Soluble and Catalytically Active Endothelin Converting Enzyme-1 is Present in Cerebrospinal Fluid of Subarachnoid Hemorrhage Patients

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Endothelin converting Enzyme-1 (ECE-1) is essential for the production of Endothelin-1 (ET-1), which is associated with vasospasm following subarachnoid hemorrhage (SAH). We have previously demonstrated the presence of a catalytically active soluble form of ECE-1 in the media of endothelial cells. We aimed to determine if this form of ECE-1 exists in vivo, in cerebrospinal fluid (CSF) of SAH patients. We examined CSF taken from SAH subjects for the presence of soluble ECE-1 using a bradykinin based quenched fluorescent substrate assay. We obtained further confirmation by characterizing the CSF mediated cleavage products of BigET-1 and BigET18–34 (6 µg/ml) using mass spectrometry. The specificity of cleavage was confirmed using the ECE-1 inhibitor CGS35066 5nmol/L. SAH CSF samples had mean ECE-1 activity of 0.127 ± 0.037 μmols of substrate cleaved/μL of CSF/24 h. The C-terminal peptides generated upon the cleavage of BigET-1 and BigET18–34 were detected 48 h after incubation of these substrates with CSF. Cleavage of these substrates was inhibited by CGS35066. Results of Western blots also produced strong evidence for the presence of truncated soluble ECE-1 in CSF. These results strongly suggest the presence of a truncated but catalytically active form of ECE-1 in the CSF of SAH subjects. Further studies are necessary to determine the biological significance of soluble ECE-1 in CSF of SAH subjects, including an association with vasospasm after SAH. Molecular & Cellular Proteomics 13: 10.1074/mcp.M113.027359, 1091–1094, 2014.

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1 The abbreviations used are: ET-1, endothelin-1; SAH, Subarachnoid hemorrhage; CSF, Cerebrospinal fluid; QFS, Quenched Fluorescent Substrate; EVD, External Ventricular Drain; MALDI, Matrix Assisted Laser Desorption Ionization; BigET-1, Big Endothelin-1; ECE-1, Endothelin Converting Enzyme-1; MCA, Methoxycoumarin.
were collected from EVD that terminated in the cerebral ventricular system. Comparison CSF samples were obtained from non-SAH subjects undergoing lumbar-drain trial for normal-pressure hydrocephalus (NPH) treatment. CSF samples from NPH subjects were collected through lumbar drains placed for clinical necessity and a small aliquot of the removed CSF was used in our study. The procedure for CSF sample collection did not impact patient care. All samples were coded and de-identified by removing all subject contact information and study data. Identifiable information was stored in secure offices and password protected computer systems accessible only to study personnel. Enzyme assays were conducted at the Department of Biochemistry & Molecular Biology (Monash University), under an amendment to the Institutional Review Board approval and a human ethics exemption granted by Partners Healthcare and Monash University Human Ethics Committee respectively.

Fluorescence Based ECE-1 Assay—ECE-1 activity measurement was based on the ability of CSF or recombinant human (rh) ECE-1 (4 ng/µl; R & D systems) to cleave a bradykinin based quenched fluorescent substrate (7-methoxycoumarin-4-yl)acetyl-Arg-Pro-Pro-Gly-Phe-Ser-Ala-Phe-Lys(2,4-dinitrophenyl), (40 µmol/L). Fluorescence was monitored over 24 h at the excitation and emission wavelengths of 320 and 405 respectively at 37 °C. The ECE-1 activity in CSF or rhECE was expressed as µmols of substrate hydrolyzed/µl of CSF or per ng of enzyme respectively over 24 h. Specific activity was calculated from a standard curve of known 7-methoxycoumarin (MCA) concentrations. The specificity of cleavage was confirmed by using the ECE-1 inhibitor CGS35066 (500 nM). The assay was conducted in ECE-1 buffer containing 150 mM NaCl, 50 mM Tris-CL; pH 6.3.

Western Blotting—CSF containing 40 µg of total protein was resolved on a 12% SDS-PAGE. ECE-1 was detected using anti-human ECE-1 monoclonal antibodies (1:1000; R & D Systems, Minneapolis, MN) followed by appropriate secondary antibodies and enhanced chemiluminescence reagent.

BigET-1 and BigET 18–34 Cleavage Assays—Cleavage of BigET-1 and BigET18–34 (a truncated form of BigET-1, MW1907 Da; DIIWNT-PEHVPYGLG) by CSF was assessed using MALDI-ToF mass spectrometry as described previously (2). But briefly, CSF containing 50 µg of total protein was mixed with substrate (6 µg/ml), and aliquots taken at time = 0 and 48 h were subjected to analysis by MALDI-TOF mass spectrometry after removing protein precipitates. The specificity of cleavage was confirmed by using CGS35066 (500 nM). Samples giving the highest ECE-1 activity based on the QFS assay above were chosen for these BigET experiments.

MALDI-TOF Mass Spectrometry—Samples were co-spotted onto the MALDI target plate with Matrix solution of 10 mg/ml a-cyano-4-hydroxy-cinnamic acid (Laser BioLabs, Sophia-Antipolis, France) in 50% Acetonitrile 0.1% TFA. The samples were analyzed on an Applied Biosystems (Foster City, CA, USA) 4700 Proteomics Analyzer MALDI TOF/TOF in reflectron mode with a mass range of 800 to 2500 Da, with the relevant focus masses at 1500 shots per spectra. The spectra were calibrated by the default calibration, which was updated by the plate model method immediately prior to sample acquisition. The spectra were processed with peak detection of 20,000 resolution at 1500 Da, minimum signal to noise threshold of 5 and monoisotopic peak de-isotoping.

RESULTS

Fluorescence Based ECE-1 Assay—An increase in fluorescence as a result of QFS cleavage provides strong evidence of ECE-1 enzyme activity in all of the SAH CSF samples (n = 6). QFS cleavage was sensitive to the ECE-1 inhibitor CGS35066. The mean ECE-1 activity in CSF of SAH subjects was 0.127 ± 0.037 and ranged from a minimum of 0.005 to a maximum of 0.248 µmols of substrate cleaved/µl of CSF (n = 6; Fig 1). The mean ECE-1 activity in CSF of comparison NPH patients was 0.135 ± 0.052, and ranged from a minimum of 0.028 to a maximum of 0.320 µmols of substrate cleaved/µl of CSF (n = 5; Fig 1). The activity of recombinant human ECE-1 was 0.139 ± 0.021 µmols of substrate cleaved/ng of enzyme.

Cleavage of BigET18–34 and BigET by CSF—BigET18–34 was incubated with CSF taken 2 days after SAH. The contents of the reaction mixture were analyzed by MALDI-ToF mass spectrometry at time = 0 h (Fig 2A) and time = 48 h (Fig 2A). At time = 0 h, the reaction mixture only contained BigET18–34 (mass/charge ratio of 1908, Fig 2A), whereas the C-terminal cleavage product BigET22–38 was detected at time = 48 h (mass/charge ratio of 1380, Fig 2B). The cleavage product was not detected at time = 48 h in the presence of CGS35066 (500 nM; Fig 2C).

The natural substrate BigET-1 (mass/charge ratio 4312) was only detected at time = 0 h. The C-terminal cleavage fragment BigET22–38 (mass/charge ratio 1808; Fig 3A) was detected after 48 h of incubation. Although this product was detected in the presence of CGS35066 (500 nM; Fig 3B), its intensity was reduced, suggesting diminished ECE-1 activity in CSF.

Detection of ECE-1 through Western Blotting—The use of antibodies directed against the C terminus of ECE-1 produced two immuno-reactive bands corresponding to 80 and 55 KDa (Fig 4). The 80 KDa band corresponds to the size of soluble ECE-1 we had previously detected in the media of endothelial cells (5). The 55 KDa band corresponds to what is most likely to
be products of proteolytic degradation of ECE-1. Full-length ECE-1 was not detected in CSF taken from SAH subjects.

**DISCUSSION**

We have detected a truncated but catalytically active form of ECE-1 in human CSF within 48 h of SAH. To the best of our knowledge, this is the first report of a catalytically active form of ECE-1 in CSF taken from SAH subjects. Catalytically active ECE-1 in CSF can convert BigET-1 to the potent vasoconstrictor ET-1. Previous studies have demonstrated the presence of both BigET-1 and ET-1 in CSF of SAH subjects (6), and an association between ET-1 and cerebral vasospasm has previously been shown (7). In this light, our discovery of soluble ECE-1 in CSF of SAH subjects indicates that the production of ET-1 can in fact occur in the CSF space. This discovery suggests that ECE-1 may be a significant source of CSF ET-1 and therefore a potential novel therapeutic target in SAH.

ECE-1 activity in CSF was measured using a QFS based assay described previously (2). ECE-1 activity was demonstrated by the increase in fluorescence over time and the specificity of cleavage was confirmed using the ECE-1 inhibitor CGS35066. Comparison of the average activity in 1 ng of ECE-1 (0.139 ± 0.021 μmols of substrate cleaved/μl CSF) with that in the CSF of SAH subjects (0.121 ± 0.012 μmols of substrate cleaved/μl) and in the CSF of NPH subjects (0.135 ± 0.052 μmols of substrate cleaved/μl) suggests that ECE-1 is present in CSF of SAH and NPH subjects at the high picogram to low nanogram level.
Further clarification for the presence of ECE-1 in CSF was obtained by analyzing the products of BigET18–34 and BigET-1 cleavage by CFSE. Our results indicate that both substrates were cleaved between Trp21 and Val22, as evidenced by MALDI analysis, which indicated the presence of C-terminal fragments BigET22–34 and BigET22–38. This cleavage was sensitive to the ECE-1 preferring inhibitor CGS35066. BigET18–34 is a truncated version of the physiological substrate BigET-1 and is known to undergo 60% cleavage by ECE-1 (8). Previously we have used this substrate to monitor ECE-1 activity in cell membrane preparations and culture media (9). The N-terminal fragment resulting from the cleavage of both BigET-1 and BigET18–34 could not be detected by MALDI ToF mass spectrometry. The lack of observation of this fragment has been reported by us in the past (2) and we believe reflect the poor ionization potential of these peptides during MALDI analysis.

Western blotting studies conducted using antibodies directed against the C-terminal domain of ECE-1 produced an immunoreactive band with a molecular mass of 80 kDa. This corresponds to the size of soluble ECE-1 produced via a proteolytic cleavage event, and previously discovered in the media of endothelial cells (5). The results of Western blotting showed no evidence for the presence of full-length ECE-1 (120 kDa) in CSF of SAH subjects. These results therefore suggest that ECE-1 found in CSF of SAH subjects, is also likely to be the result of a proteolytic cleavage event similar to that previously found in the media of endothelial cells (2).

We found comparable CSF ECE-1 catalytic activity levels in SAH patients with hydrocephalus and non-SAH patients with NPH. We do not have data on CSF ECE-1 activity in normal control subjects without known central nervous system pathology because ethical considerations prohibit CSF sampling, which requires an invasive procedure, in normal human subjects. Present and comparable CSF ECE-1 catalytic activities in both SAH and non-SAH patients with hydrocephalus raise the possibility that presence of CSF ECE-1 catalytic activity may be associated with hydrocephalus. Furthermore, this pilot study was designed to detect novel enzyme activity in a small cohort and was not powered to detect between-group differences in different patient populations. Future study with much larger sample size is necessary to determine if CSF ECE-1 catalytic activity is different between SAH and non-SAH subjects. Finally, SAH has a dynamic disease course over time and it is possible that CSF ECE-1 catalytic activity may also vary over time in this condition. We had examined CSF ECE-1 activity in an early time point (post-bleed day 2) in SAH, and it is conceivable that CSF ECE-1 activity does not show significant difference from non-SAH subjects until later in SAH course. Future study with repeated CSF ECE-1 measurements over time is necessary to answer this question.

Although clinical samples can be confounded by sample collection related protein degradation, we have minimized this effect by following a strict protocol where samples are immediately centrifuged and deep frozen upon collection. However, there are some potential limitations to this study. Discovery of ECE-1 in CSF does not exclude the possibility that the production of ET-1 in CSF was mediated by ECE-1 expressed on the surface of endothelial cells lining the CSF space. Furthermore, this study does not identify the specific ECE-1 isoform in CSF space, as human endothelial cells are known to express both ECE-1 and ECE-2 (10). This study also only used a small number of clinical samples to assess catalytically active ECE-1 levels, and as such, the data are not sufficiently extensive to determine the potential value of ECE-1 measurement as a potential biomarker. However, to the best of our knowledge, this study is the first to report the presence of a soluble form of ECE-1 in any human biological fluid. Although the presence of ECE-1 does not imply physiologic significance, the finding of this novel and catalytically active form in human CSF is highly suggestive of potential biological relevance and encourages future investigation of ECE-1 in SAH and other vascular disease states.

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