβ) fibril formation. (A) Aβ (50 μM) was incubated at 37°C for the indicated time, and Aβ fibril formation was analyzed by fluorescence spectroscopy, using thioflavin T. The data are expressed as fluorescence intensity. Amyloid fibril formation peaked at 4 h and was maintained up to 48 h. (B) To determine the effect of cystatin C on the Aβ fibril formation, 50 μM Aβ was incubated alone (empty bar), or with the indicated dose of recombinant cystatin C (gray bar) or truncated cystatin C (black bar) for 48 h. The data presented here are the means ± SEM of 3 similar experiments. Statistical significance of differences was assessed using one-way ANOVA, followed by the Bonferroni post hoc multiple comparison test. The criterion of statistical significance was $p < 0.05$, and significant differences compared with Aβ alone are indicated with an asterisk.

common mechanism observed in the formation process of amyloid fibrils is disturbed protein secretion pathways through the ER. Altered regulatory mechanisms in the protein quality control system of ER may lead to amyloidogenesis of cystatin C, transthyretin and Aβ. Although cystatin C and Aβ were determined to be colocalized intracellularly in cystatin C and Aβ co-transfected cells, the effect of cystatin C on Aβ fibril formation is unclear. It was reported that binding of cystatin C to $Aβ_{1-40}$ or $Aβ_{1-42}$ inhibited Aβ amyoloid fibril formation (27). We evaluated the involvement of cystatin C in $Aβ_{1-42}$ amyloid fibril formation with fluorescence spectroscopy using thioflavin T. Recombinant wild-type cystatin C and N-terminally 10-amino-acid-truncated cystatin C (truncated cystatin C), which lacks cathepsin B and L inhibitory activity, were produced for the study (28). After 48 h incubation of Aβ without cystatin C, amyloid fibril extension was significantly increased at 48 h (Figure 2A). Furthermore, 50 nM full-length cystatin C, but not the truncated form, significantly increased fibril formation (Figure 2B). Taken together, our results suggested that the interaction between Aβ and the N-terminal region of cyctatin C may have a pivotal role in the fibril formation. Disagreement between our findings and previous work (27) may have been due to the difference in cystatin C concentration used in fibril formation assay. We used a relatively low concentration (50 nM) of cystatin C, which is similar to the physiological concentration in human CSF, and found that this promoted fibril formation, whereas a higher concentration (50 to 200 μg/ml; about 4 to 16 μM) of cystatin C inhibited Aβ fibril formation (27). The mechanisms of the effects of cystatin C on fibril formation remain to be elucidated.

4. CONCENTRATION IN CSF

Cystatin plays a defensive role in extracellular fluids by protecting organs from the cysteine proteases produced by invading pathogens, and also endogenous cysteine proteases that escape from lysosomes (29). In CSF, these proteolytic enzymes are believed to play crucial roles in the initiation and progression of inflammatory neurological diseases (INDs). Cystatin C might play a critical role, because it is the dominant cysteine protease inhibitor in the CSF, and cystatin C levels in CSF are 5.5 times higher than those in plasma (30).

Cystatin C has been demonstrated to have a protective effect against numerous cysteine proteases in serum during systemic and local inflammation (31), in synovial fluid in inflammatory joint diseases (32), in the saliva in periodontal diseases (33) and in the sputum in bronchiectasis (34). Cathepsin B activity is blocked by cystatin C released from leukocytes or macrophages in human sputum and respiratory system (34, 35). The altered balance of these enzymes may also contribute to connective tissue remodeling or inflammatory processes in CNS diseases.

We have established a sandwich enzyme-linked immunosorbent assay (ELISA) method to measure the concentration of cystatin C in the CSF, and used it to measure the levels in various CNS diseases. We found that the concentration of cystatin C in the CSF was decreased, and cathepsin B activity was increased, in INDs such as Guillain-Barré syndrome, chronic inflammatory demyelinating polyneuropathy and multiple sclerosis (36). Furthermore, cystatin C was greatly decreased, and cathepsin B activity was remarkably elevated, in patients

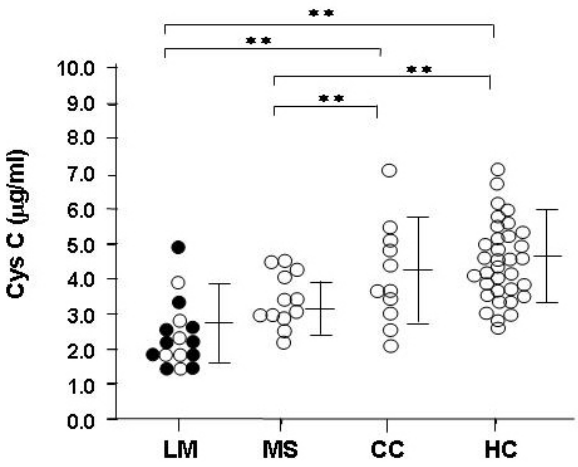

Figure 3. Cystatin C (cysC) concentration in the CSF. Cystatin C was measured with a sol particle homogenous immunoassay using colloidal gold particles coated with anti-cystatin C antibody, as recommended by the supplier, Alflesa Pharma Co. (Japan). CSF samples were collected from patients with leptomeningeal metastasis (LM: n = 16), multiple sclerosis (MS: n = 12), cancer without CNS infiltration (CC: n = 11) and healthy controls (HC: n = 34) after informed consent had been obtained. Cystatin C levels in LM and MS patients were significantly decreased compared with CC and HC, respectively. In LM patients, closed circles indicate patients with metastasis from solid tumor and open circles indicate patients with leukemia or lymphoma. ** $p < 0.05$.

with leptomeningeal metastasis from solid tumors and leukemia/lymphoma (37). Infiltrated inflammatory cells in INDs and cancer cells in leptomeningeal metastasis secrete cysteine proteases such as cathepsin B, which might lead to low levels of cystatin C through consumption by the proteases and degradation.

Recently, the level of cystatin C in serum was shown to be strongly dependent on the glomerular filtration rate (GFR) because of the low molecular weight and the stable production rate in serum (38-40). It is currently considered to be a more accurate marker of renal function than serum creatinine (41-43). A fully automated immunoassay for measuring serum cystatin C has been established (44-46). We confirmed that the system is also reliable for measuring the level in CSF, and the results were found to be similar to those obtained with the conventional ELISA system in patients with INDs or leptomeningeal metastasis (Figure 3). The measurement is helpful for diagnosing those diseases, and its relevance to other diseases of CNS is being evaluated.

Amyotrophic lateral sclerosis (ALS), which is the most common motor neuron disease, may be one of the CNS diseases in which cystatin C plays a role. Cystatin C is localized in Bunina bodies, which are a specific neuropathologic feature of ALS, being contained in degenerating motor neurons (47, 48). Proteomic profiling of CSF in ALS patients indicated that cystatin C is one of the decreased biomarkers (49). In our analysis of CSF samples, some ALS patients showed high levels of cathepsin B activity, which resulted in a significant increase in the value for all ALS, whereas no significant decrease in cystatin C levels was detected (Figure 4).

Northern blot analysis revealed that the cystatin C gene is ubiquitously expressed in human tissues, and its expression is highest in seminal vesicles (50). Several factors that influence the production and secretion of cystatin C were investigated. Dexamethasone increased cystatin C production in HeLa cells (51) and transforming growth factor β increased the secretion of cystatin C from smooth muscle cells and mouse embryo cells (15), whereas the secretion of cystatin C was decreased in monocytes and macrophages activated with lipopolysaccharide and interferon-γ (52). In the CNS, although cystatin C was expressed in neurons, astrocytes and choroid plexus (53, 54), the regulatory mechanisms remain to be elucidated. It was reported that, among CNS neoplastic tissues, astrocytomas frequently produce and secrete cystatin C (55). We stimulated human-derived astrocytes with various cytokines and proteases, and analyzed the expression levels of cystatin C (Figure 5). Cystatin C production and secretion in astrocytes were remarkably induced by a serine protease, thrombin, but not by IL-1β, TNF-α or IFN-γ. Thus, in the inflammatory milieu, thrombin could regulate the cystatin C level in CSF through the astrocyte response.

In HCHWA-I, the concentration of cystatin C in CSF is known to be lower by one-third than in normal subjects, but the mechanism seems to be different from that of the decrease observed in the INDs, leptomeningeal metastasis and ALS. Deposited cystatin C in HCHWA-I mainly consisted of variant forms, with only a small fraction of the wild-type cystatin C (56). The secretory mechanism of mutated cystatin C is the same as that of wild-type cystatin C in gene-transfected cultured cell lines, but it was demonstrated that secreted variant cystatin C is more rapidly degraded than wild-type cystatin C in stably transfected cell lines (25). The variant cystatin C readily dimerizes, which results in complete loss of its activity as a cysteine protease inhibitor. Decreased cystatin C level is a hallmark of HCHWA-I, probably resulting in increased protease activities in the CSF, which would affect the stability to remodeling or rupture of amyloid-laden vessels. The mechanism of low concentration of cystatin C detected in the CSF of sporadic CAA cases should be elucidated in the future (57, 58).

5. INVOLVEMENT IN AD

Neuropathological features of AD are the extracellular accumulation of Aβ as senile plaques and intraneuronal neurofibrillary tangles, major component of which is highly phosphorylated tau protein. Increasing evidence suggests that accumulation of Aβ in the cortex may be responsible for the neurodegeneration in AD. Immunohistochemical detection of cystatin C co-localized with Aβ in senile plaques led to the hypothesis that cystatin C might be involved in the progression of AD (59), since it is a lysosomal protease inhibitor, and lysosomal systems, such as cathepsins B and D, are upregulated at early and

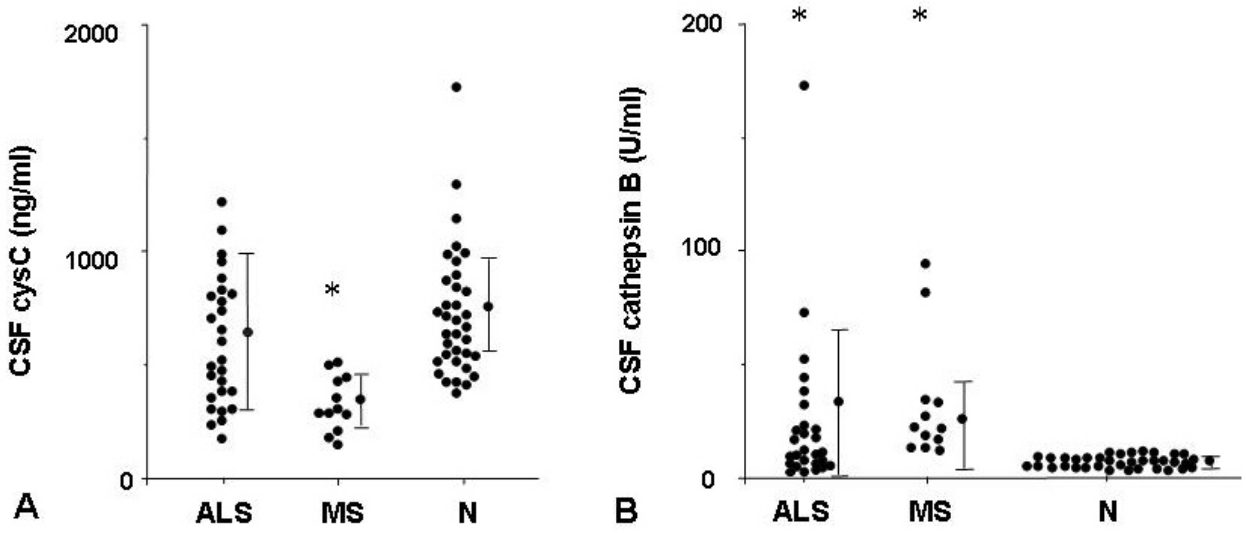

Figure 4. Cystatin C (cysC) concentration and cathepsin B activity in the CSF of patients with amyotrophic lateral sclerosis (ALS: n = 26), MS (n=12) and healthy controls (n = 34). Cystatin C levels were measured with an established ELISA method. CSF samples were collected after informed consent had been obtained, and were stored frozen until measurement. (A) Cystatin C levels in MS patients were reduced compared with those of ALS patients and normal controls. (B) Cathepsin B activity was measured with a quantitative fluorometric assay (84). Cathepsin B activities in the CSF of ALS and MS patients were increased compared with the control. $* p < 0.05$.

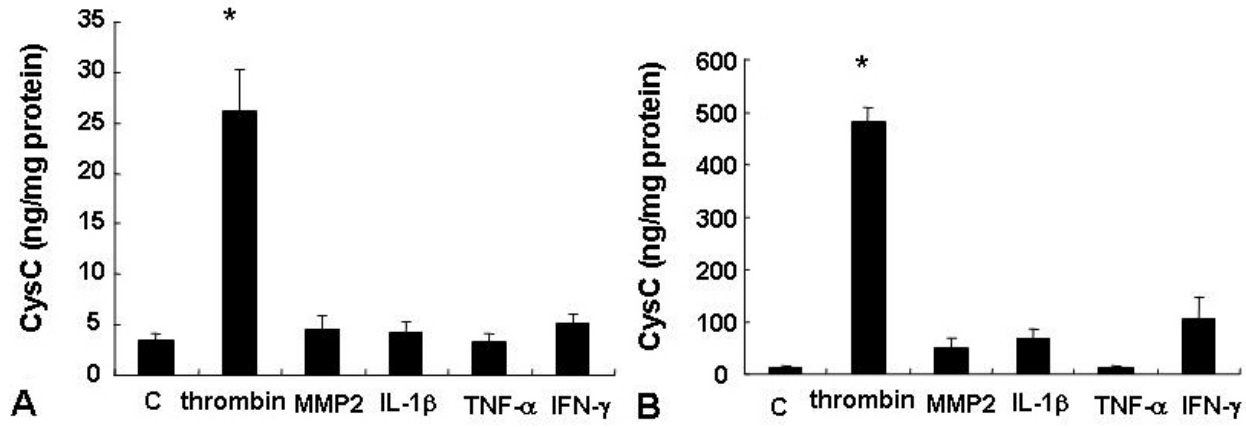

Figure 5. Effect of proteases and proinflammatory cytokines on cystatin C (cysC) protein expression in human astrocytes. Human astrocytes were incubated with medium containing 5 U/ml of thrombin, 0.5 μg/ml of MMP-2, 10 ng/ml of IL-1β, 10 ng/ml of TNF-α or 10 ng/ml of IFN-γ for 6 h and, cystatin C production was measured after 48 h. The amounts of cystatin C in culture supernatants (A) or cell lysates (B) were determined using an established ELISA method and corrected for cell protein levels. Values are the mean ± SEM (n=3). $* p < 0.05$.

late stages of AD (60, 61). *In vivo* experiments confirmed that lysozomal inhibitors led to increased β-amyloid precursor protein immunoreactivity in hippocampus (62) or hyperphosphorylated tau protein in hippocampal slice cultures (61). However, the mechanisms involved are poorly understood.

To investigate the relationship between the development of AD and cystatin C, genetic studies were conducted. The cystatin C gene (CST3) is polymorphic; G/A variation at position 73 leads to replacement of alanine with threonine as the penultimate amino acid of the signal peptide (see Figure 1) (64). The CST3-A allele that induces the Ala substitution was shown to be a risk factor for early-onset AD (65). Other studies demonstrated an association between homozygosity for the -82C/+4C/+148A haplotype and late-onset AD (66, 67). The apoprotein E (Apo) allele ε4 has been confirmed to be a risk factor for late-onset AD. A synergistic association between CST3 and Apo ε4 alleles was found in two studies (65, 68), whereas another study missed the synergisticity, finding that the two allelles were independent risk factors for AD (67).

The relationship between CST3 polymorphism and AD occurrence remains controversial, since recent

studies have found no association between CST3 polymorphism and AD (69, 70). A more thorough analysis seems to be necessary.

6. INVOLVEMENT IN NEURONAL CELL DEATH

Cystatin C has been reported to regulate cancer cell migration and metastasis in concert with cathepsins (71). Furthermore, a glycosylated form of cystatin C is necessary for proliferation of fibroblast growth factor 2 (FGF-2)-responsive neural stem cells (72). These findings indicate that cystatin C might be involved in many physiological events, perhaps including embryo implantation and placentation, by regulating cysteine proteases (73).

Altered expression of cystatin C is also seen in other CNS diseases. After stroke/ischemia, cystatin C protein expression was increased in hippocampal neurons (74). Treatment of cultured PC12 cells with 6-hydroxydopamine (6-OHDA), which is a selective neurotoxin used to induce apoptosis in catecholamine-containing neurons, increased cathepsin B, cathepsin D and cystatin C immunoreactivity in terminal dUDP nick end labeling (TUNEL)-positive cells (75). Since lysosomal function is essential for neurons and other post-mitotic cells to prevent accumulation of potentially deleterious proteins and metabolites, cystatin C is likely to be important for neuronal cell survival/death. A previous report showing that cystatin C was up-regulated in oxidative stress-induced apoptosis of cultured rat CNS neurons (76) supports the hypothesis that cystatin C is involved in neuronal cell death via apoptosis in the CNS. Although cystatin C prevents degeneration of rat dopaminergic neurons *in vitro* and *in vivo*, it remains to be elucidated whether or not cystatin C is neuroprotective under pathological conditions.

When we microinjected cystatin C unilaterally into rat hippocampus, neuronal degeneration was observed in the granule cell layer of the dentate hilus. Co-administration of cathepsin B with cystatin C significantly ameliorated the cystatin C-induced neuronal loss, indicating that the mechanism of action of cystatin C in this case may not involve amyloidogenity (77). This is consistent with previous reports, showing that lysosomal protease inhibitors induce brain aging-related materials, such as meganeurites and tangle-like structures, by inactivating cathepsins in a rat hippocampal slice culture system (51, 78). In AD pathology, cystatin C expression is elevated in pyramidal neurons in cortical layers III and IV, which are the neurons most susceptible to cell death (79). As described in Chapter 5, cystatin C may play a role in the neurodegenerative process in AD in association with abnormal protease activity in cortical neurons.

Next, we examined the effect of cystatin C on neuronal cell death in mixed cultures of human neurons and astrocytes or human-derived neuron/neuronal cell line. The A1 neuronal cell line used here is a well-established neuronal hybridoma of human fetal cerebral neurons with neuroblastoma cells, SK-SH-SY5Y, and has been confirmed to possess the characteristics of human CNS neurons (80). Cystatin C significantly increased active caspase-3 immunoreactivity in neurons of mixed cultures, increased TUNEL (+) cells and also induced DNA ladder formation in A1 cell cultures; these features are characteristic of neuronal apoptosis (81). In the present study, we quantified gene expression of proapoptotic and anti-apoptotic molecules in A1 cell cultures by means of a real-time quantitative PCR method. Cystatin C increased the proapoptotic factor bax at 8 h and decreased the anti-apoptotic factors bcl-2 and bik (Figure 6). These results are consistent with the idea that neuronal cell death in human neurons and A1 human hybrid neurons occurs through an apoptotic pathway.

Another perspective has emerged on neuronal cell death induced by cystatin C. In the acute phase of status epilepticus in mouse, cystatin C expression was mainly detected in astrocytes and microglia in the hippocampus, accompanied with neuronal cell death, whereas acute neuronal death was reduced in cystatin $C^{-/-}$ mouse (82). Rat facial nerve axonotomy increased cystatin C expression in the microglia surrounding the damaged facial nerve nucleus (83). These findings clearly indicate that cystatin C secreted from glial cells should be considered as a cause of neurodegeneration in the local disease environment.

7. CONCLUSIONS

In this review, we have highlighted the roles of cystatin C in the pathophysiology of CNS diseases, especially from the viewpoints of amyloidogenicity and inhibitory activity towards major cysteine proteases. A Leu68Gln variant of cystatin C causes extensive CAA, designated as HCHWA-I. Wild-type cystatin C augments the amyloidogenicity of Aβ *in vitro,* even at physiological concentrations. Co-deposition of wild-type cystatin C sometimes occurs with Aβ-type CAA, and increases the severity of CAA. Co-localization of cystatin C with Aβ in CAA and senile plaques suggests a relationship of cystatin C with AD, and indeed, a close relationship between them has been demonstrated by means of genetic and pathological studies. We showed that 50 nM cystatin C promoted Aβ fibril formation *in vitro*, suggesting the possible involvement of cystatin C in pathological fibril formation. The concentration of cystatin C in CSF may be regulated in balance with cysteine proteases, such as cathepsins B, H and L. Our recent findings have demonstrated that the concentration of cystatin C is decreased in the CSF of INDs and leptomeningeal metastasis, concomitantly with increased activity of cathepsin B, indicating that disturbance of cyctatin C levels in the CNS could be involved in the disease processes. Thus, the evaluation of cystatin C levels is expected to be helpful to understand the disease status, and possibly also in the diagnosis of CNS diseases.

Cystatin C is related with neuronal cell survival and is upregulated during neuronal apoptosis. Cystatin C microinjection augmented neuronal cell death in rat hippocampus, possibly through cysteine protease-inhibitory action and/or by activation of glial cells. While cystatin C

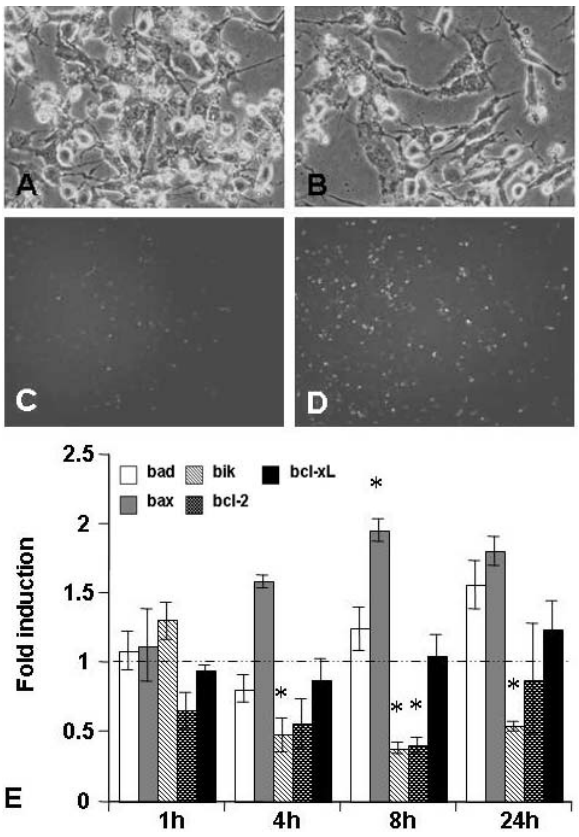

Figure 6. Effects of cystatin C on human-derived A1 hybrid neurons. A1 cells were induced to differentiate with retinoic acid, and treated with 40 nM cystatin C. After 24 h, many of the treated A1 cells (B) appeared to have lost their neurites and to be undergoing cell death, or tended to float compared with untreated A1 cells (A). When a Caspa Tag Caspase-3/7 *in situ* assay kit (Chemicon International, Temecula, CA) was used for detection of activated caspases 3 and 7, many cystatin C-treated cells (D) were positive, whereas few untreated cells were positive (C). (E) Expression of apoptotic genes after treatment with cystatin C. After stimulation for the indicated time, total RNA was isolated, reverse-transcribed and subjected to quantitative real-time PCR to analyze the expression of the bcl gene family. Cystatin C significantly increased the expression of proapoptotic bax mRNA, and inhibited that of antiapoptotic bcl-2 and proapoptotic bik mRNAs. The effects of cystatin C on bcl family gene expression was best observed at 8 h. The data presented here are the mean ± SEM of fold induction relative to the unstimulated control, from three independent experiments. Statistical significance of differences was assessed using one-way ANOVA, followed by the Bonferroni post hoc multiple comparison test. The criterion of statistical significance was $p < 0.05$, and an asterisk indicates a significant difference from the unstimulated control.

is involved in neural stem cell differentiation and cell survival, our studies using human-derived neuronal cells have revealed that cystatin C itself may induce apoptotic cell death, though the mechanism remains to be elucidated. Much recent research indicates that cystatin C is involved in various CNS disease processes, and the mechanisms of these effects will be the targets of future studies.

8. ACKNOWLEDGMENTS

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