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Autocrine and paracrine up-regulation of blood–brain barrier function by plasminogen activator inhibitor-1

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ABSTRACT

The blood–brain barrier (BBB) is the interface that separates the central nervous system (CNS) from the peripheral circulation. An increase in blood-borne substances including cytokines in plasma and brain affects BBB function, and this is associated with the development of pathogenesis of a number of diseases. Plasminogen activator inhibitor (PAI)-1 regulates the plasminogen activator/plasmin system as a serpin in the periphery and the CNS. We investigated whether PAI-1 alters BBB function using in vitro models of the BBB consisting of rat primary brain endothelial cells (RBECs) alone and co-cultured with pericytes. We found that PAI-1 increased the tightness of the brain endothelial barrier in a time- and dose-dependent manner, as shown by an increase in the transendothelial electrical resistance (TEER) and a decrease in the permeability to sodium fluorescein (Na-F). RBECs responded equally to PAI-1 in the blood-facing and brain-facing sides of the brain, leading to a decrease in Na-F permeability. In addition, RBECs constitutively released PAI-1 into the blood-facing (luminal) and brain-facing (abluminal) sides. This release was polarized in favor of the luminal side and facilitated by serum. The neutralization of PAI-1 by an antibody to PAI-1 in RBEC/pericyte co-culture more robustly reduced TEER of RBECs than in RBEC monolayers. These findings suggest that PAI-1 derived from the neurovascular unit and peripheral vascular system participates as a positive regulator of the BBB in facilitating the barrier function of the endothelial tight junctions.

Introduction

The blood–brain barrier (BBB), which is composed of brain endothelial cells, pericytes, and astrocytes, regulates plasma components from the cerebral microcirculation entering the central nervous system (CNS). The main machinery underlying barrier function is composed of tight junctions formed between brain endothelial cells and various efflux transporters. All structural components of the BBB, neurons, and non-neuronal cells (e.g., microglia) form a neurovascular unit to contribute to dynamic and continuous regulation of cerebral microvascular permeability, synaptic transmission, and neurogenesis (Hawkins and Davis, 2005; Zlokovic, 2008). Pericytes and astrocytes are required to induce and maintain barrier properties by secreting various soluble factors including neurotrophic factors (Abbott et al., 2006; Zlokovic, 2008). Moreover, brain endothelial cells, which act as an interface between blood and brain parenchyma, can directly communicate with blood-borne substances that lead to the alteration of BBB function.

Plasminogen activator inhibitor-1 (PAI-1) is a member of the serine protease inhibitor (serpin) superfamily and the primary inhibitor of tissue- and urokinase-type plasminogen activator (tPA and uPA, respectively). The serine protease proteolytically converts plasminogen into active plasmin in the blood. PAI-1 regulates both tPA and uPA activity, indicating that PAI-1 plays a role in tissue remodeling and fibrinolysis in the periphery. Endothelial cells and smooth muscle cells are a major source of PAI-1. Regulation of PAI-1 production is inducible rather than constitutive (Juhan-Vague et al., 2003). The proinflammatory mediators, tumor necrosis factor (TNF)-α and interleukin (IL)-1, are inducers of PAI-1 and are released from adipose tissue (Juhan-Vague et al., 2003). Increased PAI-1 causes impaired fibrinolysis and enhanced thrombosis leading to microvascular thrombosis and the development of atherosclerotic lesions (Sobel, 1999). Clinical studies have suggested that PAI-1 is associated with metabolic syndrome (Ingelsson et al., 2007) and vascular diseases in diabetes (Agirbasli, 2005).

While tPA is widely expressed in the CNS and plays a role in synaptic remodeling and neuronal plasticity (Adibhatla and Hatcher, 2008), exogenous tPA facilitates excitotoxic neuronal death (Lo et al., 2004) and disruption of the BBB (Yepes et al., 2003). PAI-1 prevents this tPA-induced neuronal degeneration and BBB disruption (Nagai et al., 2005; Abu Fanne et al., 2010). In contrast, elevated levels of PAI-1 in the

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brain are known to be associated with the pathogenesis of Alzheimer's disease (Jacobsen et al., 2008; Zlokovic, 2008). PAI-1 derived from astrocytes, a major source of PAI-1 in the brain (Buisson et al., 1998), protects against neuronal apoptotic death (Soeda et al., 2004). Besides astrocytes, brain endothelial cells and pericytes express PAI-1 mRNA (Kose et al., 2007). Thus, PAI-1 in the brain is highly likely to act as a mediator for cross-talk among the elements of the neurovascular unit to regulate endothelial barrier function. However, the role of PAI-1 in BBB function is incompletely understood.

In the present study, we demonstrated that PA-1 released from brain endothelial cells and pericytes increases the tightness of the brain endothelial barrier.

Materials and methods

Isolation of rat brain microvascular endothelial cells (RBECs) and pericytes

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 RBECs and pericytes were prepared from 3-week-old Wistar rats, as previously described (Dohgu et al., 2005; Takata et al., 2008; Sumi et al., 2010). In brief, the meninges were carefully removed from the forebrain and gray matter was minced into small pieces in ice-cold Dulbecco's modified Eagles medium (DMEM; Wako, Osaka, Japan), and then digested in DMEM containing collagenase type 2 (1 mg/ml; Worthington, Lakewood, NJ), DNase I (15 μg/ml; Sigma, St. Louis, MO, USA), 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, Mannheim, Germany) 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* for 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 (Sigma) supplemented with 10% bovine plasma derived serum (Animal Technologies, Tyler, TX), 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 to construct in vitro BBB models.

Brain pericytes were obtained by a prolonged culture of isolated brain microvessel fragments under selective culture conditions (Dohgu et al., 2005; Takata et al., 2009; Nakagawa et al., 2007). Briefly, the obtained brain microvessel fragments were placed in an uncoated culture flask in DMEM supplemented with 20% fetal bovine serum (FBS; Biowest, Nuaillé, France) (FBS-DMEM), 100 units/ml penicillin, and 100 μg/ml streptomycin (Nacalai Tesque, Kyoto). After 7 days in culture, rat pericytes overgrew brain endothelial cells and typically reached 80–90% confluency. The cells were used at passage 2.

Preparation of the in vitro BBB models

Preparation of the in vitro BBB models co-culturing RBECs and brain pericytes has been described previously (Dohgu et al., 2005). Brain pericytes (4×10^4 cells/well) were seeded in wells of 24-well culture plates (Costar, Corning, NY). After 2-3 days, RBECs (5×10^4 cells/well) were seeded on the upper side of a fibronectin-collagen IV (0.1 mg/ml)-coated polyester membrane (0.33 cm^2, 0.4 μm pore size) of a Transwell®-Clear insert (Costar) placed in the well of a 24-well culture plate containing layers of brain pericytes (RBEC/pericyte co-culture). This co-culture system allows cells to communicate with each other through soluble factors. A monolayer system was made with RBECs alone (RBEC monolayer). Cells were cultured in RBEC medium II supplemented with 500 nM hydrocortisone (Sigma) at 37 °C with a humidified atmosphere of 5% CO_2/95% air until the in vitro BBB models reached confluency.

Pretreatment of RBECs with PAI-1

The median plasma levels of PAI-1 in healthy control subjects and patients with venous thrombosis were more than 100 ng/ml (Meltzer et al., 2010). Based on these data, we treated RBECs with various doses of PAI-1 up to 100 ng/ml. Recombinant rat PAI-1 (Calbiochem, La Jolla, CA) was supplied in solution and this original solution was diluted with a solution containing 100 mM sodium acetate, 100 mM NaCl, 1 mM EDTA, pH 5.0 to obtain 100-fold concentrations for experiments. These PAI-1 solutions and an anti PAI-1 mouse monoclonal antibody (100 μg/ml; Affinity BioReagents, Golden, CO) were diluted with serum-free RBEC medium II containing 500 nM hydrocortisone in the luminal and/or abluminal side of the Transwell insert to expose RBECs. In all experiments, controls were RBECs treated with the corresponding amount of mouse IgG (Sigma) or the solvent for PAI-1.

Measurement of transendothelial electrical resistance (TEER)

TEER across the monolayers grown on the filter membranes was measured by an EVOM resistance meter in an EndOhm tissue resistance measurement chamber (World Precision Instruments, Sarasota, FL). Values are presented as $\Omega\times cm^2$ culture insert. The TEER of cell-free inserts was subtracted from the obtained values.

Measurement of transendothelial transport of sodium fluorescein (Na-F)

Endothelial barrier function was evaluated by measuring permeability of RBECs to Na-F as previously described (Dohgu et al., 2004a, 2005). To initiate the transport experiments, the medium was removed and then physiological buffer (141 mM NaCl, 4 mM KCl, 2.8 mM $CaCl_2$, 1.0 mM $MgSO_4$, 1.0 mM NaH_2PO_4, 10 mM HEPES and 10 mM d-glucose, pH 7.4) containing 100 μg/ml Na-F (Sigma) was loaded in the luminal chamber of the insert (0.1 ml). Samples (0.4 ml) were removed from the abluminal chamber at 15, 30, 45, and 60 min and immediately replaced with fresh physiological buffer. The concentration of Na-F was determined using a fluorescence multiwell plate reader (Ex(λ) 485 nm; Em(λ) 530 nm; CytoFluor Series 4000; PerSeptive Biosystems, Framingham, MA). The permeability coefficient and clearance were calculated as previously described (Dehouck et al., 1992; Dohgu et al., 2005).

Measurement of PAI-1 in culture supernatants

RBEC monolayers were incubated in RBEC medium II with or without 10% bovine plasma-derived serum for 24 h. Culture supernatants were collected from the luminal and abluminal chambers, and stored at -80 °C until use. Levels of PAI-1 in culture supernatants were measured with the total rat PAI-1 antigen ELISA kit (Innovative Research Inc., Novi, MI) by following the manufacturer's instructions.

Statistical analysis

Values are expressed as means ± SEM. The Student's *t*-test was applied to compare the two groups. One-way and two-way analyses