Cystatin C in Cerebrospinal Fluid is Upregulated in Elderly Patients With Chronic Osteoarthritis Pain and Modulated Through Matrix Metalloproteinase 9–Specific Pathway

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Objectives: Although high abundant cystatin c (CysC) in cerebrospinal fluid (CSF) is well known, its ambiguous role associated with pain still remains unclear. This study evaluated the effects of intrathecal CysC content from chronic pain caused by osteoarthritis (OA) and the novel relationship with matrix metalloproteinases 2 and 9 (MMP2 and MMP9) in CSF.

Methods: Samples of CSF were obtained from 8 elderly patients (65 y and above) with OA with lower limb pain for at least 6 months (OA group) and 8 sex-matched and age-matched relatively healthy elderly individuals without any pain problems (control group). The intrathecal CysC, MMP2, and MMP9 were examined by Western blotting. The analysis of CysC cleavage under different conditions was performed through silver staining and using mass-spectroscopy (SELDI-TOF) on 2 groups.

Results: Expression of full-length CysC and pro-MMP2 proteins showed statistically significant upregulation ($P = 0.0004$ vs. 0.03), and expression of MMP9 protein showed downregulation ($P = 0.007$) in the OA group. Both MMP9 and MMP2 initiated the mechanism for full-length CysC cleavage only in the presence of CSF. However, MMP9 showed greater ability than MMP2 for CysC cleavage in control and OA groups in sliver staining. Incubation of CSF with the MMP9 inhibitor led to the suppression of CysC cleavage in SELDI-TOF.

Discussion: These findings provide the first in vivo evidence on a relationship between CysC and gelatinases (MMP2 and MMP9), and could facilitate further investigation of novel interactions among these proteins within the proteomics field, especially protein-protein interactions involved in pain.

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Pain is regarded as an alarm system that helps to prevent the body from further injury.[1] However, abnormal repeated stimuli or damage to the nervous system can both lead to the persistent hyperalgesia that defines the clinical experience of chronic pain.[2] Peptidergic neurotransmission plays a key role in the development and maintenance of chronic pain (CP).[3] The varying intracellular signaling pathways and interaction of distinct transcription factors can cause long-term changes in neural plasticity and maintenance of hyperalgesia.[4] Studies have recently identified the importance of intercellular signaling in CP and the involvement of non-neuronal cells in the microenvironment.[5] Upon activation, glial cells release a variety of algesic substances that can potentiate pain transmission by neurons.[6] Of these glial products, the candidate mediators include proinflammatory cytokines, chemokines, and reactive oxygen species.[7]

Most previous research on CP was conducted on animal models or cell cultures. However, investigators have also described clinical observations in human cerebrospinal fluid (CSF) and identified its constituents.[8–12] The CSF provides buoyancy and protection to the brain and spinal cord, transports biological substances within the central nervous system (CNS), and provides a means of excreting toxic and waste substances.[13] In addition, it directly contacts and communicates with the extracellular fluid of the CNS.[14] The reciprocal changes in CSF components can reflect the biological status or transmitter fluctuations within the CNS.[15] The CSF is accessible and irreplaceable and provides an effective means of monitoring changes in different clinical conditions.

Cystatin C (CysC) is an endogenous cysteine protease inhibitor belonging to the type 2 cystatin superfamily.[16,17] It is found in almost all biofluids. More than 90% of the total body CysC content is distributed in the CSF, with a negligible plasma-derived fraction.[18] Results from previous studies indicate that CysC plays a role in neurological disorders involving amyloid, such as Alzheimer disease,[19] cerebral amyloid angiopathy,[20] and multiple sclerosis.[21] Prior studies have also evaluated the association of intrathecal CysC with pain.[22,23] However, the mechanism by which CysC is involved in pain pathophysiology remains unclear.

Gelatinases are a subfamily of matrix metalloproteinases (MMPs) containing matrix metalloproteinase 2 (MMP2) and matrix metalloproteinase 9 (MMP9).[24] These proteins are involved in a wide range of biological

events including cancer cell metastasis,[25] angiogenesis,[26] tissue repair,[27] and inflammation.[28] Recent studies identified the involvement of these 2 MMPs in certain CNS diseases,[29] and their association with pain perception.[30]

The purpose of the present study is to evaluate the changes in CysC and gelatinase expression in human CSF in a CP condition deriving from osteoarthritis (OA). The results provide the first evidence of a relationship between CysC and gelatinases. These data indicate the effects of fluctuations in CysC in the CSF on pain progression, and the initiation of metabolism of CysC by gelatinases.

MATERIALS AND METHODS

This case-controlled, clinical-observed study followed protocol CT9660, approved by the Institutional Review Board, Cathay General Hospital. The experiments were performed in accordance with Good Clinical Practice Guidelines and the Declaration of Helsinki. All patients received information on the study procedures and they provided written personal consent for participation before their samples were collected.

Participants and Study Design

This study enrolled 16 consecutive patients, 8 controls and 8 CP patients with OA in the lower extremities and receiving spinal anesthesia for operation, (Supplemental Figure 1, http://links.lww.com/CJP/A68). All enrolled patients were older than 65 years of age. Compared with the general population, this age group is associated with a higher prevalence of pain problems, lower complication rate of spinal puncture, and larger CSF volume. Exclusive criteria included CNS tumor, CNS infection, CNS traumatic history, or known abnormal CSF protein expression diseases, such as dementia, Alzheimer disease, multiple sclerosis, and Creutzfeldt-Jakob disease. Only patients reporting OA pain in the lower limbs, but without other visceral, neuropathic pain, cancer pain, or unknown pain origins, were selected. If OA pain had been sustained over 6 months and surgical intervention suggested, then the patients were categorized into the OA group. To minimize the variance of OA patients, we adjusted for possible confounders (Visual Analog Scale [VAS], x-ray finding, and clinical evaluation tool, such as Western Ontario and McMaster Universities Osteoarthritis index,[31] [WOMAC]). Criteria for selection in x-ray finding were: a definite joint space narrowing, marked sclerosis change, and bony end deformity. When applied to the Kellgren-Lawrence classification, these criteria correspond to a severity (for an OA patient) of worse than grade III. Thresholds for selection by WOMAC, standardization from 0 to 100 in 3 independent dimensions were: above 30 in pain, 50 in stiffness, and 50 in physical function. The age-matched and sex-matched patients assigned to the control group had experienced no pain symptoms, especially lower back or limb pain. All clinical data were collected, including active or resting pain scores, locations and referring areas, pain characteristics, alleviated factors, aggravating factors, history of disease, medication lists, among others.

Patient CSF was collected during anesthesia. To avoid bias, the same anesthesiologist performed the lumbar puncture procedures to collect all samples. The patient was placed in the lateral decubitus position and the puncture site (between L3 and L5) marked. A #25 spinal needle was inserted into the intrathecal space and approximately 4 mL CSF was collected. To avoid contamination by blood, the specimen was discarded if its appearance was turbid or bloody. The CSF samples were aliquoted and immediately stored at $-80^{\circ}C$ until use. Each aliquot, therefore, underwent only 1 freeze-thaw cycle.

Western Immunoblotting

The concentration of protein in the CSF was determined using the Bradford assay (Bio-Rad Laboratory, Hercules, CA). Each CSF protein (5 μg) was mixed with the same volume of 2× SDS sample buffer (63 mM Tris-HCl [pH 6.8], 10% glycerol, 2% SDS, 0.0025% bromophenol blue) and boiled for 10 minutes. The SDS and Western blotting followed standard procedures. The antibodies used were as follows: monoclonal antibody against His-tag (1:1000, #70796; Novagen/Merck, Darmstadt, Germany), against *N*-terminal of human CysC (1:500, #sc-73878; Santa Cruz Biotechnologies Inc., Santa Cruz, CA), antibody specific to MMP2 (1:1000, #LV1714332; Millipore, Temecula, CA) and MMP9 (1:1000, #sc-21733; Santa Cruz Biotechnologies Inc.). The Western blotting images were captured and quantified using Alpha FluroChem FC2 with Alpha View Software (Version 2.0.1.1; Alpha Innotech, Santa Clara, CA).

CysC Cleavage Assay

Recombinant human MMP9 protein (#911-MP; R&D Systems, Minneapolis, MN) and recombinant human MMP2 protein (#902-MP; R&D Systems) were activated using activation buffer (1 mM p-aminophenylmercuric acetate, 50 mM Tris-HCl, [pH 7.0], 10 mM $CaCl_2$, 150 mM NaCl, and 0.05% Brij-35) at 37°C for 2 hour. For immunoblotting, 8 pmol recombinant His-tagged human CysC protein (#1196-PI; R&D Systems) and 1 μg CSF were coincubated with 1 pmol recombinant human MMP9 protein or 1 pmol recombinant human MMP2 protein in 10 μL assay buffer (50 mM Tris-HCl [pH 7.0], 10 mM $CaCl_2$, 150 mM NaCl, and 0.05% Brij-35) at 37°C for 16 hours. For silver staining analysis, 80 pmol recombinant His-tagged human CysC proteins and 0.1 μg CSF were coincubated instead. The reactions were terminated by adding 2× SDS sample buffer, then boiling for 10 minutes. The reactants were separated using 15% SDS/PAGE after standard silver staining analysis or immunoblotting procedures described in the above section.

Inhibition of CysC Cleavage

Activated MMP9 protein (1 pmol) was added to 1 μg CSF collected from the OA group and incubated in the presence or absence of MMP9 inhibitor (0.1 mM, #444278; Merck). The EDTA-Free Protease Inhibitor Cocktail (ROCHE, Basel, Switzerland) was used to inhibit the serine and cysteine proteases in the CSF. All reaction mixtures were incubated for 6 hours at 37°C, and the resulting reactants were separated using 15% SDS/PAGE and then immunoblotted.

SELDI-TOF Mass Spectrometry

Each of the prepared reaction mixtures (1 μL) described in the previous paragraph was spotted onto a NP Proteinchip (Bio-Rad Laboratory, Fremont, CA). The chip was first equilibrated with distilled water for 5 minutes before the adding of reactants. After the addition of reactants, 1 μL energy-absorbing matrix solution (α-CHCA in 50% ACN and 0.5% TFA) was added before drying. The chips were loaded into the Proteinchip SELDI System,

Personal Edition (Bio-Rad Laboratory) for protein profile analysis. A nitrogen laser (337 nm) was used to generate nanosecond laser pulses. Spectra were obtained from the average collection deriving from 200 laser shots. The peaks in the spectra represented proteins of specific mass over charge present in the samples.

Statistical Analysis

Statistical analysis was conducted using SPSS 13.0 for Windows (SPSS, Chicago, IL, http://www.spss.com). The *P*-values for differences in CysC, MMP9, and MMP2 protein levels between OA and control groups were calculated using the Student *t* test. A *P*-value <0.05 was considered significant.

RESULTS

All 16 participants received spinal anesthesia according to original surgical decision and had their CSF samples taken before injection of local anesthetics. No serious adverse events were recorded, such as backache, postpuncture headache, or neurological complications.

Patient Characteristics

From the sample set of 16 participants, the OA group contained 8 patients diagnosed as OA according to clinical criteria (5 with knee OA and 3 with hip OA). The control group contained 8 age-matched and sex-matched patients (4 with inguinal hernia and 4 with benign prostate hypertrophy). Table 1 displays the demographics of the included patients. The characteristics of the OA group were as follows (mean ± SD): age, 72.5 ± 6.1 years; female distribution 5/8; BMI, 26.6 ± 3.5. The characteristics of the control group were as follows: age, 69.3 ± 8.5 years; female distribution, 5/8; BMI, 24.6 ± 3.2. Age ($P = 0.396$) and BMI ($P = 0.245$) did not significantly differ between the 2 groups. The characteristics of disease in the OA group were as follows: mean resting VAS, 1.4 ± 1.8; mean active VAS, 4.5 ± 2.1; mean duration of disease, 26 ± 16.7 months; WOMAC in pain, 48.6 ± 12.8 mm; WOMAC in stiffness, 62.5 ± 11.3 mm; WOMAC in function, 64.1 ± 8.5 mm.

TABLE 1. The Demographic and Disease-related Characteristics of Patients

	Osteoarthritis	Controls
N	8	8
Mean age (SD)* (y)	72.5 ± 6.1	69.3 ± 8.5
% Female (n)	62.5 ± 5	62.5 ± 5
BMI (SD)*	26.6 ± 3.5	24.6 ± 3.2
Mean active VAS (SD)	4.5 ± 2.1	0
Mean resting VAS (SD)	1.4 ± 1.8	0
Mean duration of disease (SD) (mo)	26 ± 16.7	0
WOMAC in pain (mm)	48.6 ± 12.8	N/A
WOMAC in stiffness (mm)	62.5 ± 11.3	N/A
WOMAC in function (mm)	64.1 ± 8.5	N/A

*P-value >0.05, no significant difference.

BMI indicates body mass index; N/A, not available; VAS, visual analog scale (0-10); WOMAC, Western Ontario and McMaster Universities index of osteoarthritis (0-100 mm).

Protein Profile

CysC

Immunoblotting quantified the expression of full-length CysC protein in the CSF in the 2 groups using a monoclonal antibody specific for the *N*-terminal region of CysC (Fig. 1A). The blotted CysC band represented the expression of full-length CysC; the cleaved *N*-terminal of the CysC was not probed by the monoclonal antibody. Figure 1B displays the quantified expression of full-length CysC in the 2 groups. The results showed an average 1.6-fold upregulation of full-length CysC expression in the OA group compared with the control group. A *P*-value of 0.000425 demonstrated that the differences in full-length CysC expression between the 2 groups were highly significant. Lower expression of full-length CysC protein in the control group than in the OA group suggested that a protease might be responsible for CysC processing.

MMP2 and MMP9

Using terminal amine isotopic labeling of substrates (TAILS), Kleifeld et al[32] identified several possible substrates of MMP2 including CysC. MMP2 and MMP9 proteins belong to the gelatinase subfamily and share relatively conserved catalytic pockets and enzymatic substrates.[33] To investigate the possibility that MMP2 and MMP9 might be responsible for the cleavage of full-length CysC in CSF, MMP2 and MMP9 protein expression profiles in the CSF of the 2 groups were examined. Immunoblotting was used to evaluate MMP9 expression (Fig. 2A, upper panel), with the bands representing its pro forms and active forms. Figure 2B (upper rows) displays the quantified

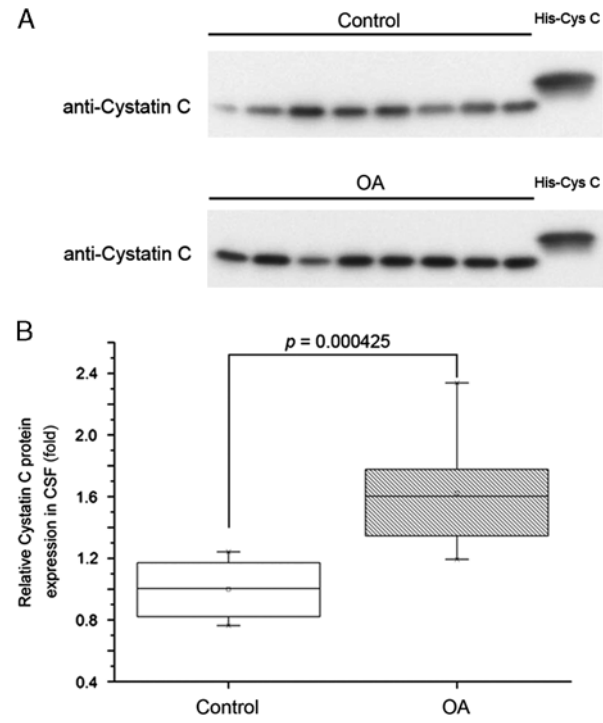

FIGURE 1. Full-length cystatin C protein expression in CSF from control and OA patients. The presence of full-length CysC in the CSF was identified using monoclonal antibody specific for the *N*-terminal of human CysC. The CSFs were loaded with 25 μg in all samples. Recombinant His-tagged CysC (His-CysC, 0.1 μg) provided a control. A, The specific band representing CysC protein expression. B, Quantified levels of CysC. Expression of CysC protein showed significant upregulation in the OA group compared with the control group. The Student *t* test calculated the *P* value. CSF indicates cerebrospinal fluid; CysC, cystatin c; OA, osteoarthritis.

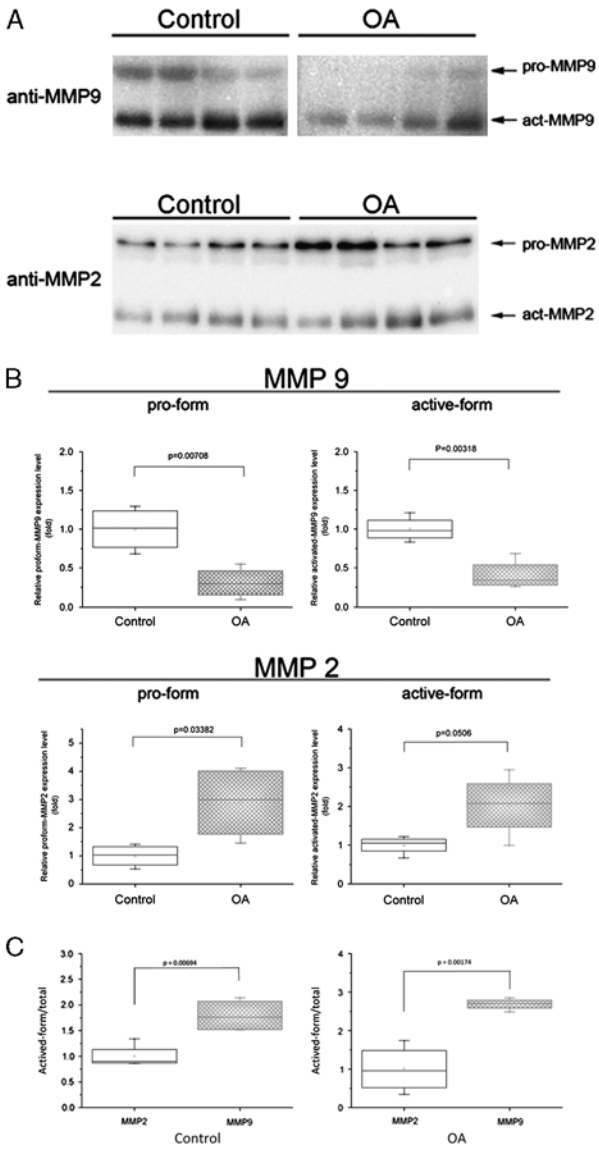

FIGURE 2. Expression of MMP9 and MMP2 proteins in CSF of control and OA patients. A, Immunoblotting using specific antibodies identified MMP9 and MMP2 protein expression in the CSF of 8 selected patients. All lanes were loaded with 20 μg total protein. B, Quantified expression of the pro forms and active forms of MMP9 and MMP2. In the OA group, expression of the pro form of MMP9 was 30.9% of that of the control group; expression of the active form of MMP9 was 40.6% of that of the control group. The pro form and active form of MMP9 all showed significantly reduced expression in the OA group compared with the control group ($P < 0.01$). The pro form of MMP2 showed a 2.9-fold significant increase in expression in the OA group compared with the control group ($P < 0.05$); the active form of MMP2 showed a 2.0-fold increase in expression in the OA group compared with the control group. C, Quantified activated and total MMP9 and MMP2 expressions. The average activation ratio for MMP2 was 39.8% in the control group and 31.3% in the OA group. The average activation ratio for MMP9 was 71.4% in the control group and 84.1% in the OA group. The activation status of MMP9 was significantly higher than that of MMP2 in both control and OA groups. The Student *t* test calculated the *P* value. CSF indicates cerebrospinal fluid; MMP, matrix metalloproteinase; OA, osteoarthritis.

expressions of these forms. The 2 MMP9 forms demonstrated significantly reduced expression in the OA group compared with the control group. The expression of MMP2 protein in the CSF of the 2 groups was also evaluated (Fig. 2A, lower panel) and quantified (Fig. 2B, lower rows). The levels of the pro form of MMP2 were significantly higher in the OA patients than in controls ($P < 0.05$). However, the expression of active MMP2 protein showed no significant differences between the groups ($P > 0.05$). The average MMP2 activation ratio was 39.8% in the control group and 31.3% in the OA group. The average MMP9 activation ratio was 71.4% in the control group and 84.1% in the OA group. In both groups, the activation status of MMP9 was significantly higher than that of MMP2. Although the expression of MMP9 protein was downregulated in the OA group, the MMP9 showed an unchanged activation mechanism. However, expression of MMP2 protein was upregulated in the OA group despite the MMP2 activation mechanism being limited in both control and OA groups.

The differences in protein expression and activation between MMP2 and MMP9 suggest that these proteins have different physiological roles. In the OA group, the significant downregulation of MMP9 expression correlated with the upregulation of CysC protein expression. In the control group, the trends displayed in MMP9 expression were opposite to those in the OA group. In addition, the expression of the active form of MMP2 was lower than that of the active form of MMP9. The MMP2 and MMP9 proteins, therefore, displayed variations in protein expression profiles and also in activation statuses.

Cleavage of Full-Length CysC

To examine the abilities of MMP9 and MMP2 proteins to cleave CysC, the recombinant full-length CysC containing His-tag at the N-terminal was incubated with activated recombinant MMP9 or MMP2 protein for 16 hours, in the presence or absence of CSF collected from the control group. The resulting reactants were separated using SDS/PAGE and then immunoblotted (Fig. 3A) or silver stained (Fig. 3B). To avoid interference from the endogenous CysC protein, the full-length CysC with His-tag in its *N*-terminal was used and the integrity of the CysC protein was verified by probing with anti-His tag antibody. Immunoblotting results indicated that CysC cleavage did not occur after incubation of the activated MMP9 or MMP2 protein with CysC alone. However, if CSF was added to the reactants, CysC underwent cleavage. Cleavage occurred to a greater extent after the addition of activated MMP9 than after the addition of activated MMP2 (Fig. 3A). These results indicate that MMP9 and MMP2 can both initiate CysC cleavage with the assistance of some factors in CSF, and that MMP9 shows greater CysC-cleaving ability than MMP2. Silver staining SDS/PAGE findings further validated these results. Reduced full-length CysC and the presence of several processed peptides in the MMP9 lane (Fig. 3B) showed that MMP9 had initiated the cleavage process to obtain multiple cleavage sites within CysC. The MMP2 lane, in contrast, showed reduced full-length CysC band without the presence of processed peptides. The differing expression profiles of MMP9 and MMP2 (Fig. 2) and the lower CysC cleavage activity of MMP2 than MMP9 (Fig. 3) combined to indicate lesser involvement of MMP2 than MMP9 in CysC cleavage in the

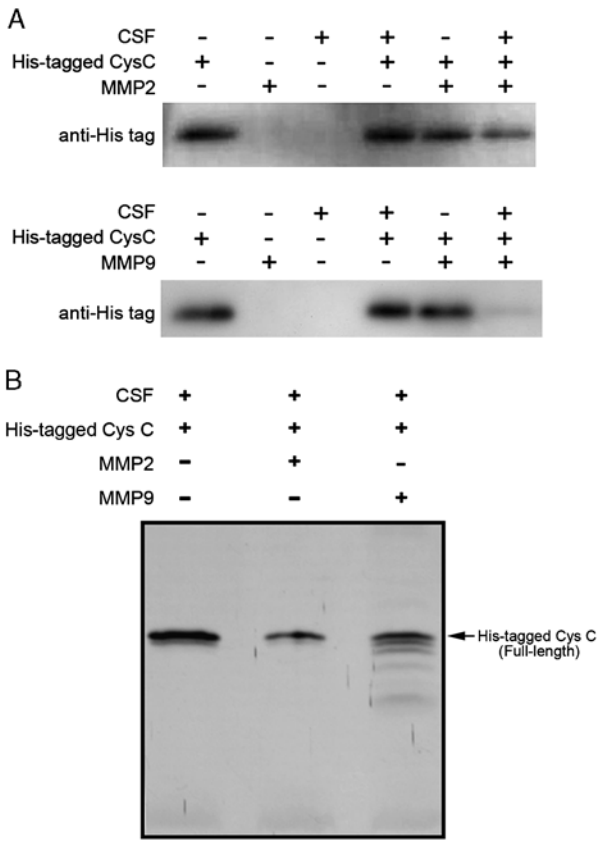

FIGURE 3. Activated MMP9 and MMP2 proteins initiated cleavage of cystatin C to different extents in CSF. A, Activated MMP9 (1 pmol) and activated MMP2 (1 pmol) were incubated with His-tagged CysC (8 pmol). CSF (1 μg) was added. The mixtures were incubated for 16 hours at 37°C, separated using 15% SDS/PAGE, and transferred to a PVDF membrane. Immunoblotting was performed by probing the membrane with anti-His tag antibody. Incubation of activated MMP9 or MMP2 protein alone with full-length CysC did not result in CysC cleavage. However, after the addition of CSF to the mixture, MMP9 and MMP2 both initiated the mechanism for CysC processing within the CSF. The MMP9 and MMP2 proteins showed differing abilities to initiate cleavage of CysC. B, For silver staining, activated MMP9 (1 pmol) and activated MMP2 (1 pmol) were incubated with His-tagged CysC (80 pmol). All lanes contained 0.1 μg CSF and the reactants were incubated for 16 hours at 37°C. The resulting reactants were separated using 15% SDS/PAGE and silver stained. The results indicated that MMP9 induced CysC digestion to a greater extent than MMP2 because of lesser amounts of CysC residue and ladder distrubution of cleavage. CSF indicates cerebrospinal fluid; CysC, cystatin c; MMP, matrix metalloproteinase.

CSF. The results demonstrated that MMP9 plays an important role in the cleavage of CysC in vitro.

MMP9 on CysC Metabolism

To investigate the CysC cleavage ability of MMP9 in vivo, CSF from the OA group was incubated with activated recombinant MMP9, in the presence or absence of MMP9 inhibitor, for 6 hours and the reactants were separated using SDS/PAGE. The complete EDTA-Free Protease Inhibitor Cocktail was used to inhibit the serine and cysteine proteases in the CSF. Expression of CysC protein expression was identified using anti-CysC monoclonal antibody (Fig. 4A). Addition of MMP9 (Fig. 4A, second lane) resulted in the downregulation of CysC in the CSF; the addition of MMP9 inhibitor (Fig. 4A, third lane) reversed this effect. Comparison of the second and fourth lanes revealed limited involvement of serine or cysteine protease in CysC downregulation. Addition of the EDTA-Free Protease Inhibitor Cocktail did not lead to suppression of CysC cleavage by MMP9 (fourth lane), indicating that the MMP9 protein has a more important role than endogenous serine and cysteine proteases in the CysC cleavage process. The fifth lane, containing MMP9 inhibitor in the absence of recombinant MMP9 protein, showed a stronger CysC signal. This suggested that the endogenous MMP9 protein in the CSF is also significantly resposible for cleavage of CysC in vivo. Protein expressions in each reaction mixture were then profiled using SELDI-TOF MS (Fig. 4B). The mixtures were spotted onto a NP chip using α-CHCA as a matrix. A 13.3 kDa full-length and *N*-terminal truncated CysC with molecular weight of 12.5 kDa in the CSF both noted in the control group (first row, Fig. 4B) and the profiled protein peaks demonstrated the only presence of 13.3 kDa full-length CysC protein in the CSF of the OA group (second row, Fig. 4B). After the addition of activated MMP9 protein to the CSF for 6 hours, some processing of the 13.3 kDa CysC in the CSF to its 12.5 kDa form occurred (third row, Fig. 4B). After the addition of MMP9 inhibitor, the processed 12.5 kDa CysC was undetectable (fourth row, Fig. 4B). Control CysC protein purified from urine confirmed the molecular weight of truncated 12.5 kDa CysC (fifth row, Fig. 4B). These results suggested that the MMP9 protein initiates the cleavage of CysC in CSF in vivo. The MMP9 inhibitor (PubChem ID: 26759113) used in Figure 4B was purchased from Calbiochem/Merck. According to the datasheet provided by the vendor the agent also inhibit abilities on MMP1 (IC_{50} = 1.05 μM) and MMP13 (IC_{50} = 113 nM) besides MMP9 (IC_{50} = 5 nM). A possible explanation of what was shown in Figure 4B concerning the peak at 14 kDa MMP1/MMP13 was activated in the OA group but not in control group.

DISCUSSION

The in vivo and in vitro analyses in the present study further evaluated the relationship between MMP9 and CysC in CSF. Results indicated that MMP9 initiated the cleavage of full-length CysC in the CSF; addition of MMP9 inhibitor confirmed the cleavage specificity. The SELDI mass spectrum indicated the cleavage of full-length CysC (13.3 kDa) into its truncated form (12.5 kDa) by MMP9. The results also suggested the presence of higher full-length CysC content in the CSF of the OA group than in CSF of the control group because of reduced active MMP9 protein to initiate CysC cleavage. This provides the first evidence to suggest that pain-induced variations in CysC in the CSF might be caused by changes in MMP9 activities. The present study evaluated the role of CysC in CSF and its potential use as a pain biomarker. One novel finding was the interaction between CysC and gelatinases in the CSF.

CysC

CysC is expressed by all nucleated cells and distributed in human body fluids such as plasma, saliva, ascites, colostrum, CSF, and seminal fluid.[34] The CysC gene is stably produced and commonly regarded as a housekeeping type gene.[18] Mussap and Plebani[35] reviewed the functions

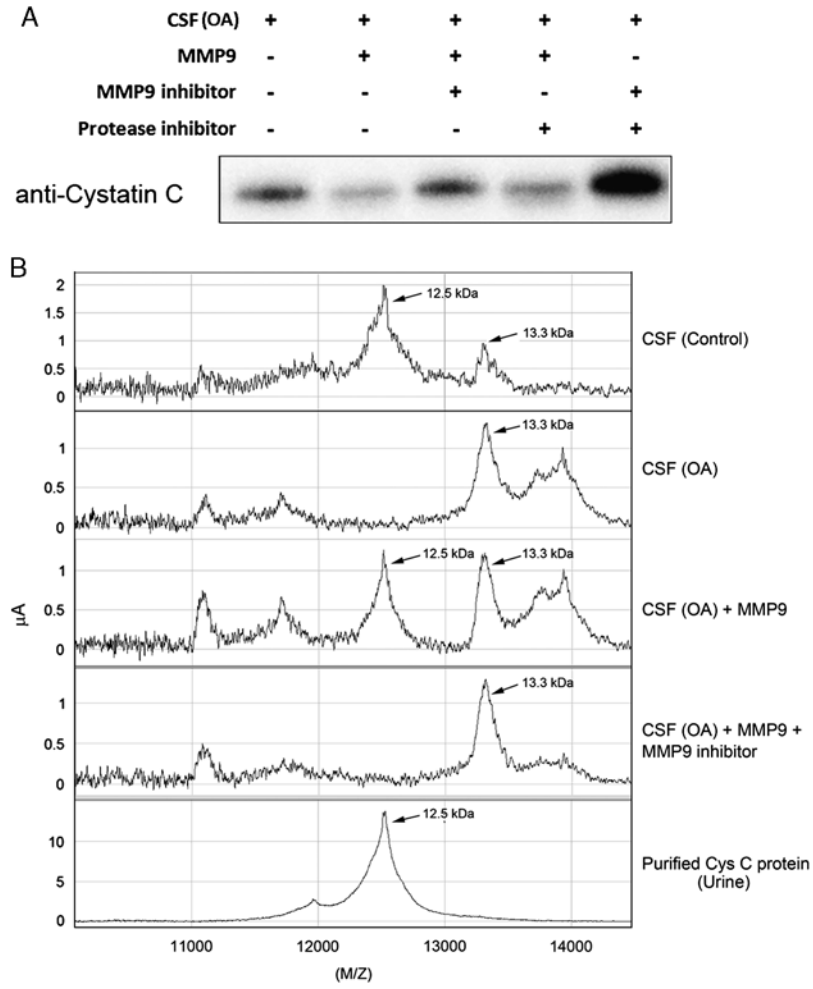

FIGURE 4. Activated MMP9 protein initiated the processing of full-length CysC in CSF. A, Activated MMP9 protein (1 pmol) was added to CSF (1 μg) collected from OA patients. This mixture was then incubated in the presence or absence of MMP9 inhibitor (0.1 mM). The EDTA-Free Protease Inhibitor Cocktail was used to inhibit the serine and cysteine proteases in the CSF. Reaction mixtures were incubated for 6 hours at 37°C, and the resulting reactants were separated using 15% SDS/PAGE and immunoblotted. Expression of CysC was identified using monoclonal anti-CysC antibody. Expression of CysC protein decreased after the addition of MMP9 protein (second lane); MMP9 inhibitor negated these effects (third lane). The protease inhibitor cocktail (Complete EDTA-Free) did not suppress the cleavage of full-length CysC by MMP9 (fourth lane). Incubation of MMP9 inhibitor with protease inhibitor cocktail provided the lowest levels of CysC cleavage (fifth lane). B, The expression profiles of CysC proteins in CSF were detected using SELDI-TOF MS. In the control group, the CysC occurred in the processed 12.5 kDa form with reduced expression of the 13.3 kDa form (first row); in CSF of the OA group, the CysC protein retained its 13.3 kDa form (second row). After the addition of activated MMP9 protein to the CSF of the OA group, CysC was cleaved into its 12.5 kDa form (third row). The addition of MMP9 inhibitor suppressed this cleavage (fourth row). A urine-purified Cys C protein was used as an indicator of processed Cys C (fifth row). CSF indicates cerebrospinal fluid; CysC, cystatin c; MMP, matrix metalloproteinase; OA, osteoarthritis.

of CysC, classifying them into 4 categories: (1) direct inhibition of cysteine proteases, (2) immune modulation, (3) antipathogen activity, and (4) pathologic response in the CNS. Several studies have shown that blood CysC levels are not influenced by age, sex, muscle mass, diet, or physiological activities.[36,37] Hence, it has been used as a biomarker for renal function because it seems superior to [Cre] or other estimating equations.[38,39] Since 2004, the fluctuation of CysC concentration in blood has been proved that it is associated with the prognosis of coronary heart disease[40] or heart failure.[41] Furthermore, if we want to study the exact role of CysC on pain, its distribution has to be validated in other body fluids. In our study, chronic pain from OA stimulates CNS, which has 1 constant effect on CysC expression. Hence, CSF is an ideal and necessary target for analysis. For blood, chronic pain signals are transmitted through nerve fibers, not vessels. In addition, as 90% of CysC is distributed in CSF, the fraction of CysC in plasma may be ignored due to effect from CSF. CysC levels in synovial fluid have been studied in OA,[42] gouty arthritis,[43] and rheumatoid arthritis.[42,44] However, due to anatomic discontinuance we did not take any sample from synovial fluid. On the basis of the same rationale, 2 studies observed changes in CysC in the CSF among patients with distinct pain symptoms. Mannes et al[23] collected CSF from pregnant women, with 1 group experiencing persistent labor pain for >8 hours (VAS >5) and a control group receiving cesarean section without pain. The results showed that

CysC protein expression was significantly higher in the labor pain group than in the control group. However, Eisenach et al[22] examined CSF samples from control, labor pain, cesarean section, and chronic neuropathic pain, and observed no significant differences in CysC expression among the 4 patient groups. These inconsistencies indicate the need for further advanced experiments to elucidate possible variables other than total CysC in CP conditions. It is possible that significantly increased full-length CysC in the CSF of the OA group might have resulted from reduced CysC cleavage, caused by an unknown enzymatic mechanism.

MMPs

Gelatinases are involved in neuroinflammation and associated with several CNS diseases, including amyotrophic lateral sclerosis,[45] multiple sclerosis,[46] brain trauma,[47] and stroke.[48] Kawasaki et al[49] used a rat spinal nerve injury model to identify marked increases in MMP9 expression, followed by upregulation in MMP2 after MMP9 overexpression. The authors concluded that intracellular MMP2 protein might be involved in the maintenance of neuropathic pain. The present study's analyses also indicated that the CSF of OA patients displays upregulated MMP2 protein expression in CSF (Fig. 2). This suggested that nociception could influence the expression of gelatinases and CysC. The relationship between these proteins remains under investigation.

The main substrates of gelatinases are type IV collagen and gelatin.[24] However, 2 previous studies used proteomic strategies, including iTRAQ[50] and TAILS[32] to identify CysC as a novel substrate for MMP2. Gelatinase might, therefore, be the enzyme responsible for the cleavage of the *N*-terminal of CysC in the CSF. However, results from in vitro analyses indicated that MMP2 or MMP9 alone is unable to cleave recombinant full-length CysC. The cleavage of CysC by gelatinases occurred in vivo and in the presence of CSF in vitro. This suggested that CysC might not be the direct enzymatic substrate of MMP9 or MMP2. Gelatinases might initiate the mechanism for cleavage of CysC in the CSF, in manners different between MMP9 and MMP2 (Figs. 3A, B). Comparing to MMP9, MMP2, with less active form quality and weaker enzymatic activity, was deemed unlikely to be the candidate enzyme causing intrathecal CysC fluctuation in CSF. These results suggest that intrathecal MMP9 has a more important role in cleavage of CysC than MMP2.

Combinations

The CSF has no isolated microenvironment or compartment-like cells; therefore, changes in protease activity are dependent on inhibitor binding. The activation of a specific enzyme might occur through the cleavage of its inhibitor, such as in hemostasis and the complement system in serum. The expression of CysC, a cysteine protease inhibitor, inversely reflects the activity of its target protease. Nagai et al[51] reported that CysC is tightly bound to cathepsin B and H. CysC, which has truncated 11 *N*-terminal amino acids, shows a much lower affinity for cysteine proteinases than the intact inhibitor.[52] Interaction of valine-10 in CysC with the S2 subsite of cathepsin B is crucial for their interaction.[53] The present study's findings showed the existence of a mechanism in the CSF in which MMP9, more efficiently than MMP2, cleaves the *N*-terminal of full-length CysC to obtain its truncated form. In addition, the MMP9-driven mechanism processes the 11 *N*-terminal amino acids after the activation of cathepsin B. In vitro analyses indicated that the addition of recombinant-activated MMP9 changes the existing protease-inhibitor balance in the CSF, resulting in increased CysC cleavage. The body might adjust its CysC protein levels to accommodate the appropriate protease activities in response to physiological stimuli. An MMP9-driven mechanism within CSF was therefore proposed (Fig. 5). Chronic musculoskeletal pain can induce signals that reduce MMP9 protein expression, resulting in increased CysC protein expression and the inhibition of its target protease. The proteins in these pathways might potentially provide future therapeutic or pharmacological targets for the diagnosis or treatment of chronic pain.

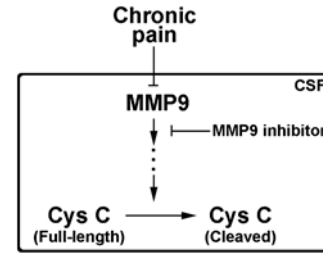

FIGURE 5. Proposed MMP9-driven mechanism for CysC processing within the CSF. Chronic musculoskeletal pain regulates intrathecal CysC expression through MMP9-specific initiating mechanism. In fact, CysC cleavage can be modified by active MMP9, inhibited by chronic pain stimuli. The whole digestion process will be performed in the presence of CSF. Therefore, increased expression of intrathecal CysC will be associated with chronic musculoskeletal pain with MMP9-specific cleavage mechanism. CSF indicates cerebrospinal fluid; CysC, cystatin c; MMP, matrix metalloproteinase.

From our present results, we found that the abundance of CysC in the CSF was anticorrelated with MMP9 activity in patients with CP. Clinical application awaits evidence that the accumulation of full length of CysC, or the lowering of MMP9 expression, is the cause of central sensitization from pure non-neuropathic pain. An established causal relation between CysC/MMP9 and pain can be clinically applied, such as an evaluation of CP by observing intrathecal CysC, or the slowing down of the process of central sensitization by enhancing MMP9 expression.

LIMITATIONS

Certain limitations need to be deliberated in the interpretation of the present findings. First, we investigated the difference of intrathecal CysC and MMP9 between chronic pain and controls. To minimize possible effect of small sample size we did rigorous due diligence in our patient recruitment by adjusting for possible confounders including sex, ASA status, age, and comorbidity. The total sample size of 16 patients allowed us to only detect distinct differences, the trend of which was confirmed by both in vivo and in vitro results. A larger sample size is not expected to change this result, but would have allowed the performance of additional within-group analysis, possibly leading to more detailed results. Second, we did not do multiple testing in this study. Because of the observational design of our study, the results obtained provide only preliminary evidence regarding causal influences. In such observational studies such as the present one, adjustment for multiple testing is not recommended.[54] We are planning future confirmatory studies including multiple testing.

Finally, we found that pain-induced variation in the abundance of CysC in the CSF was anticorrelated with MMP9 activity. However, it is still unclear whether the accumulation of full length of CysC is the cause or the result of chronic OA pain. We believe establishing that evidence required animal model studies, such as the manipulation of MMP9 or/and CysC expression levels in the CSF using gene silencing techniques and observing the resulting pain intensity in the model.

Despite these limitations, this is the first study that has simultaneously compared intrathecal CysC and MMP9 differences in chronic pain patients and controls. Making a distinction between these 2 proteins with CP stimulation in the future is of undoubted relevance.

CONCLUSIONS

The present study conducted preliminary research on the fluctuations in gelatinase in the CSF among control and OA patients, and the relevance of these fluctuations to CysC variations in the CSF. A major finding was the unique relationship between MMP9 and CysC, as verified using several in vivo and in vitro examinations. The results indicated that active MMP9 protein, transformed by natural activators in the CSF, initiates a mechanism for cleavage of full-length CysC protein into its truncated product in the CSF. The fluctuations in CysC in the CSF might be caused by variations in the MMP9 activities involved in chronic musculoskeletal pain. These findings could explain previous studies' observations of an association between CysC in the CSF and distinct pain conditions. Results also indicated the mechanism of CysC involvement in pain perception and might facilitate future study of CSF proteomics in pain medicine.

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