ORIGINAL ARTICLE

Presence of secretory cellular apoptosis susceptibility protein in cerebrospinal fluids of patients with intracerebral hemorrhage caused by stroke and neurotrauma

Jai-Nien Tung [1,2], Tang-Yi Tsao [3], Shun-Liang Chen [4], Cheng-Jeng Tai [5], Shing-Chuan Shen [6], Ya-Wen Cheng [1*], Ming-Chung Jiang [5*]

1 Institutes of Medicine, Chung Shan Medical University, Taichung, Taiwan, R.O.C.
2 Department of Surgery, Tungs' Taichung MetroHarbor Hospital, Taichung, Taiwan, R.O.C.
3 Department of Pathology, Tungs' Taichung MetroHarbor Hospital, Taichung, Taiwan, R.O.C.
4 Division of Clinical Pathology, Tungs' Taichung MetroHarbor Hospital, Taichung, Taiwan, R.O.C.
5 Department of Medicine, Section of Hematology-Oncology, Taipei Medical University and Hospital, Taipei, Taiwan, R.O.C.
6 Graduate Institute of Medical Science, Taipei Medical University, Taipei, Taiwan, R.O.C.

**Correspondence to:* Ming-Chung Jiang, PhD.
Section of Hematology-Oncology, Department of Medicine, Taipei Medical University and Hospital, 252 Wu-Hsing St, Hsing-Yi Dist. Taipei 110, Taiwan, R.O.C.
TEL: +886-2-27372181 ext. 3903, E-MAIL: jiangmwd@gmail.com

Ya-Wen Cheng, PhD.
Institute of Medicine, Chung Shan Medical University,
No. 110, Sec. 1, Chien-Kuo N. Rd., Taichung 40203, Taiwan, R.O.C.
TEL: +886-4-24730022-11690, E-MAIL: yawen@csmu.edu.tw

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Abstract

OBJECTIVE: The blood-brain barrier (BBB) is a specialized structure that separates blood vessels from the central nervous system (CNS) and restricts the entry of biomolecules and cells into the brain. Matrix metalloproteinase-2 (MMP-2) produced by interferon-γ-activated microglia (brain macrophages) is essential for disrupting the glia limitans of BBB, which is critical for lymphocytes penetration into brain capillaries in various CNS disorders. The cellular apoptosis susceptibility (CSE1L/CAS) protein has been shown to regulate MMP-2 secretion. **METHODS:** We examined if CSE1L played a role in regulating the progression of intracerebral brain hemorrhage disorders. **RESULTS:** CSE1L was detected by immunoblotting in cerebrospinal fluids (CSFs) of patients with intracerebral hemorrhage brain disorders, including stroke and neurotrauma. Interferon-γ treatment induced CSE1L expression and increased the secretions of CSE1L and MMP-2 by U937 macrophages. Moreover, tranfection of U937 macrophages with siRNA that targeted CSE1L inhibited interferon-γ-induced CSE1L and MMP-2 secretion by U937 macrophages. The numbers of lymphocytes in CSF were correlated with the levels of CSE1L and MMP-2 in patients' CSF. **CONCLUSIONS:** Our results suggest that CSE1L plays a role in regulating MMP-2-mediated BBB breakdown and it may be a target for control of BBB permeability in intracerebral brain hemorrhage disorders.

Abbreviations:

BBB - blood-brain barrier
CNS - central nervous system
CSF - cerebrospinal fluid
DMEM - Dulbecco's modified Eagle's medium
FBS - fetal bovine serum
MMP - matrix metalloproteinase
PBS - phosphate buffered saline

INTRODUCTION

Activation of microglia, or resident brain macrophages, and the subsequent migration of leukocytes from the blood vessels into the CNS are essential for controlling ongoing pathophysiological processes and promoting neuroprotection or regeneration in many brain injuries (Streit *et al.* 2005). The glia limitans seals the surface of the CNS and extends into brain tissue along the perivascular space and creates the blood-brain barrier (BBB) (Ballabh *et al.* 2004). Loss of BBB integrity causes fluid to leak into the brain interstitial space and leading to vasogenic cerebral edema (Walski & Gajkowska 2001). Microglial cells are crucial for maintaining CNS homeostasis under both normal physiological and pathological conditions, such as phagocytosis, antigen presentation and post-inflammation renewed growth of neural tissue (Chew *et al.* 2006).

Matrix metalloproteinases (MMPs) play key roles in modulating the migration and invasion of immune cells including monocytes to break through the blood vessel wall and infiltrate the target organ during disease progression (del Zoppo *et al.* 2007). MMP-2 is involved in disrupting the tight junctions between cerebral endothelial cells in brain capillaries in various CNS disorders; MMP-2 produced by activated microglia can disrupt the glia limitans of the BBB to facilitate leukocytes penetrate the capillaries and enter the inflamed CNS (Hartung & Kieseier 2000). Interferon-γ is a pro-inflammatory T helper (Th)-1 type cytokine produced by T-lymphocytes, microglia, and reactive astrocytes in the brain (Folkerth *et al.* 2004; Kawanokuchi *et al.* 2006; Lee *et al.* 2007). Interferon-γ is an activator of microglia and is involved in the pathogenesis of various CNS disorders (Millward *et al.* 2007; Moran *et al.* 2007). One of the neuro-inflammatory responses of interferon-γ-activated microglia is the production of proteases, including MMP-2, for BBB disruption and blood lymphocytes penetration (Gottschall & Deb 1996; Grau *et al.* 1997).

The cellular apoptosis susceptibility (CSE1L/CAS) protein is the human homologue of the yeast chromosome segregation gene product, CSE1 (Brinkmann *et al.* 1995). CSE1L (or CAS) regulates apoptosis induced by chemotherapeutic drugs (Jiang *et al.* 2007; Liao *et al.* 2008b,c). Our recent studies showed that CSE1L also regulates the secretion of human colorectal cells and the metastasis of cancer cells (Liao *et al.* 2008a; Tsao *et al.* 2009), and that CSE1L is a secretory protein present in sera of cancer patients (Tung *et al.* 2009; Tsai *et al.* 2010). CSE1L has been shown to regulate cellular translocation and secretion of MMP-2 (Liao *et al.* 2008a; Tsao *et al.* 2009; Tung *et al.* 2009). Interferon-γ treatment can increase CSE1L expression in human HT-29 colorectal cancer cells (Jiang *et al.* 2001). We report here that secretory CSE1L is present in the CSF of patients with intracerebral hemorrhage brain disorders including stroke and neurotrauma. Our studies also show that CSE1L modulates interferon-γ-induced MMP-2 secretion of U937 macrophages. CSE1L may play a role in regulating the progression of intracerebral brain hemorrhage disorders and it may be a target for the control of intracerebral brain hemorrhage.

MATERIALS AND METHODS

Antibodies

Antibodies used in the experiment were anti-CSE1L (clone 3D8) (Abnova, Taipei, Taiwan), anti-MMP-2 (H-76) and CD68 (sc-59103) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-β-actin (Ab-5) (Neomarker, Westinghouse Drive, CA), and goat anti-mouse (or anti-rabbit) IgG secondary antibodies coupled to Alexa Fluor 488 (or Alexa Fluor 568) (Molecular Probes, Eugene, OR).

Cells

U937 macrophages and U-87 MG brain cancer cells were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Cells were cultured in DMEM medium supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamate at 37 °C under a humidified 5% CO_2 atmosphere as described previously (Tsai *et al.* 2009).

Immunoblotting Samples were resolved with 8% SDS-polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes (Amersham Pharmacia, Buckinghamshire, UK) and immunoblotting was performed using specific antibodies and an ECL Western blotting detection system (Amersham Pharmacia) as previously described (Tsai *et al.* 2009).

Collection of cerebrospinal fluids

The study protocol was approved by the Ethics Review Committee of the Tungs' Taichung MetroHarbor Hospital, Taichung, Taiwan and samples were obtained from patients following the Institutional Review Board-approved guidelines (IRB no: 98014). All experiments on human subjects were conducted in accordance with the Declaration of Helsinki and all experimental procedures were carried out with the adequate understanding and written consent of the patients. CSF samples (each 4–8 ml) were collected from the patients admitted to the hospital during standard diagnostic lumbar puncture. The samples were immediately analyzed for

CSF cell populations within 30 min after lumbar puncture. For characterization of cell populations, CSF was immediately centrifuged after lumbar puncture at 3000 rpm for 10 minutes. The cell pellets were re-suspended in Liu's staining solution, and the cells were classified and counted under a microscope. The supernatant were stored at –80 °C for further diagnostic purposes. Baseline characteristics of the CSF samples are shown in Table 1.

Immunofluorescence

Briefly, cells grown on coverslips were washed with PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.1% Triton X-100. Coverslips were incubated with primary antibodies, followed by secondary antibodies coupled to Alexa Fluor 488 or 568. Labeled cells were observed with a Zeiss Axiovert 200M inverted fluorescence microscope (Carl Zeiss, Jena, Germany).

Immunohistochemistry and immunofluorescence histochemistry

Immunohistochemistry and immunofluorescence histochemistry were performed on 4-μm thick, formalin-fixed/paraffin-embedded brain tissue sections using a 50-fold dilution of specific antibodies. Immunohistochemical detection was performed using a labeled streptavidin-biotin method with the Histostain kit according to the manufacturer's instructions (Zymed, San Francisco, CA). Sections were developed with diaminobenzidine, washed with distilled water, and counterstained with Mayer's hematoxylin. For immunofluorescence histochemistry, samples were incubated with primary antibodies, followed by secondary antibodies coupled to Alexa Fluor 488 or 568. Labeled cells were observed with a Zeiss Axiovert 200M inverted fluorescence microscope (Carl Zeiss, Jena, Germany).

Small interfering RNA (siRNA) experiments

The Silencer siRNA CSE1L (s3590), a siRNA sequence targeting the human *CSE1L* (GenBank accession number U33286) mRNA sequence, and the Silencer Negative Control siRNA (AM4611), a non-targeting negative control siRNA, were obtained from Ambion (Austin, TX, USA). The *CSE1L* mRNA target sequence was 5'-GGCUUAUUGGAGCUUAATT-3' (sense) and 5'-UUAAGAGCUCCAAUAAGCCGG-3' (antisense). U937 macrophages (3×10^5 cells/ml) cultured in DMEM without serum and antibiotics were transfected with 35 nM Silencer siRNA CSE1L or Silencer Negative Control siRNA using the Lipofectamine 2000 Stealth/siRNA transfection Unit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. After transfection, the macrophages were cultured for 12 h and then changed with serum-free DMEM media containing 100 U/ml interferon-γ (PeproTech, Rehovot, Israel). After cultured for an additional 48 h, the macrophages were centrifuged at 3000 rpm for 10 minutes. The conditioned media were harvested and the cell numbers were determined. The macrophages were washed with PBS and lysed in RIPA buffer. The total cell lysates and the cell number standardized conditioned media were resolved in 10% SDS-PAGE and the levels of CSE1L and MMP-2 in cells and in media were analyzed by immunoblotting with anti-CSE1L and anti-MMP-2 antibodies.

RESULTS

Cell protrusions are active sites where secretion of cells takes place (Burnham & Williams 1982). Immunofluorescence showed vesicle-like staining of CSE1L in the protrusions and the long fibers of U-87 MG brain cells (Figure 1). Immunohistochemical studies with brain tissue sections showed positive CSE1L staining in the cytoplasm and the extracellular areas of astrocytes in brain tissues (Figure 1). These results indicate that CSE1L may be secreted by brain cells in the CNS.

CSF samples collected from patients with intracerebral hemorrhage brain disorders including stroke and neurotrauma were analyzed for secretory CSE1L by immunoblotting with anti-CSE1L antibodies. The results showed many sharp and intense CSE1L protein bands on the immunoblots of patients' CSF samples (Figure 2). Immunoblotting assays also showed that the levels of MMP-2 in the patients' CSF samples were essentially in proportion to the levels of CSE1L (Figure 2). Analyses for lymphocytes in patients' CSF samples showed that the numbers of lymphocytes were also correlated with the levels of CSE1L and MMP-2 (Figure 2). Thus, secretory CSE1L is present in CSF from patients with intracerebral hemorrhage induced by stroke or neurotrauma and is correlated with the presence of MMP-2 and lymphocytes in their CSF.

Double-staining immunofluorescence with antibodies against CSE1L and MMP-2 showed co-localization of CSE1L with MMP-2 in U-87 MG cells (Figure 3). The results of immunohistochemistry showed positive staining for CSE1L and MMP-2 in the cytoplasm of giant cells around or close to the glia limitans of the BBB in the brain capillaries (Figure 3). Immunofluorescence histochemistry with antibodies against CSE1L and a marker for microglia, CD68, showed CSE1L expression in microglia (Figure 3). We investigated if interferon-γ modulated CSE1L expression and regulated MMP-2 secretion by U937 macrophages. U937 macrophages were treated with 100 U/ml interferon-γ for 16 h and the results of immunoblotting showed that interferon-γ treatment increased CSE1L expression in U937 macrophages (Figure 4). U937 macrophages were then transfected with either the small interfering RNA (siRNA) sequence targeting *CSE1L* mRNA (Silencer siRNA CSE1L) or the non-targeting negative control siRNA (Silencer Negative Control siRNA). The results of immunoblotting showed that CSE1L siRNA transfection reduced CSE1L expression in U937 macrophages as compared to the level of CSE1L in control

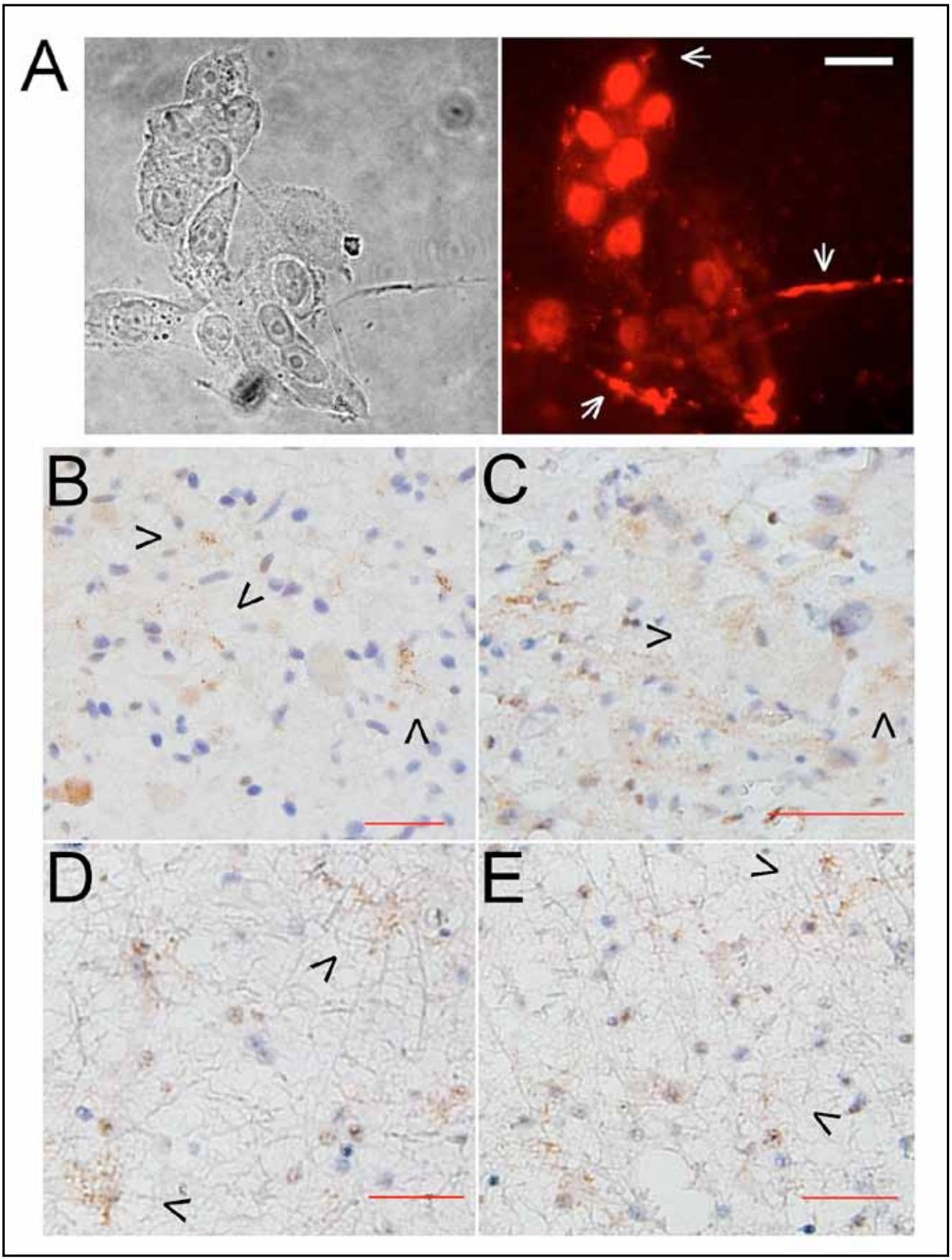

Fig. 1. CSE1L staining in the extracellular areas of brain tissue. (**A**) Immunofluorescent localization of CSE1L in U-87 MG brain cells. Note the vesicle-like staining of CSE1L (arrows) in the protrusions and the long fibers of cells. Scale bar = 10 μm. (**B**-**E**) Representative immunohistochemistry results showed CSE1L staining in the extracellular areas of astrocytes in brain tissues (arrowheads). Scale bars = 50 μm.

Tab. 1. Baseline characteristics of the cerebrospinal fluid samples.*

Clinicopathological parameter	Sample 1	Sample 2#	Sample 3	Sample 4	Sample 5	Sample 6
Patient's sex	Male	Male	Male	Male	Male	Male
Patient's age (years)	46	72	68	72	82	50
Appearance	Clear	Clear	Turbid	Turbid	Clear	Turbid
Color	Colorless	Yellow	Brown	Bloody	Yellow	Yellow
RBCs (cells/μl)	69	910	735	5000	862	196
WBCs (cells/μl)	2	4	960	6	2	11
Lymphocytes (%, per 100 WBCs)	2	3	10	3	2	7
PMN (%, per 100 WBCs)	0	1	90	3	0	4
Protein (mg/dl)	51	55	477	81	83	499
Glucose (mg/dl)	60	100	119	92	95	38
Syndrome	Cerebral embolism induced by stroke	Multifocal intracranial hemorrhage induced by trauma	Cerebral edema induced by stroke	Multifocal intracranial hemorrhage induced by trauma	Aging brain-induced cerebellar hemorrhage	Intracerebral hemorrhage induced by stroke

RBCs: red blood cells, WBCs: white blood cells, PMN: polymorphonuclear neturophils.
*No pathogen infection was assayed in all of the samples.
#Sample 2 and 4 were collected from a same patient at different times.

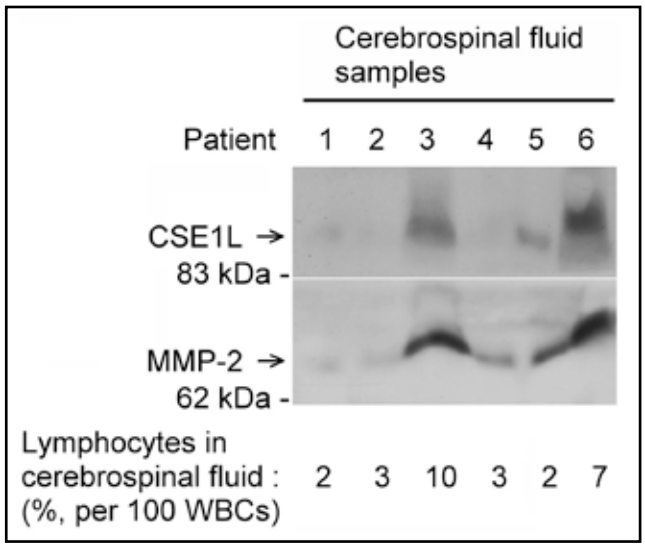

Figure 2. Secretory CSE1L is present in CSF from patients with intracerebral hemorrhage brain disorders and is correlated with the presence of MMP-2 and lymphocytes. The presences of CSE1L and MMP-2 in patient CSF samples were evaluated by immunoblotting using anti-CSE1L and anti-MMP-2 antibodies, and lymphocytes were analyzed by microscopy after lumbar puncture CSF collection**.** Note CSE1L in the patient CSF samples and its level correlated with the presence of MMP-2 and lymphocytes in each sample. Each well was loaded with 80 μl of CSF sample.

siRNA-transfected U937 macrophages (Figure 4B, left panel). We then treated the CSE1L siRNA-transfected U937 macrophages and the control siRNA-transfected U937 macrophages with 100 U/ml interferon-γ for 48 h. The levels of CSE1L and MMP-2 expression in macrophages and the levels of CSE1L and MMP-2 secretion in conditioned media were analyzed with immunoblotting. The results showed that interferon-γ treatment increased CSE1L expression in U937 macrophages, and CSE1L siRNA transfection reduced interferon-γ-induced CSE1L expression in U937 macrophages (Figure 4B, right panel). The results of our experiments also showed that U937 macrophages secreted CSE1L and MMP-2 proteins, and that CSE1L siRNA transfection reduced interferon-γ-induced CSE1L and MMP-2 secretion by U937 macrophages (Figure 4). The expression of MMP-2 in U937 macrophages was not affected by interferon-γ treatment or CSE1L siRNA transfection (Figure 4). Therefore, CSE1L regulated interferon-γ-induced MMP-2 secretion of U937 macrophages.

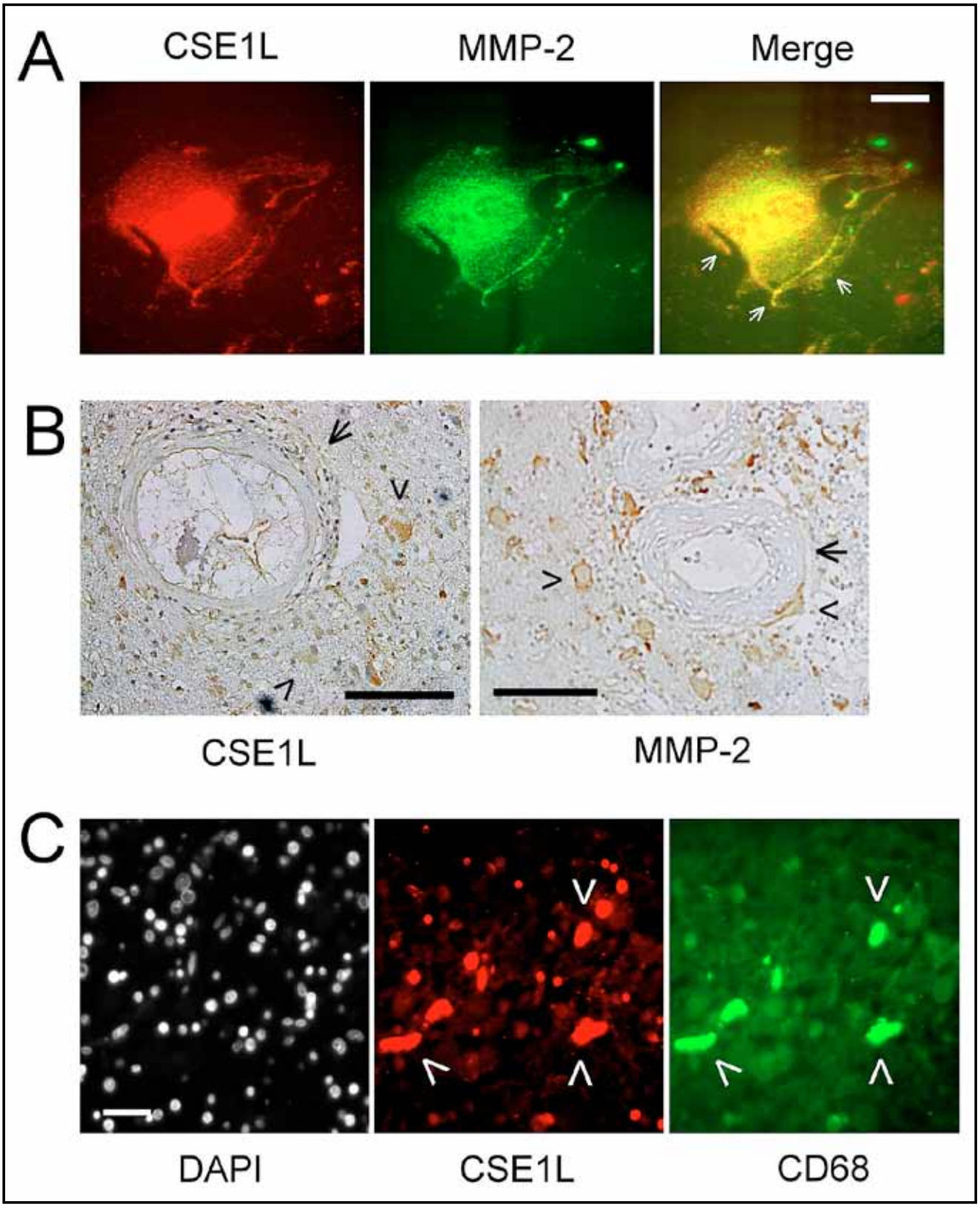

Fig. 3. Immunostaining of CSE1L and MMP-2 in cells surrounding brain capillaries. (**A**) Immunofluorescent of CSE1L and MMP-2 in U-87 MG cells.The localizations of CSE1L and MMP-2 in U-87 MG cells were analyzed by immunofluorescence with anti-CSE1L and anti-MMP-2 antibodies. Arrows indicate co-localization of CSE1L with MMP-2 in the protrusions and the long fibers of U-87 MG cells. Scale bar = 10 μm. (**B**) CSE1L and MMP-2 staining in cells around brain capillaries. Representative immunohistochemistry results with anti-CSE1L and anti-MMP-2 antibodies showed positive staining for CSE1L and MMP-2 in cells around the BBB of brain capillaries in brain tissues. Note the multiple rings-like layers of glia limitans (arrows) surrounding the brain capillaries and cells (arrowheads) surround glia limitans. Scale bars = 10 μm. (C) CSE1L expression in microglia. Representative immunofluorescence histochemistry with formalin/paraffin-embedded brain section showed staining of CSE1L in CD68-positive cells. The cell nuclei were stained with DAPI. Scale bar = 25 μm.

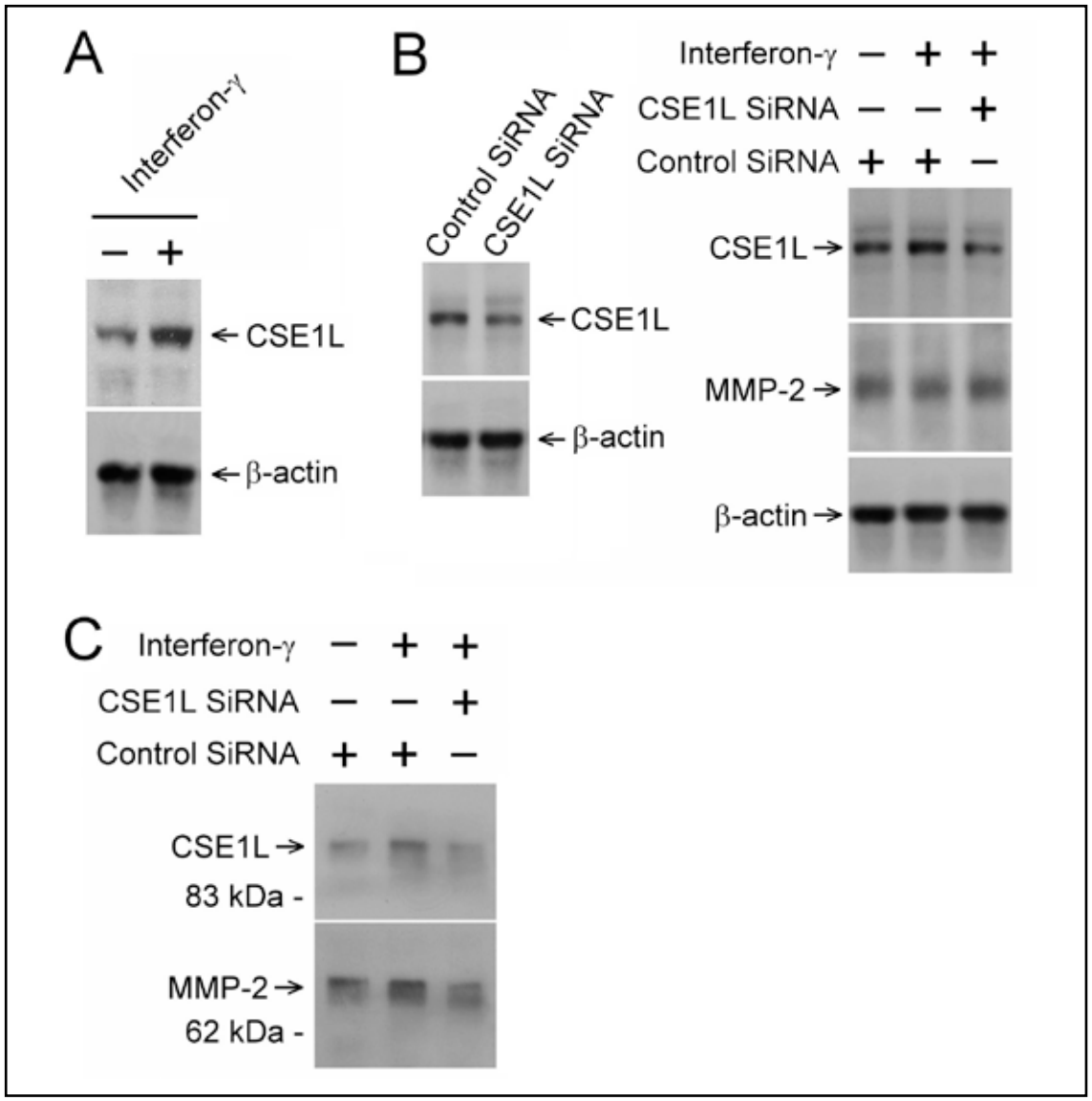

Fig. 4. Interferon-γ regulates CSE1L expression and MMP-2 secretion by U937 macrophages. (**A**) Interferon-γ treatment increased CSE1L expression in U937 macrophages. U937 macrophages were treated with or without 100 U/ml interferon-γ for 16 h and CSE1L protein levels were analyzed by immunoblotting with anti-CSE1L antibodies. β-actin levels were assayed using anti-β-actin antibodies as a control. (**B**) CSE1L siRNA transfection reduced interferon-γ-induced CSE1L expression by U937 macrophages. U937 macrophages transfected with CSE1L siRNA or non-targeting control siRNA were treated with or without 100 U/ml interferon-γ for 48 h as indicated. Cellular CSE1L and MMP-2 levels were analyzed by immunoblotting with anti-CSE1L and anti-MMP-2 antibodies. β-actin levels were assayed with anti-β-actin antibodies as a control. (**C**) CSE1L siRNA transfection reduced interferon-γ-induced CSE1L and MMP-2 secretion by U937 macrophages. U937 macrophages transfected with CSE1L siRNA or non-targeting negative control siRNA were treated with or without 100 U/ml interferon-γ for 48 h as indicated. Levels of CSE1L and MMP-2 protein in conditioned media were analyzed by immunoblotting with anti-CSE1L and anti-MMP-2 antibodies. The assays were repeated 3 times and showed similar results; representative immunoblots are shown here.

DISCUSSION

Microglia are resident macrophages in the brain that act as the first and primary form of active immune defense in the CNS. U937 macrophages have been extensively used to characterize many biological functions pertaining to brain microglia (Chabot *et al.* 2001). Also, interferon-γ-treated U937 cells show similarities to microglia in their interactions with activated T lymphocytes (Chabot *et al.* 2001). Secretory CSE1L was present in CSF from patients with intracerebral hemorrhage brain disorders induced by stroke or neurotrauma (Figure 2). The level of CSE1L in patients' CSF correlated with the presence of MMP-2 and lymphocytes in the CSF samples (Figure 2). CSE1L modulated interferon-γ-induced MMP-2 secretion by U937 macrophages (Figure 4). These results suggest that CSE1L regulates MMP-2 secretion in the brain and may play an important role in regulating the progression of intracerebral hemorrhage brain disorders.

Because CSF is closely associated with the brain, changes in CSF protein composition may reflect the expression of a CNS disorder (Raedler & Wiedemann, 2006). A large variety of CNS disorders require CSF analyses in order to evaluate disease conditions or to rule out relevant differential diagnoses (Qureshi *et al.* 2000; Stuerenburg *et al.* 2005; Bik *et al.* 2008; Bougoin *et al.* 2008; Valis *et al.* 2008; Jiang *et al.* 2009; Starka *et al.* 2009; Talab *et al.* 2009). We have recently reported that CSE1L is a secretory protein present in the sera of cancer patients (Tung *et al.* 2009; Tsai *et al.* 2010). Our immunohistochemical studies with brain tissue sections also showed positive CSE1L staining in the extracellular areas of brain astrocytes, indicating that CSE1L could be secreted by astrocytes in the brain (Figure 1). Our present results showed that secretory CSE1L was detectable by immunoblotting in the CSF of patients with intracerebral hemorrhage brain disorders induced by stroke or neurotrauma (Figure 2). Our results also showed that U937 macrophages secreted CSE1L and that interferon-γ treatment increased CSE1L secretion by U937 macrophages (Figure 4). Microglia are brain macrophages and interferon-γ can activate microglia during brain inflammation (Gottschall & Deb 1996; Grau *et al.* 1997; Moran *et al.* 2007). Brain astrocytes and their secreted substances also participate in regulation of BBB permeability during the progression of various neurodegenerative disorders (Aschner 1998). Therefore, CSE1L in the CSF of patients with intracerebral hemorrhage brain disorders might derive from either CSE1L secreted by brain astrocytes or interferon-γ-activated microglia. Thus, analysis of CSE1L in patients' CSF samples may be useful for appraising disease progression in the CNS.

Interferon-γ has been shown to increase matrix metalloproteinase-2 secretion by rat mesangial cells (Xue *et al.* 2007). Our results showed that interferon-γ treatment increased CSE1L expression in U937 macrophages and that CSE1L modulated interferon-γ-induced MMP-2 secretion (Figure 4). In addition to MMP-2, interferon-γ-activated microglia also secrete other inflammatory cytokines and proteinases for responding the onset of inflammatory or immune-mediated brain diseases (Rock *et al.* 2005). CSE1L probably regulates cellular translocation of MMP-2-containing vesicles and thus increase MMP-2 secretion (Liao *et al.* 2008a; Tsao *et al.* 2009; Tung *et al.* 2009). CSE1L might not only mediate the cellular translocation and secretion of vesicles-containing MMP-2. Hence, it will be worthwhile to study the CSE1L-mediated inflammatory cytokines and proteinase secretion by microglia in more detail for a more understanding of microglia activation.

The normal BBB restricts the entry of therapeutic agents into the brain and limits the treatments of many severe CNS disorders. Therefore, there has been much interest in devising methods for controlling the opening or the closing of the BBB. Our results suggest that CSE1L is involved in interferon-γ-mediated MMP-2 secretion by microglia and thereby the disruption of BBB. Thus, CSE1L may be a target for regulating the progression of intracerebral brain hemorrhage disorders.

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