Cystatin C promotes cognitive dysfunction in rats with cerebral microbleeds by inhibiting the ERK/synapsin Ia/Ib pathway

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Abstract. Although higher serum level of cystatin C (CysC) was observed in patients with cerebral microbleeds, its associated role in the disease has not been elucidated. In this work, a rat model of cerebral microbleeds was created with the aim of investigating effects of CysC on cognitive function in rats with cerebral microbleeds and the underlying mechanism. Serum samples of patients with cerebral microbleeds and healthy people of the same age were collected. Levels of cystatin C expression in these samples were measured using CysC kits. Moreover, 48 spontaneously hypertensive rats (SHRs) bred under specific pathogen-free (SPF) conditions were randomly divided into 4 groups: sham surgery control group (sham), model group (CMB), model + empty vector control group (CMB + vehicle), and model + cystatin C overexpression group (CMB + CysC). Expression levels of CysC in hippocampus of rats in each group were measured by western blot analysis. The Y-maze was used to evaluate cognitive function of rats. Hippocampal long-term potentiation (LTP) in rats was assessed by the electrophysiological assay. Alterations in levels of p-ERK1/2 and p-synapsin Ia/b proteins associated with cognitive function were identified by western blot analysis. The serum levels of CysC in patients with cerebral microbleeds were significantly upregulated (P<0.001). After injection of CysC, its expression levels in rat hippocampus were significantly increased (P<0.001), which enhanced the decline in learning and memory function, as well as the decrease of LTP in the rat model of cerebral microbleeds (P<0.001). Western blot results showed that injection of CysC further reduced the levels of p-ERK1/2 and p-synapsin Ia/b in the rat model of microbleeds (P<0.001). CysC was upregulated in serum of patients with cerebral microbleeds. It promoted cognitive dysfunction in rats with microbleeds by inhibiting ERK/synapsin Ia/Ib pathway.

Introduction

Cerebral microbleeds (CMB) are subclinical lesions caused by deposition of hemosiderin and other substances in the brain parenchyma as a result of leakage of intravascular components into the surrounding space following damage of the cerebral small vessel wall (1). The prevalence rate of CMB in healthy adults is ~5% (2). CMB is rarely detected in young people (aged <40 years) (3), but the prevalence rate and case number increase with age. In a Rotterdam Scan study, the prevalence rate of CMB increased from 18% in people aged 60-69 years to 38% in people aged ≥80 years (4). CMB occurred to 34% of patients with ischemic stroke and 60% of patients with intracerebral hemorrhage (2), indicating that CMB is associated with the severity of underlying vascular diseases. Association of CMB with stroke was reported in many studies (5,6). Among patients with dementia, the prevalence rate of CMB is ~14% in patients with mild cognitive impairment and 23% in patients with Alzheimer’s disease (7,8). The increased prevalence rate of CMB in the elderly indicates its association with conditions of deep perforating vessels and cerebral amyloid angiopathy. It was reported that CMB is spatially associated with amyloid deposition areas in the brain (9,10), and thus it was speculated that CMB may pose a risk of developing dementia (4-11). Two hypotheses were proposed for possible mechanism. Firstly, CMB may have an impact on the complex cortical connections, leading to disruption of the neural network (12,13). Secondly, it may induce conditions of deep perforating vessels and cerebral amyloid angiopathy (14). As far as we know, there are few reports on specific molecular mechanisms.

Cystatin C (CysC) is a member of the type 2 cystatin superfamily of cysteine protease inhibitors. It is a small protein (13 kDa) produced and secreted by almost all karyocytes in the body (15,16). Cystatin C is a potent competitive cysteine protease inhibitor that primarily modulates extracellular protease activity. Secreted extracellular cystatin C (CysC) can also regulate target tissue homeostasis after in vivo cellular uptake (17,18). CysC is a negative regulator of angiogenesis.
and endothelial cell homeostasis both in vitro and in vivo (16). Clinically, the levels of CysC in serum of patients with CMB and patients with stroke were found significantly higher than the normal level in healthy people (19-22). Elevated expression of CysC is a common response of the body to injury. However, researchers have not yet reached a consensus about its implication and mechanism. It was reported that CysC played a neuroprotective role in preclinical disease models (23,24). It was also reported that CysC was negatively correlated with cognitive function (25,26), and expression of CysC in the elderly was higher than that in young people (27,28). As far as we know, there has been no report on the role of CysC in cognitive development in CMB patients.

In this study, the difference in expression of CysC in serum of patients with CMB and healthy subjects was confirmed. CMB model mice were treated with CysC drug, followed by investigation of the effect of CysC on the cognitive function of CMB mice and the molecular mechanism.

Materials and methods

Materials

Subjects. Serum samples of 60 patients with cerebral microbleeds, including 32 males and 28 females, were collected. Furthermore, serum samples of 60 healthy subjects of similar age were collected, including 29 males and 31 females. Fasting blood samples were drawn in the morning from all subjects after an 8-h overnight fast. All subjects signed informed consent. Patients who met the following criteria (29) were eligible for the study: i) patients whose age was ≥18 years but <65 years; ii) patients who were diagnosed with brain microbleeds (CMB) by MRI in accordance with the diagnostic criteria of CMB; and iii) patients' family agreed to participate in the study and signed an informed consent form. Patients who met the following criteria were excluded from this study: i) patients who did not take an MRI exam; ii) patients who had intracerebral hemorrhage due to abnormal structures in the brain; iii) patients who were experiencing parenchymal hemorrhage due to intracranial aneurysm rupture; iv) patients who had cerebral bleeding due to traumatic brain injury; v) patients who had circulatory system diseases; vi) patients who had moyamoya disease; vii) patients who were pre-treated with anticoagulant therapy; and viii) patients who had severe respiratory diseases, advanced cancers, severe liver and kidney dysfunction, severe heart dysfunction, hyperthyroidism or severe endocrine system diseases. The study was approved by the Ethics Committee of The Third Affiliated Hospital of Qiqihar Medical University (Qiqihar, China).

Animals. Spontaneously hypertensive rats (SHR) were purchased from Shanghai Experimental Animal Center affiliated with Chinese Academy of Sciences. The animals were given a batch number of SYXK Black 2008004 and were raised and reproduced in our institution's laboratory animal center.

Reagents. Materials and reagents used in this study were purchased from commercial sources: Human cystatin C kit (item no. ab179883) from Abcam; CysC drug from Enzo Life Sciences; RIPA lysis buffer from Beyotime Biotechnology Co., Ltd.; sucrose, glucose, KCl, NaHCO3, NaH2PO4, CaCl2 and MgCl2 from Sigma-Aldrich; Merck KGaA; rat brain stereotaxic device from Anhui Zhonghua Biological Instrument Co., Ltd.; primary antibodies to cystatin C (item no. ab109508), p-extracellular signal-regulated kinase 1/2 (ERK1/2) (item no. ab223500), synapsin I (item no. ab8), and p-synapsin-I-S549 (item no. ab19370) from Abcam; and β-actin primary antibody (item no. 66009-1-Ig) from Proteintech.

Methods

Blood sample collection and processing. Venous blood was drawn from subjects into a 15 ml tube after more than 8 h fast. Immediately after collection, the blood was centrifuged at 1,006.2 x g at room temperature (25-28°C) for 20 min. The supernatant was carefully collected, aliquoted into 200 µl, and stored at -80°C. Multiple freeze-thaw cycles were avoided. In accordance with the CysC kit manual, absorbance of the sample was measured at 450 nm using a microplate reader, and the expression level of CysC in the serum was calculated.

Animal housing and handling. Following conditions were provided in SHR housing. All the SHRs were housed in a specific pathogen-free room maintained at 18-26°C with a daily temperature difference of ≤3°C. The housing was kept at a relative humidity of 40-70%, a noise level of ≤60 dB, and a 12 h light (150-300 Lux)/12 h dark cycle. The animals were maintained with free access to food and water. All experimental procedures were consistent with experimental animal welfare and ethical principles.

The animals were randomly divided into 4 groups: sham surgery control group (sham), model group (CMB), model + empty vector control group (CMB + vehicle), and model + cystatin C overexpression group (CMB + CysC). Of each group, 12 animals were used for behavioral experiment, 6 for electrophysiological experiment, and the remaining 6 for molecular biology experiments.

Following protocol was for establishing the animal model. Forty-eight specific pathogen-free SHRs at 10 weeks of age were selected, of which 36 were randomly chosen for the rat model with brain microbleeds. A published protocol was followed (30). Rats were first restricted from food and water for 8 h prior to the experiment. The rats were anesthetized with 1% sodium pentobarbital via intraperitoneal injection at 0.6 ml/10 g. After induction of deep anesthesia, the rats were fixed on a stereotactic instrument. The skin area in the head was prepared and disinfected for surgery. Two holes were drilled 5 mm behind the anterior fontanelle, one at 2 mm left and the other at 2 mm right of the midline. To elicit microbleed formation, the rat brain was pierced perpendicularly to a depth of 4 mm from the dura mater using stainless steel needles of 474 and 159 µm in diameter, respectively, at both sides of the midline. Bone wax was used to patch the holes, and the skin over it was closed with sutures. The other 12 rats were used for sham surgery which was the same as the procedure described above except that there was no piercing of stainless-steel needles into the brain.

To elicit cystatin C overexpression, following steps were performed. The drug CysC was dissolved in physiological saline to a final concentration of 50 µg/ml (31). The lateral ventricle was injected with 10 µl of the drug solution. The same amount of saline was injected into the brain of rats in
the negative control group. The lateral ventricle injection was at 0.8 mm behind the anterior fontanelle, 1.5 mm distal to the midline, and 4.5 mm deep.

Y-maze test. Behavioral experiment was conducted 5 days after rat model creation. Y-maze test was performed using a Y-maze apparatus. The Y-maze as a labyrinth was composed of three arms of equal length, i.e. arm I, arm II and arm III, as well as their junction area. The bottom of the box was covered with an electric grid (0.2 cm in wire diameter, 14 cm in length and 1 cm in grid spacing). There were multiple buttons on the Y-maze control panel such as I, II, III and 0, as well as a voltage adjusting knob and a time control knob. When button I, II or III was pressed, a signal light corresponding to that arm became on, indicating the arm was not energized and therefore was a safe zone, while the other two arms and the junction area were dark and energized and therefore were non-safe zones. When the button 0 was pressed, the junction area was energized while the three arms were not. Five seconds after the signal light was on, electricity (60 v) was on in the grid. Each rat was tested 20 times a day for 2 days. Day 1 test was for study, and day 2 test was for memory retention. Safe zones were changed in a random and alternating way. In the beginning of the test, two arms including the one where the rat hid were energized. The animal would escape to the light area after receiving an electric shock. A test was completed when the rat stayed in the safe zone for 30 sec. Then next test started from where the rat was. It would be a correct reaction if the rat fled directly to the safe zone after an electric shock or ran to the safe zone within 10 sec after the shock. It would be an incorrect reaction if the rat escaped to any other arm without lights.

Testing indicators are listed below: i) Error number (EN) was defined as the number of incorrect reactions in all reactions. ii) Total reaction time (TRT) was defined as sum of the time required to complete correct reactions and incorrect reactions. Reaction time referred to the time taken from when the signal light was on to when the rat escaped to the safe zone. iii) Active avoidance rate (AAR) was defined as the percentage of times a rat completed its escape reaction within 5 sec after the light was on but the arm was not energized.

Electrophysiology
Preparation of acute hippocampal slices. Artificial cerebrospinal fluid (ACSF) for anatomical use was made containing 210 mM sucrose, 12 mM glucose, 2 mM KCl, 24 mM NaHCO3, 1 mM NaH2PO4, 0.5 mM CaCl2 and 7 mM MgCl2. A mixed gas of 5% CO2/95% O2 was bubbled through the ACSF for at least half an hour at an osmotic pressure of 310-320 Osm, followed by pH adjustment to 7.4. The well-cut brain slices were incubated in the ACSF for recording use at room temperature for at least one hour to restore brain slice activity. A slice was selected, and after it was placed on the MED64 probe, the slice was infused continuously. At this stage it was ready for recording. A stimulation site in the hippocampal CA3 region was selected. All the sites in the CA1 region were for recording. An input-output (I-O) curve was constructed. The current corresponding to 30-50% of the maximum excitatory post-synaptic potential amplitude was chosen as the subsequent stimulation intensity. High-frequency stimulation was delivered to the brain slice after baseline recording. The change in amplitude was recorded during the 60 min period after stimulation.

Western blot analysis. Cells were lysed with ultrasound in RIPA lysis buffer. Protein concentration was measured using the BCA kit. Samples were subjected to electrophoresis, membrane transfer, blocking, incubation with antibodies and image development. Incubation with primary antibodies, i.e. anti-cystatin C (dilution factor, 1:20,000), p-ERK1/2 (dilution factor, 1:400), synapsin I (dilution factor, 1:1,000), and β-actin (dilution factor, 1:10,000), was maintained at 4˚C overnight. After washing, the membrane was incubated with corresponding secondary antibody (dilution factor, 1:4,000), followed by exposure and image development. Quantity One software was used to analyze the image in Grayscale format.

Statistical analysis. Experimental data were expressed as mean ± standard deviation (mean ± SEM). Statistical analysis was performed using SPSS 16.6 statistical software. The results were analyzed using one-way ANOVA. A Bonferroni test was used for comparison between groups. A difference was statistically significant at P<0.05, P<0.01 and P<0.001. GraphPad Prism 5 software was used for graph drawing.

Results
Measurement of CysC level in serum of patients with cerebral microbleeds (CMB). CysC expression levels in serum of clinically healthy subjects and patients with cerebral microbleeds were measured. The results in Fig. 1 show that compared with the control group, expression level of CysC in serum of patients with cerebral microbleeds increased significantly. The difference was statistically significant.

CysC expression in rat hippocampus. Expression levels of CysC in hippocampus of rats in each group were measured.
As shown in Fig. 2, expression level of CysC in the CMB model group increased slightly after model creation. When rats in the CMB model group were administered with CysC, the expression level of CysC in hippocampus increased significantly.

Cognitive function evaluation of rats in each group. Cognitive functions, including learning and memory, were evaluated in each group using the Y-maze test. Results showed that compared with the sham group, the number of incorrect reactions (Fig. 3A and B) and the total reaction time (Fig. 3C and D) increased, while the active avoidance rate (Fig. 3E and F) decreased in learning and memory test in the CMB model group, indicating impairment of rat learning and memory function. After administration of drug CysC, the number of incorrect reactions and the total reaction time (Fig. 3A-3D) further increased, while the active avoidance rate (Fig. 3E and F) further decreased, indicating that CysC aggravated impairment of learning and memory function in the model rats.

Recording rat hippocampal LTP. LTP is widely considered one of the major cellular mechanisms that underlies learning and memory. In this study, changes in LTP were recorded in each group using slice electrophysiological techniques. As shown in Fig. 4, compared with the sham group, LTP of the CMB model group was significantly reduced. Compared with the CMB model group, LTP of the cystatin C overexpression group showed further reduction as well.

ERK/synapsin Ia/b pathway expression. Western blot analysis was performed to observe changes in expression of p-ERK and p-synapsin Ia/b (Ser549) which are associated with cognitive functions. As shown in Fig. 5, compared with the sham group, expression levels of p-ERK and p-synapsin Ia/b (Ser549) were significantly reduced in the CMB model group. Compared with the CMB model group, CysC overexpression led to a further reduction in expression levels of p-ERK and p-synapsin Ia/b (Ser549).

Discussion

In clinic, expression level of cystatin C (CysC) was found significantly higher in serum of patients with cerebral microbleeds (CMB) than in healthy subjects (Fig. 1). In this study, a rat CMB model was created, and was injected with CysC in the hippocampus aimed at studying the impact of CysC on rat cognitive functions (Fig. 2). In behavioral study, cognitive dysfunction was observed in the CMB model rats. After administration of CysC, the learning and memory functions were further deteriorated in the rats (Fig. 3). In electrophysiological study, a decrease in hippocampal LTP was found in the CMB model rats. The hippocampal LTP was further decreased after administration of CysC in the model rats (Fig. 4). Among biomarkers associated with learning and memory functions, it was found that the expression levels of phosphorylated ERK1/2 and phosphorylated synapsin Ia/b (Ser549) were reduced in hippocampus of the CMB model rats. After administration of CysC, these levels were further reduced (Fig. 5). These findings suggested that overexpression of CysC can promote cognitive dysfunction in rats with cerebral microbleeds by inhibiting the ERK/synapsin Ia/b pathway.

CMB is the main cause of mild vascular cognitive impairment and vascular dementia (33). The underlying mechanism may be that CMB affects specific cognitive domain functions such as executive functions and attention, and CMB may also damage the cerebral white matter association fiber bundles, cholinergic fiber bundles, and frontal subcortical circuitry (34). In addition, CMB is often associated with neurodegenerative diseases such as Alzheimer's disease, aggravating cortical atrophy and increasing the risk of cognitive impairment (35). The present study demonstrated learning and memory dysfunction in CMB model rats through Y-maze learning and memory tests, indicating association of CMB with rat cognitive functions. Our findings were consistent with related literature reports. For example, CMB was reported to cause reduced cognitive ability and increased risk of dementia (36,37). CMB may play a role in development of cerebrovascular diseases and neurodegenerative diseases. Its detection rate in Alzheimer's disease is twice that in the same age control group (38). In electrophysiology, LTP is a synaptic plasticity mechanism underlying learning and memory (39-40). In this study, LTP in the CA1 region of rat hippocampus was recorded using slice electrophysiological techniques. It was found that CMB caused a decrease in LTP, which was consistent with the behavioral test results.

CysC is an important endogenous inhibitor of cysteine protease activity (41). Its function is still unclear in the brain, but it is associated with neuronal degeneration and nervous system repair. CysC is highly expressed in patients with epilepsy and neurodegenerative diseases. According to literature, high expression was also found in facial nerve transection, perforation pathway transection, pituitary resection, and animal models of transient cerebral ischemia and epilepsy (42). There is an opinion that high CysC expression in injury or disease may imply an intrinsic neuroprotection that counteracts disease progression. It was also reported that CysC exerts a protective effect when neurons are under attack by inducing autophagy (31,43). Several studies have demonstrated that upregulated expression of CysC is
Figure 2. Expression of CysC in hippocampus of rats in each group: (A) Western blot image of CysC expression in rat hippocampus. (B) Bar charts indicating CysC expression in hippocampus of rats in each group. *P<0.05, ***P<0.001 vs. the sham group; ###P<0.001 vs. the CMB group; n=6 per group. CysC, cystatin C; CMB, cerebral microbleeds.

Figure 3. Y-maze test: (A) Number of incorrect reactions in the learning test. (B) Number of incorrect reactions in the memory test. (C) Total reaction time in the learning test. (D) Total reaction time in the memory test. (E) Active avoidance rate in the learning test. (F) Active avoidance rate in the memory test. *P<0.05, ***P<0.001 vs. the sham group; ##P<0.01, ###P<0.001 vs. the CMB group; n=6 per group. CMB, cerebral microbleeds.
positively correlated with cerebrovascular diseases (44,45). In this study, overexpression of CysC in rat serum and hippocampus was achieved by injection of external CysC aimed at studying its impact on cognitive functions in...
CysC further decreased the level of p-synapsin Ia/b (Ser549), indicating that synapsin I was involved in the promoting process of cognitive dysfunction in CMB rats due to CysC overexpression.

Although this work demonstrated the promoting effect of CysC on cognitive impairment and the involvement of the ERK-synapsin I signal pathway, it does not rule out the possibility of CysC having other mechanisms of promoting cognitive dysfunction in the CMB model rats. Thus, further work is still necessary for clarification.

In conclusion, this study preliminarily demonstrated that CysC overexpression can promote cognitive dysfunction in rats with cerebral microbleeds by inhibiting the ERK/synapsinIa/b pathway. Based on these findings, it is postulated that reducing the expression level of CysC in serum of CMB patients may help alleviate the symptoms and slow down disease progression of cognitive impairment.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

GY wrote the manuscript. XS and LL conceived and designed the study. LH and HL were responsible for the collection and analysis of the experimental data. SW interpreted the data and drafted the manuscript. ZR and YZ revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ethics Committee of The Third Affiliated Hospital of Qiqihar Medical University (Qiqihar, China). All subjects signed informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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