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Relationship of Cystatin C With the Pathophysiology of CNS Diseases

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Cystatin C（cysC）is a low molecular weight basic protein（13kDa）belonging to the cystatin superfamily. It inhibits lysosomal cysteine proteases, such as cathepsins B, H, and L, and is present in all human body fluids at physiologically significant concentrations, being particularly abundant in the cerebrospinal fluid. While cysC Leu68Gln variant is known to induce amyloid deposition in cerebral arterioles, resulting in cerebral amyloid angiopathy（CAA）with amyloidosis, Icelandic type（HCHWA-I）, wild-type cysC deposition is also observed in solitary cerebral amyloid angiopathy involving amyloid β protein（Aβ）, which may progress to cerebral hemorrhage or white matter disturbance. Wild-type cysC also tends to form dimers and accelerates the amyloidogenicity of Aβ under certain conditions in vitro. The high cysC concentration in cerebrospinal fluid（CSF）is thought to be important for modulating cysteine protease activities to maintain central nervous system（CNS）homeostasis. In inflammatory neurological diseases（INDs）, leptomeningeal metastasis（LM）and some in amyotrophic lateral sclerosis（ALS）, low cyC levels are accompanied with high activities of cathepsins in the CNS. CysC is localized in the cytoplasm of every cell type in the CNS and is thought to be involved in the nerurodegenerative processes. Analysis of genetic polymorphism indicated that the G/A variation at position 73 may be a risk factor for Alzheimer's disease（AD）onset. Our recent findings revealed that cysC might be involved in the degenerative processes of spinal motor neurons in ALS. CysC is an interesting protein for further investigation because it is involved in inflammation and cell death processes by means of two aspects of its amyloidogenicity and lysosomal cysteine protease. This article reviews the relationship of cysC with pathophysiology of CNS diseases, including our clinical and research findings.

Key words: cystatin C, protease inhibitor, cerebrospinal fluid, inflammatory neurological diseases, neurodegenerative diseases

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Cystatin C expression in CNS

CysC is a 13-kDa protein mainly located in the lysosome, where it acts as an inhibitor of cysteine proteases such as cathepsin B, cathepsin H, and cathepsin L［1-4］. It is also found in body fluids as a secreted protein, especially at high levels in CSF［5］. Northern blot analysis revealed that the cysC gene is ubiquitously expressed in human tissues and its expression is highest in seminal vesicles［6］. Several factors that influence the production and secretion of cysC were investigated. Dexamethasone increased cysC production in HeLa cells［7］and transforming growth factor β increased the secretion of cysC from smooth muscle cells and mouse embryo cells［8］, whereas the secretion of cysC was decreased in monocytes and macrophages activated with lipopolysaccharide and interferon-γ［9］. In the CNS, although cysC was expressed in neurons, astrocytes and choroid plexus［10, 11］, the regulatory mechanisms remain to be elucidated. It was reported that, among CNS neoplastic tissues, astrocytomas frequently produce and secrete cysC［12］. We stimulated human-derived astrocytes with various cytokines and proteases, and analyzed the expression levels of cysC. CysC production and secretion in astrocytes were remarkably induced by a serine protease, thrombin, but not by IL-1β, TNF-α or IFN-γ［13］. Thus, in the inflammatory milieu, thrombin could regulate the cysC level in CSF through the

astrocyte response.

CysC has also been reported to regulate cancer cell migration and metastasis in concert with cathepsins [14]. Furthermore, a glycosylated form of cysC is necessary for proliferation of fibroblast growth factor 2 (FGF-2)-responsive neural stem cells [15]. These findings indicate that cysC might be involved in many physiological events, perhaps including embryo implantation and placentation, by regulating cysteine proteases [16].

Cystatin C-type cerebral amyloid angiopathy

The deposition of 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 common form of CAA is the Aβ type that frequently accompanies AD. In AD, both parenchymal amyloid and vascular deposition are seen. Mutated cysC deposition was also observed in hereditary CAA with amyloidosis, Icelandic type (HCHWA-I) [17]. 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 [18], whose rupture often leads to recurrent brain hemorrhage and obstruction induce infarction in cortical and subcortical regions.

Cause of HCHWA-I has been clarified by molecular biological methods, and the mutation of the Leu68Gln variant of cysC (Fig. 1) [19] is found in those patients, where truncated form of cysC, lacking the first 10 N-terminal amino acids [20, 21] was deposited in CAA. They suffered of recurrent young-onset cortical hemorrhage or subcortical infarction, leading to death.

On the other hand, wild-type cysC has been found even in the sporadic type of CAA, with Aβ deposition, in a ratio of about 1:100 [22]. Immunohistochemical studies revealed that cysC 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 [23-26]. The co-localization of both proteins in CAA was associated with fatal subcortical hemorrhage [25]. A further study analyzing biopsy cases showed that severe cysC immunoreactivity was a risk factor for the occurrence and enlargement of cerebral hemorrhage, with loss of vascular smooth muscle [27]. In human and mouse atheroma, increased expression of cysteine and aspartic proteases correlated with decreased cysC [28, 29]. Decrease of cysC in the lesions was closely related to the incidence of collagen and elastic lamina degradation in the vessel walls, leading to aneurysms [8, 30]. Thus, it is postulated that cysC is an intrinsic factor that influences the stability of CAA and the occurrence of stroke.

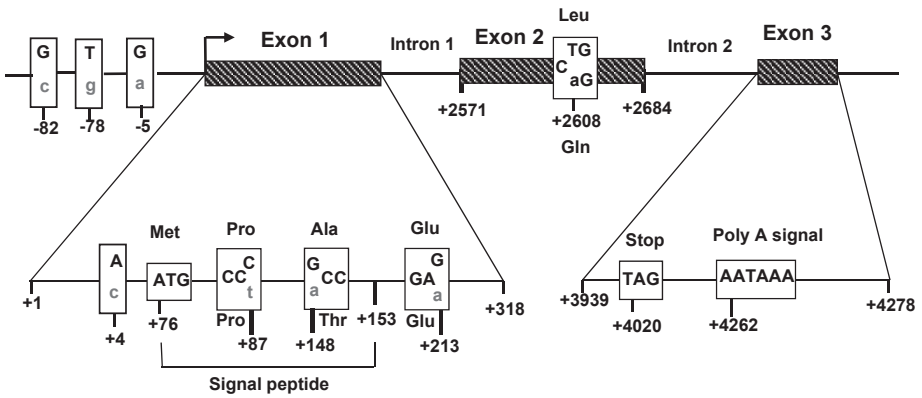

Fig. 1. CysC gene structure and sequence variations. The genetic mutation A for T at position 2608 causes the amino acid substitution of Leu (CAG) for Gln (CTG), as seen in HCHWA-I. Only G/A at position +148 among seven polymorphs causes an amino acid mutation, Ala/Thr, which is at position 73, and may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

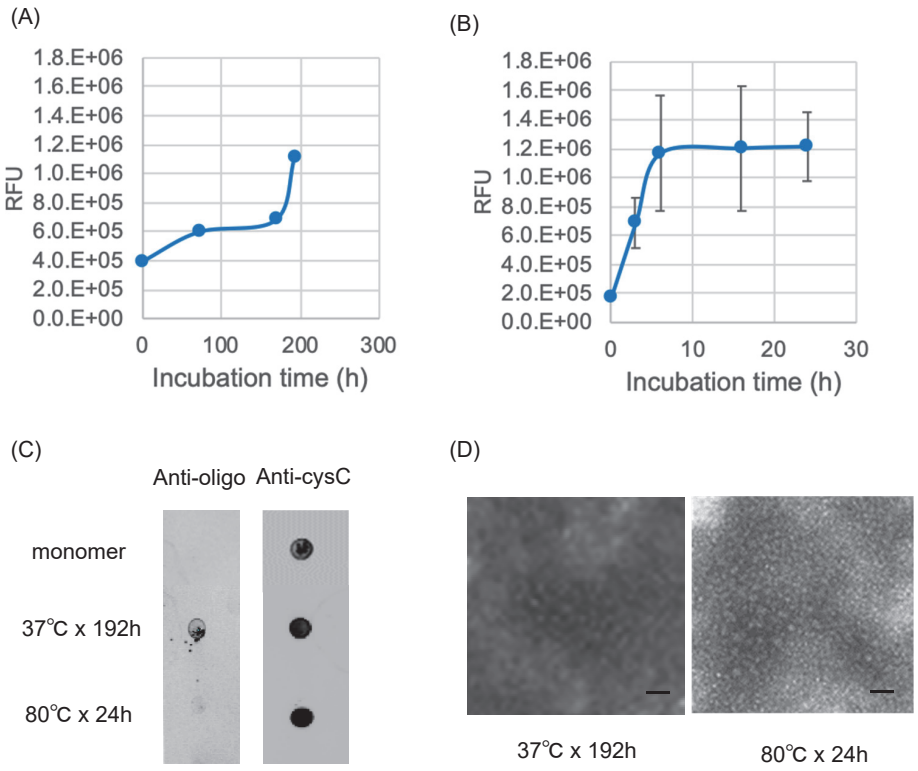

Fig. 2. *In vitro* aggregation of wild type cysC. Aggregation was induced by incubating recombinant cysC (1.3 mg/ml) at 37℃ with agitation (A) or 80℃ without agitation (B). Subsequently the volume and formation of aggregates were analyzed by ProteoStat protein aggregation assay kit measured with fluorescence intensity (A and B) and dot blot immunoassay (C) using oligomer specific antibody (A11) and anti-cysC antibody. Morphology of CysC aggregates was checked by transmission electron microscopy (TEM) in each condition.

Amyloid fibril formation of cystatin C

Amino-terminally truncated cysC lacking the first 10 amino-acid residues is deposited as amyloid in CAA in patients with HCHWA-I [21], where the mutation of L68Q may cause the reduced stability of the proteins and acquire amyloidogenity. The Leu68Gln mutation causes cysC to be more unfolded than the wild-type when exposed to denaturing agents, low pH or high temperature *in vitro*. In fact, cysC monomer and dimer were detected in the serum and cerebrospinal fluid (CSF) of HCHWA-I patients, whereas only monomer was detected in control subjects [31].

However, more reasonable explanation for cysC amyloidogenesis is necessary because full-length cysC was also deposited in patients with non-hereditary CAA [32]. Crystal structure analysis revealed that the protein refolds to produce very tight 2-fold symmetric dimers, retaining the secondary structure of the monomeric form [33]. The dimerization occurs through 3-dimensional domain swapping, which could lead to infinite linear polymerization and amyloid fibril formation [34]. Our recent analysis proved that phenomenon. It revealed that cysC was aggregated after incubation with continuous agitation for 192 h at physiological condition (37℃, pH7.4, Fig. 2A) compared with de-stabilized condition (80℃, pH7.4, Fig. 2B). Aggregated oligomer of cysC were most detected at physiological condition (Fig. 2C). Transmission electron microscopy (TEM) study also confirmed aggregated cysC adopted a small globular structure (Fig. 2D).

We also evaluated the involvement of cysC in $A\beta_{1\text{-}42}$ amyloid fibril formation with fluorescence spectroscopy using thioflavin T. Recombinant wild-type cysC and N-terminally 10-amino-acid-truncated cysC (truncated cysC), which lacks cathepsin B and

L inhibitory activity, were produced for the study [35]. After 48 h incubation of Aβ without cysC, amyloid fibril extension was significantly increased at 48 h (Fig. 3A). Furthermore, 50 nM full-length cysC, but not the truncated form, significantly increased fibril formation (Fig. 3B).

Taken together, our results suggested that cysC itself has the amyloidogenic properties and the interaction between Aβ and the N-terminal region of cyctatin C may accelerate the fibril formation, which may play an important role in the occurrence of sporadic CAA, where both Aβ and cysC were deposited. Since there remains to be elucidated clear mechanisms. further studies need to be demonstrated.

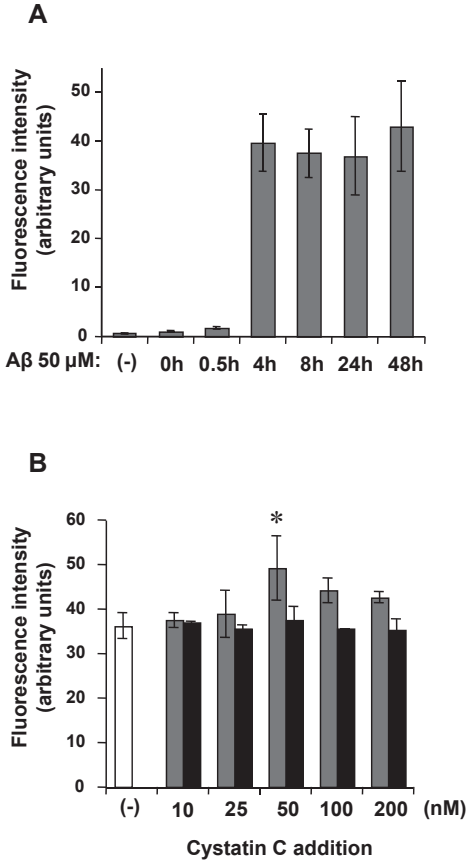

Fig. 3. Effect of cysC on amyloid β (Aβ) fibril formation. (A) Aβ (50 μM) was incubated at 37℃ for the indicated time, and Aβ fibril formation was analyzed by fluorescence spectroscopy, using thioflavin T. The data are expressed as fluorescence intensity. Amyloid fibril formation peaked at 4 h and was maintained up to 48 h. (B) To determine the effect of cysC on the Aβ fibril formation, 50 μM Aβ was incubated alone (empty bar), or with the indicated dose of recombinant cysC (gray bar) or truncated cysC (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.

Concentration in CSF

Cystatin superfamily 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 [36]. Since, in CSF, these proteolytic enzymes are involved in the initiation and progression of INDs, cysC has a protective effect as a dominant cysteine protease inhibitor in the CSF, at 5.5 times higher levels than that in plasma [37].

CysC has been demonstrated to have a protective effect against numerous cysteine proteases in serum during systemic and local inflammation [38], in synovial fluid in inflammatory joint diseases [39], in the saliva in periodontal diseases [40] and in the sputum in bronchiectasis [41]. Cathepsin B activity is blocked by cysC released from leukocytes or macrophages in human sputum and respiratory system [41, 42]. 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 cysC in the CSF and used it to measure the levels in various CNS diseases. We

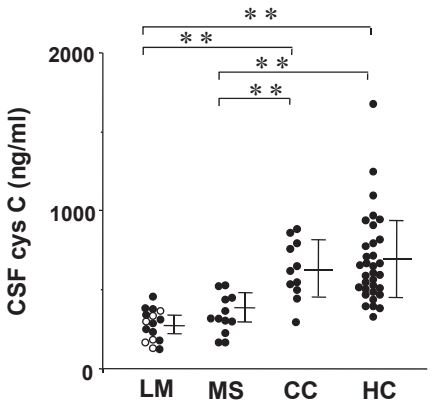

Fig. 4. CysC concentrations in the CSF of patients with leptomeningeal metastasis (LM: n = 16), multiple sclerosis (MS: n = 12), cancer control (CC: n = 11), and healthy control (HC: n = 34). CysC concentrations were measured using the established ELISA methods. In LM patients, closed and open circles indicate patients with metastasis due to solid tumor and leukemia or lymphoma, respectively. The vertical bars indicate mean ± SD. The mean CSF cysC levels were decreased in patients with LM compared to CC and HC. The MS patients had low levels of cysC compared to CC and HC. ** $p < 0.01$.

found that the concentration of cysC in the CSF was decreased, and cathepsin B activity was increased, in INDs such as multiple sclerosis (MS), Guillain-Barré syndrome and chronic inflammatory demyelinating polyneuropathy (Fig. 4) [43]. Furthermore, cysC was greatly decreased, and cathepsin B activity was remarkably elevated, in patients with leptomeningeal metastatis from solid tumors and leukemia/lymphoma (Fig. 4) [44]. 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 cysC through consumption by the proteases and degradation.

Amyotrophic lateral sclerosis (ALS), which is the most common motor neuron disease, may be one of the CNS diseases in which cysC plays a role. CysC is localized in Bunina bodies, which are a specific neuropathologic feature of ALS, being contained in degenerating motor neurons [45, 46]. Proteomic profiling of CSF in ALS patients indicated that cysC is one of the decreased biomarkers [47]. 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 cysC levels was detected (Fig. 5).

In HCHWA-I, the concentration of cysC 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, LM and ALS. Deposited cysC in HCHWA-I brain mainly consisted of variant forms, with only a small fraction of the wild-type cysC [48]. The secretory mechanism of mutated cysC is the same as that of wild-type cysC in gene-transfected cultured cell lines, but it was demonstrated that secreted variant cysC is more rapidly degraded than wild-type cysC in stably transfected cell lines [49]. The variant cysC readily dimerizes, which results in complete loss of its activity as a cysteine proteinase inhibitor. Decreased cysC 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.

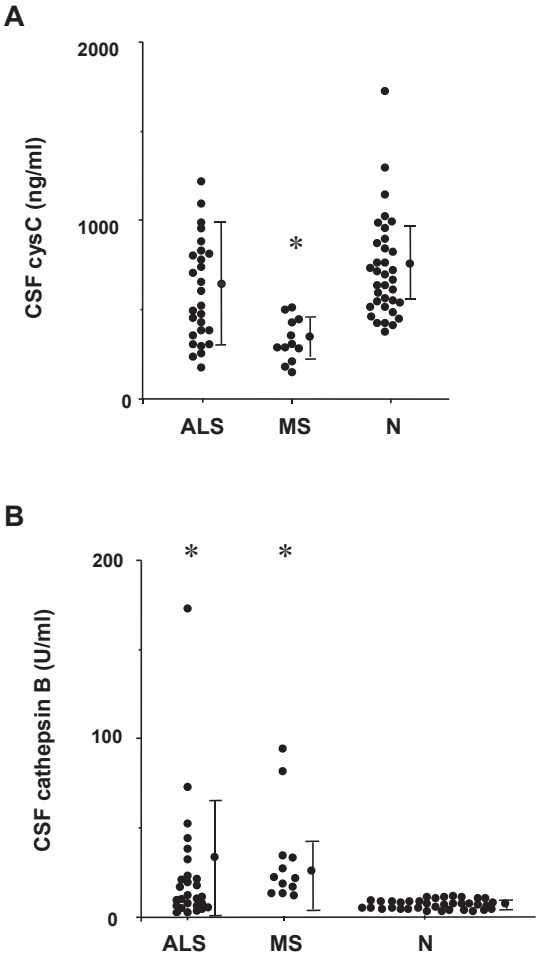

Fig. 5. CysC concentration and cathepsin B activity in the CSF of patients with ALS (n = 26), MS (n = 12) and healthy controls (n = 34). CysC levels were measured with an established ELISA method. CSF samples were collected after informed consent had been obtained, and were stored frozen until measurement. The vertical bars indicate mean ± SD. (A) CysC 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 [68]. Cathepsin B activities in the CSF of ALS and MS patients were increased compared with the control. $* p < 0.05$.

Involvement in the neurodegenerative diseases

Altered expression of cysC is also seen in other CNS diseases. After stroke/ischemia, cysC protein expression was increased in hippocampal neurons [50]. 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 cysC immunoreactivity in terminal

dUDP nick end labeling (TUNEL)-positive cells [51]. Since lysosomal function is essential for neurons and other post-mitotic cells to prevent accumulation of potentially deleterious proteins and metabolites, cysC is likely to be important for neuronal cell survival/death. A previous report showing that cysC was up-regulated in oxidative stress-induced apoptosis of cultured rat CNS neurons [52] supports the hypothesis that cysC is involved in neuronal cell death via apoptosis in the CNS.

We examined the effect of cysC 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-SN-SY5Y, and has been confirmed to possess the characteristics of human CNS neurons [53]. CysC 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 [54]. In the study, we quantified gene expression of proapoptotic and anti-apoptotic molecules in A1 cell cultures by means of a real-time quantitative PCR method. CysC increased the proapoptotic

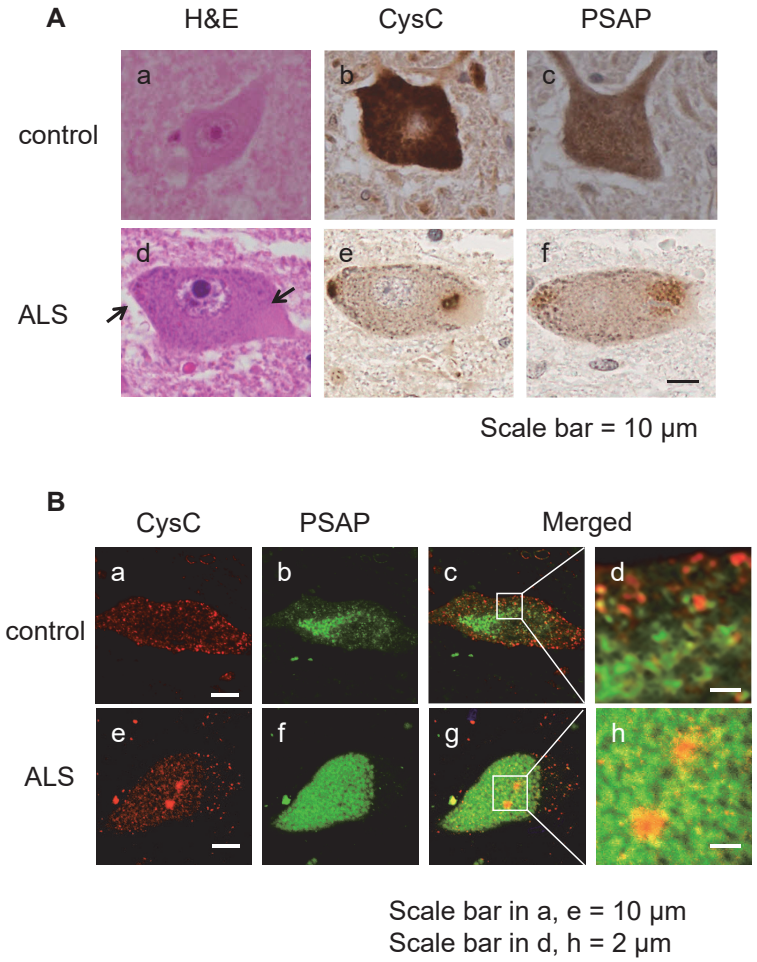

Fig. 6. Immunohistochemical detection of CST3 and PSAP in the spinal cord motor neurons of control subjects and patients with ALS. (A) Consecutive tissue sections of the spinal cord were immunostained for CST3 and PSAP using specific antibodies. For identification of Bunina bodies, H&E staining was performed. A-a, -b and -c are the photomicrographs of control spinal cord, and A-d, -e and -f are the same from an ALS patient, respectively. A-a, and -d are photomicrographs of H&E staining, A-b and -e are CST3 immunostaining photomicrographs, and A-c and -f are PSAP immunostaining photomicrographs. (B) CST3 and PSAP were further analyzed by double immunofluorescence staining of control (a, b, c and d) and an ALS patient (e, f, g and h) spinal cords. CST3 immunostaining is shown in B-a and -e, PSAP in B-b and -f, and the corresponding merged pictures are shown in B-c and -g. To demonstrate co-localization, the boxed areas of c and g are enlarged, and shown in d and h, respectively. Photomicrographs of a, b, c and d are from the spinal cord of a control subject and e, f, g and h are from the spinal cord of an ALS patient. Scale bar in (A) and (B-a-c and B-e-g) = 10 μm, and (B-d, and h) = 2 μm.

factor bax at 8 h and decreased the anti-apoptotic factors bcl-2 and bik. These results are consistent with the idea that neuronal cell death in human neurons and A1 human hybrid neurons occurs through an apoptotic pathway [55].

Neuropathological features of AD are the extracellular accumulation of Aβ as senile plaques and intraneuronal neurofibrillary tangles as deposition of tau protein. Increasing evidence suggests that accumulation of Aβ in the cortex may be responsible for the neurodegeneration in AD. Immunohistochemical detection of cysC co-localized with Aβ in senile plaques led to the hypothesis that cysC might be involved in the progression of AD [56] since it is a lysosomal protease inhibitor, and lysosomal systems, such as cathepsins B and D, are upregulated at early and late stages of AD [57, 58]. In AD pathology, cysC expression is also elevated in pyramidal neurons in cortical layers III and IV, which are the neurons most susceptible to cell death [59]. CysC may play a role in the neurodegenerative process in AD in association with abnormal protease activity in cortical neurons.

To investigate the relationship between the development of AD and cysC, genetic studies were conducted. The cysC 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 Fig. 1) [60]. The CST3-A allele that induces the Ala substitution was shown to be a risk factor for early-onset AD [61]. Other studies demonstrated an association between homozygosity for the -82C/+4C/+148A haplotype and late-onset AD[62, 63]. 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 [61, 64], whereas another study missed the synergisticity, finding that the two allelles were independent risk factors for AD [63]. The relationship between CST3 polymorphism and AD occurrence remains controversial since recent studies have found no association between CST3 polymorphism and AD [65, 66].

Since it is possible that cysC is involved in the pathogenesis of ALS from our CSF study, we searched the proteins bound with cysC *in vitro* and in ALS spinal cord [67]. As a result, we found prosaposin (PSAP) as a candidate protein, confirmed the binding of both proteins by immunoprecipitation-based in vitro assay. Further immunohistochemical results investigating spinal motor neurons of ALS patients revealed that both proteins co-localized mainly in the lysosomes and CST3 was immunopositive in the inclusions of ALS motor neurons, where it was closely associated, and sometimes co-localized, with PSAP (Fig. 6). Since CST3 and PSAP are lysosomal protein where they interact with each other, these proteins might make complex and incorporate to Bunina body through autophagolysosome, and show co-localization at the periphery of the inclusion bodies. The co-localization of both proteins was detected in cultured cells and in Bunina body (Fig. 6-d and -e)-containing motor neurons from patients with ALS, suggesting that they might be involved in the process of Bunina body formation in the degenerated motor neurons.

CONCLUSIONS

In this review, we have highlighted the roles of cysC in the pathophysiology of CNS diseases. Although a Leu68Gln variant of cysC causes extensive CAA, designated as HCHWA-I, wild-type cysC augments the amyloidogenicity of Aβ *in vitro*, even at physiological concentrations. Co-deposition of wild-type cysC sometimes occurs with Aβ-type CAA, and increases the severity of CAA. Co-localization of cysC with Aβ in CAA and senile plaques suggests a relationship of cysC with AD. The concentration of cysC in CSF may be regulated in balance with cysteine proteases, such as cathepsins B, H and L. Our findings have demonstrated that the concentration of cysC is decreased in the CSF of INDs, LM and some of ALS, concomitantly with increased activity of cathepsin B, indicating that disturbance of cysC levels in the CNS could be involved in the disease processes.

Since cysC is found to be involved in the neuronal cell death process possibly as an amyloidogenic protein and a cysteine protease inhibitor, it is interesting to investigating the relationship of cysC with various CNS disease processes, especially in neurodegenerative diseases.

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