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PROTECTIVE EFFECTS OF THROMBOMODULIN ON MICROVASCULAR PERMEABILITY AFTER SUBARACHNOID HEMORRHAGE IN MOUSE MODEL

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Abstract—The enhanced vascular permeability is a major early brain injury following subarachnoid hemorrhage (SAH). However, its mechanism is not clear yet. In this work, we explored its potential mechanism and investigated the roles of thrombomodulin (TM) in maintaining microvascular integrity after SAH. SAH models were established in adult male ICR mice (28–32 g) by endovascular perforation. TM was immediately administered by femoral vein injection following SAH. The brain water content, Evans Blue content and neurological functions were evaluated. Brain edema was also detected by magnetic resonance imaging (MRI) (T2 map). The siRNA technique, enzyme-linked immunosorbent assay (ELISA), immunofluorescence staining and western blotting were performed to explore the potential mechanism of TM treatment. The number of microthrombi in the hippocampus microvessels was also recorded. TM significantly decreased brain water content and Evans Blue content, alleviated brain edema and neurological deficits after SAH. The plasma concentration of activated protein C was increased after TM treatment. In addition, the levels of phospho-p38MAPK, phospho-p53, cleaved caspase-3, phospho-NF-κB (p65) were markedly decreased. Additionally, the loss of VE-cadherin and Occludin (markers of vascular integrity) and the number of microthrombi in the hippocampus were also reduced. Our results indicated that TM has protective effects on preserving microvascular integrity following SAH partly through preserving endothelial junction proteins and quenching apoptosis/inflammation in endothelial cells via blocking p38MAPK-p53/NF-κB (p65) pathway.

Key words: microvascular permeability, mouse, subarachnoid hemorrhage, thrombomodulin.

INTRODUCTION

Subarachnoid hemorrhage (SAH) is a devastating neurological injury, it causes severe morbidity and mortality. Early brain injury (EBI), such as enhanced vascular permeability and cellular apoptosis within 72 h after SAH (Park et al., 2004; Yan et al., 2011), was considered a major factor significantly contributing to the clinical outcomes of SAH. The connections of endothelial cells mainly include tight junctions (TJs), adherent junctions (AJs) and gap junctions (GJs). TJs and AJs constitute the blood–brain barrier (BBB) to maintain the microvascular integrity. Occludin and VE-cadherin are two important proteins located on TJs and AJs, respectively (Sandoval and Witt, 2008). The damage of these proteins will lead to severe BBB disruption (Liu et al., 2012; Yan et al., 2013). Additionally, apoptosis mediated by proapoptosis protein (such as p53 (Zhou et al., 2013)) and inflammation induced by NF-κB (Sun et al., 2013) in endothelial cells will result in BBB breakdown after SAH.

Thrombin, a multifunctional serine protease, is derived from its zymogen, prothrombin, and converts fibrinogen into fibrin, activates platelets, and stimulates the proliferation of vascular smooth muscle cells (Davey and Luscher, 1967; Davie et al., 1991; McNamara et al., 1993). After SAH, the plasma concentration of thrombin is markedly increased (Fujii et al., 1995, 1997; Nina et al., 2001).Through its protease-activated receptor located on the endothelial membrane, it can lead to inflammation, apoptosis of endothelial cells (Kitaoka et al., 2002), finally results in BBB disruption and brain edema (Yan et al., 2013). It was reported that thrombin played roles in BBB disruption and brain edema after stroke and its antagonist argatroban ameliorated EBI and improved neurological outcomes after SAH (Sugawara et al., 2009).

Thrombomodulin (TM) is a membrane protein mainly expressed by endothelial cells and critically modulates endothelial anticoagulant activity. It binds to thrombin with high affinity and decreases the negative effects of thrombin. Additionally, TM-thrombin complex promotes the conversion of protein C to activated protein C (APC) (Esmon, 1989; Dittman and Majerus, 1990), whose cytoprotective roles include anti-apoptotic and anti-

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Abbreviations: AJs, adherent junctions; ANOVA, analysis of variance; APC, activated protein C; BBB, blood–brain barrier; EBI, early brain injury; ELISA, enzyme-linked immunosorbent assay; MRI, magnetic resonance imaging; PAR-1, protease-activated receptor-1; PBS, phosphate-buffered saline; ROI, region of interest; SAH, subarachnoid hemorrhage; TJs, tight junctions; TM, thrombomodulin.

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inflammatory effects, protection of endothelial barrier function (Mosnier et al., 2014). The TM analog Solulin promotes reperfusion and reduces infarct volume in a thrombotic model of stroke (Su et al., 2011). APC can preserve BBB integrity after cerebral venous sinus thrombosis (Nagai et al., 2010). However, the effects of TM on the BBB permeability after SAH are still to be cleared.

In this study, we had observed the effects of TM on the microvascular integrity after SAH and explored its potentially protective mechanisms.

EXPERIMENTAL PROCEDURES

Animals

All animal experiments were conducted by the Animal Care and Use Committee at Peking University Health Science Center and the Guidelines for the Use of Animal in Neuroscience Research by the Society for Neuroscience.

All male ICR mice were purchased from the Department of Laboratory Animal Science of Peking University Health Science Center, and housed in a 12-h light/dark cycle at a controlled temperature and humidity with free access to food and drink environment.

SAH model

The endovascular perforation model of SAH was established in male ICR mouse (28–32 g) (Bederson et al., 1995). Briefly, the animals were anesthetized by 4% isoflurane in 60%/40% medical-air/oxygen and maintained with 2% isoflurane. A sharpened 5-0 nylon suture was introduced into the right internal carotid artery through the external carotid artery until resistance was felt (approximately 10–12 mm from the common carotid bifurcation). The suture was then pushed further to perforate the bifurcation of the anterior and middle cerebral arteries and withdrawn immediately. In sham-operated animals, the suture was inserted into the carotid artery, however, no perforation was performed. After suture removal, the incision was closed. The animals were individually housed until recovery. Body temperature was monitored by rectal probe, and normothermia was maintained by a heating pad. After operation, the animals were allowed food and drink *ad libitum*.

Experimental groups and treatment

Firstly, 23 mice were randomly divided into four groups (finally, 20 mice were used, described as 20/23, same below): sham, SAH + N.S, SAH + TM 1 μg/kg, SAH + TM 10 μg/kg except the dead and unqualified animals (see inclusion criteria below). The dose-dependent effects of TM were observed at 24 h after SAH (Fig. 1A).

Next, 63/75 mice were randomly divided into three groups: sham, SAH + N.S, SAH + TM 10 μg/kg. The effects of TM treatment after SAH were determined by magnetic resonance imaging (MRI) examination and analysis of Evans Blue contents and plasma APC concentration. The impact of TM on the mortality following SAH was also evaluated (Fig. 1B).

Furthermore, 25/33 mice were randomly divided into five groups: sham, SAH + N.S, SAH + TM + control siRNA, SAH + TM + protein C siRNA and SAH + TM + PAR-1 siRNA groups. The siRNA was injected into the right lateral ventricle at 24 h before SAH operation. The brain water content was determined at 24 h following SAH (Fig. 1C).

Lastly, 45/54 mice were randomly divided into three groups: sham, SAH + N.S, SAH + TM 10 μg/kg. The potentially protective mechanism of TM treatment was explored using immunofluorescence and western blot methods (Fig. 1D).

SAH grade

The SAH severity was evaluated as previously reported (Sugawara et al., 2008). The basal cistern of brain was divided into 6 segments: left and right frontal, left and right temporal, and upper and lower brain stem. Each part was allotted a score from 0 to 3. 0: no subarachnoid blood; grade 1: minimal subarachnoid blood; grade 2: moderate blood clot with recognizable arteries; grade 3: blood clot obliterating all arteries within the segment. A total score (0–18, sham group = 0) of each region was recorded.

In this study, the mice with subdural/extradural hemorrhage or mild hemorrhage (grade score < 12) were excluded when they were sacrificed at 24 h after SAH, only mice with severe hemorrhage (grade score > 12) were recruited to perform further study.

Drug administration

Recombinant human TM/BDCA-3 (Catalog # 3947-PA, R&D system, Minneapolis, MN, USA) was diluted in N.S, and two doses (1 μg/kg or 10 μg/kg) of TM were immediately administered through femoral vein after SAH. The same volume of N.S was used as a vehicle control. The siRNA was mixed with the transfection reagent (both 5 μl, Santa Cruz Biotechnology, MO, St. Louis, USA) immediately before application. The siRNA mixture was injected through a 28-G needle on a syringe into the right lateral ventricle (1.0 mm lateral to the midline, 0.2 mm posterior to the bregma, 2.5 mm in depth) in 10 min at 24 h before SAH surgery, as reported by others(Du et al., 2014).

Neurobehavioral evaluation

Neurological functions (3–18) were blindedly evaluated by the Garcia method at 24 h after SAH (Garcia et al., 1995). The evaluation consists of six tests (spontaneous activity, symmetry in the movement of all four limbs, forepaw outstretching, climbing, body proprioception and response to whisker stimulation) that are scored 0–3 or 1–3.

A 6-point neurological evaluating system was also performed as following (Zausinger et al., 2000): 5 = normal motor function, no neurological deficit; 4 = flexion of torso and contralateral forelimb when lifted by the tail; 3 = decreased resistance to lateral push without circling; 2 = circling to contralateral side against resistance when tugged by the tail on a flat surface; 1 = circling