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Research article

Brain pericytes are the most thrombin-sensitive matrix metalloproteinase-9-releasing cell type constituting the blood–brain barrier in vitro

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HIGHLIGHTS

- Thrombin induced MMP-9 release from brain pericytes.
- This MMP-9 release from pericytes was higher than that from other BBB cells.
- Pericytes expressed high and moderate levels of PAR1 and PAR4 mRNA, respectively.
- PAR1 inhibitor blocked the thrombin-induced MMP-9 release from pericytes.
- Thrombin-PAR1/PAR4 axis in pericytes may mediate ICH-associated BBB disruption.

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ABSTRACT

In the acute phase of intracerebral hemorrhage (ICH), hemorrhagic transformation and brain edema are associated with blood–brain barrier (BBB) disruption. Elevated levels of thrombin, a coagulation factor, contribute to the development of brain edema during ICH through matrix metalloproteinase (MMP)-9 production. Thrombin directly induces a variety of cellular responses through its specific receptors known as protease-activated receptors (PARs). However, it remains unclear which cell types constituting the BBB mainly produce MMP-9 in response to thrombin. Here, we compared the MMP-9 release induced by thrombin using primary cultures of rat brain microvascular endothelial cells, astrocytes, and pericytes. Brain pericytes exhibited the highest levels of MMP-9 release due to thrombin stimulation among the BBB cells. The pattern of PAR mRNA expression in pericytes was characterized by high expression of PAR1 and moderate expression of PAR4. Heat-inactivated thrombin failed to stimulate pericytes to release MMP-9. A selective PAR1 inhibitor SCH79797 blocked the thrombin-induced MMP-9 release from pericytes. These findings suggest that both PAR1 and PAR4 mediate thrombin-induced MMP-9 release from pericytes. The present study raises the possibility that brain pericytes could play a pivotal role as a highly thrombin-sensitive and MMP-9-producing cell type at the BBB in brain damage including ICH.

1. Introduction

Intracerebral hemorrhage (ICH) occurs spontaneously in strokes or complications after severe ischemic stroke and shows high morbidity and mortality in about 15% of stroke patients [1]. ICH outcome depends on hemorrhagic transformation and brain edema during acute phase [2]. These events are thought to occur due to the blood–brain barrier (BBB) disruption [3].

Thrombin, which is increased in the brain during ICH, is an essential component in the coagulation cascade producing fibrin clots [4]. This increased thrombin is associated with the development of acute cerebral edema following ICH [4–6]. The injection of thrombin into the basal ganglia produced BBB breakdown, leading to brain edema formation [7]. Lowered activity of thrombin reduced the ICH-induced brain edema and neuronal cell death [8,9]. In addition to a role in the coagulation process, thrombin acts as a serine protease to produce a variety of

Abbreviations: ICH, intracerebral hemorrhage; MMP-9, matrix metalloproteinase-9; BMECs, brain microvascular endothelial cells; RBECs, rat brain endothelial cells.

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cellular responses through its specific receptors known as protease-activated receptors (PARs). PAR1, PAR3 and PAR4 are activated by thrombin, but not PAR2. The latter is activated by trypsin and mast cell tryptase [10]. PARs are widely expressed in various cell types in the brain, including microglia, astrocytes, neurons, and oligodendrocytes [11]. PARs mediate the development of acute cerebral edema following ICH or ischemic stroke. This acute edema is attenuated by the administration of PAR1 inhibitor [12] and by the deficiency of PAR1 or PAR4 [13,14]. Therefore, thrombin likely induces brain edema through an activation of PARs.

Thrombin injection into the brain parenchyma induces matrix metalloproteinase (MMP)-9 expression in rats [15]. MMPs, a family of zinc-dependent endopeptidases, degrade the components of the extracellular matrix under physiological and pathological conditions [16,17]. After ICH, MMP-9 becomes upregulated and degrades BBB-maintaining substrates, leading to the sequential events of edema, inflammatory cell infiltration, and parenchymal damage in the brain of ICH model mice [18,19]. These neurologic deficits and infiltration of leukocytes can be attenuated by an MMP-9 inhibitor [18]. MMP-9 acts synergistically with thrombin to exacerbate ICH injury [19]. Brain microvascular endothelial cells (BMECs) and astrocytes release MMP-9 due to thrombin stimulation [20,21]. Wang et al. elucidated the localization of active MMP-9 in the various types of cells including neurons, astrocytes, neutrophils, microglia/macrophages, and endothelial cells [18]. MMP-9 was detected in a subpopulation of neurons but not in other cells. Most of the MMP-9 localization was observed along brain capillaries. We previously reported that brain pericytes, which cover capillaries and share a common basement membrane with BMECs, are highly sensitive to TNF-a in releasing MMP-9 among the BBB cells: BMECs, astrocytes, and brain pericytes [17]. Therefore, in the present study, we examined whether thrombin induces MMP-9 release from pericytes and compared the sensitivity to thrombin for MMP-9 release among the BBB cells.

2. Methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM) was purchased from Wako (Osaka, Japan). DMEM/Ham's nutrient mixture F-12 medium (DMEM/F12) and thrombin from bovine plasma were purchased from Sigma (St. Louis, MO, USA). Fetal bovine serum (FBS) and bovine plasma-derived serum (PDS) were purchased from Biowest (Nuaillé, France) and Animal Technologies Inc. (Tyler, TX, USA), respectively. SCH79797 (PAR1 inhibitor) was purchased from Tocris Bioscience (Bristol, UK).

2.2. Cell culture

All procedures involving experimental animals adhered to the Law (No. 105) and Notification (No. 6) of the Japanese Government, and were approved by the Laboratory Animal Care and Use Committee of Fukuoka University.

Primary cultures of rat brain microvascular endothelial cells (RBECs) and brain pericytes were prepared from 3-week-old Wistar rats, as previously described [22–24]. In brief, the meninges were carefully removed from the forebrain and gray matter was minced into small pieces in ice-cold DMEM, then digested in DMEM containing collagenase type 2 (1 mg/mL; Worthington, Lakewood, NJ, USA), DNase I (15 μg/mL; Sigma), and gentamicin (50 μg/mL; Sigma) for 1.5 h at 37 °C. The cell pellet was separated by centrifugation in 20% bovine serum albumin (BSA; Sigma)-DMEM (1000 × *g*, 20 min). The microvessels obtained in the pellet were further digested with collagenase/dispase (1 mg/mL; Roche, Basel, Switzerland) and DNase I (6.7 μg/mL) in DMEM for 1 h at 37 °C. Microvessel endothelial cell clusters were separated on a 33% continuous Percoll (GE Healthcare, Buckinghamshire, UK) gradient (1000 × *g*, 10 min), collected, and washed in DMEM before plating on culture dishes coated with collagen type IV and fibronectin (both 0.1 mg/mL; Sigma). RBEC cultures were maintained in DMEM/F12 supplemented with 10% bovine PDS, basic fibroblast growth factor (1.5 ng/mL; Roche), heparin (100 μg/mL; Sigma), insulin (5 μg/mL), transferrin (5 μg/mL), sodium selenite (5 ng/mL) (insulin–transferrin–sodium selenite media supplement; Sigma), gentamicin (50 μg/mL), and puromycin (4 μg/mL; Sigma) (RBEC medium I) at 37 °C in a humidified atmosphere of 5% CO_2/95% air, for 2 days. On the third day, the cells received a new medium that contained all the components of RBEC medium I except for puromycin (RBEC medium II). When the cultures reached confluency, the purified endothelial cells were passaged and used for experiments.

Brain pericytes were obtained by a prolonged culture of isolated brain microvessel fragments under selective culture conditions [22,25,26]. Briefly, the obtained brain microvessel fragments were placed in an uncoated culture flask in DMEM supplemented with 20% FBS (FBS–DMEM), 100 U/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesque, Kyoto, Japan). After 7 days in culture, rat brain pericytes overgrew brain endothelial cells and typically reached 80–90% confluency. The cells were used for experiments at passage 2.

Primary astrocytes were prepared from the cerebral cortex of 1- to 3-day-old Wistar rats according to the method of McCarthy and de Vellis [27] with a slight modification. Briefly, after removing the meninges and blood vessels, the forebrains were minced and gently dissociated by repeated pipetting in DMEM containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, and filtered through a 70-μm cell strainer. Cells were collected by centrifugation (800 × *g*, 6 min), resuspended in 10% FBS DMEM, and cultured in 75-cm^2 flasks (BD Biosciences, Franklin Lakes, NJ, USA) in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Cells were fed every 2–3 days by changing the medium. After 10–14 days in culture, floating cells and weakly attached cells of the mixed primary cultured cell layer were removed by vigorous shaking of the flask. Then, astrocytes at the bottom of the culture flask were trypsinized and seeded into new culture flasks. The primary cultured astrocytes were maintained in 10% FBS/DMEM. They were grown in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Cells at the second or third passage were used for experiments.

Astrocytes, pericytes, and RBECs in primary culture were identified by immunostaining with antibodies against glial fibrillary acidic protein, platelet endothelial cell adhesion molecule-1, and α-smooth muscle actin, respectively. Almost all cells in each cell culture showed positive immunoreactivities for each corresponding marker protein (data not shown).

2.3. Quantitative Real-time RT-PCR analysis

Total RNA of RBECs, astrocytes, and brain pericytes were extracted using Sepasol (Nacalai Tesque) according to the manufacture's protocol. qRT-PCR was performed in a one-step reaction using Brilliant III Ultra-Fast SYBR Green QRT-PCR Master Mix (Agilent Technologies, Santa Clara, CA, USA) supplemented with 100 ng of isolated RNA as the template and 250 nM primers on a MX3000P real-time PCR system (Agilent Technologies). The cDNAs were synthesized at 50 °C for 10 min and subsequently denatured at 95 °C for 3 min. Then, the cDNAs were amplified through 40 cycles (denaturation, 95 °C and 20 s; annealing/extension, 60 °C and 20 s). Primers for qRT-PCR were purchased from Takara perfect real-time support system (Shiga, Japan). All mRNA measurements were normalized to the internal control gene, GAPDH. Rat brain cerebral cortex total RNA