SUPPLEMENTAL MATERIAL

White matter injury after subarachnoid hemorrhage: role of blood-brain barrier disruption and matrix metalloproteinase-9

Supplemental Methods

Mouse SAH model

Anesthesia was induced by inhalation of 4% isoflurane in a nitrous oxide/oxygen mixture (70/30) and maintained by 2% isoflurane administered through a facemask. A midline skin incision was made in order to expose the left common carotid artery. The external carotid artery (ECA) and its branches were isolated and coagulated; thereafter, an 5-0 nylon monofilament (Ethicon, Somerville, NJ), with heat blunted-tip, was introduced into the left internal carotid artery (ICA) through the ECA stump up to the left ACA near the anterior communicating artery, where resistance was encountered. Then the suture was advanced 3 mm further to perforate the artery and was immediately withdrawn through the ICA into the ECA, allowing reperfusion and producing SAH. Sham control mice underwent the same surgical procedure, without insertion of suture. Body temperature of all animals was maintained at 37.5°C with a feedback-controlled heating pad throughout these procedures. After the surgery, mice were housed in heated cages until recovery.

Evaluation of SAH severity and neurological scores

The basal brain including brainstem was divided into 6 segments to evaluate SAH severity. Each segment was assigned a grade from 0 to 3 depending on the amount of SAH as follows: Grade 0, no SAH; Grade 1, minimal SAH; Grade 2, moderate SAH with recognizable

arteries; and Grade 3, SAH covering the cerebral arteries. The animals assigned a total score ranging from 0 to 18 after summating the scores from all 6 segments.

The evaluation of neurological score consisted of six tests that could be scored 0 to 3 or 1 to 3. These six tests consist; symmetry in the movement of all four limbs; forelimbs outstretching; climbing; side stroking; and response to vibrissae (whisker stimulation). Mice were given a score of 3 to 18 in 1-number step (higher scores indicate greater function).

Transmission electron microscopy

Mice were anesthetized and underwent transcardiac perfusion with 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1mol/L Sorensen's buffer (pH 7.4). Brains were removed and a 1-mm thick coronal slice was cut with a blade 1mm posterior to bregma. Slices were immersed in the same fixative overnight at 4°C and then post-fixed with 1.0% OsO4 and dehydrated in graded ethyl alcohol. After dehydration, samples were infiltrated with propylene oxide, embedded in Epon, and sectioned. Ultra-thin sections were then stained with uranyl acetate and Raynold's lead citrate, and evaluated using a Philips CM 100 TEM (FEM Company, Hillsbolo, OR) and digitally imaged using a CCD camera (Hamamatsu ORCA-HR; Hamamatsu Photonics, Hamamatsu, Shizuoka, Japan).

Gelatin zymography for measurement of MMP activity

Brains from sham or SAH mice were removed, and a 1-mm thick coronal slice was cut with a blade 1mm posterior to bregma, and the white matter carefully separated from cortex and basal ganglia. They were then lysed, homogenized and protein concentration measured. Forty micrograms of total proteins were subjected to electrophoresis in polyacrylamide gels containing 0.5 mg/mL gelatin in the presence of SDS under nonreducing conditions, washed twice in 2.5% Triton X-100 for 1 h, rinsed briefly, and incubated at 37°C for 48 hours in 100

mmol/L Tris-HCl (pH7.4) and 10 mmol/L $CaCl_2$. Then they were stained with Coomassie Brilliant Blue R-250 and destained in a solution of 5% acetic acid and 30% methanol. Zones of enzymatic activity appeared as clear bands against a blue background and their signal intensity were measured using Image-J software (version 1.47; National Institutes of Health, Bethesda, MD).

Immunohistochemistry and histochemistry

Mice were anesthetized with pentobarbital (60mg/kg; i.p.) and underwent transcardiac perfusion with 4% paraformaldehyde in 0.1mol/L phosphate-buffered saline (PBS; pH 7.4). Forebrains were removed, fixed in 4% paraformaldehyde for 48 h, immersed in 30% sucrose for 48 hours at 4°C, and then frozen. Fresh frozen forebrains were sliced into 10-μm-thick coronal sections by using a cryostat vibratome (Leica CM 1900; Leica Microsystems, Wetzler, Germany), and sectioned tissues were placed onto individual slides. For immunohistochemistry, anti-albumin (Bethyl Laboratories Inc, Montgomery, TX; 1:1000), anti-glial fibrillary acid protein (GFAP; Millipore, Billerica, MA; 1:400), **anti-platelet-derived growth factor receptor alpha (PDGFRα, Abcam; 1:100)** and anti-Iba-1 (Wako, Osaka, Japan; 1:400) antibodies were used. Luxol fast blue (LFB) staining was performed to evaluate myelin integrity according to the manufacturer's instructions (American Mastertech, Rodi, CA).

Immunofluorescence

For immunofluorescent labeling, the primary antibodies were anti-MMP-9 (Millipore; 1:100), anti-GFAP (Millipore; 1:400), **anti-PDGFRα (Abcam; 1:100)**, anti-Iba-1 (Wako; 1:400), anti-CD-31 (BD Biosciences, San Jose, CA; 1:200) and anti-GST-π (BD Biosciences; 1:200). The anti-GST-π antibody was used as a marker of mature oligodendrocytes. The appropriate

Alexa-Flour conjugated antibodies (Molecular Probes, Eugene, OR; 1:400) were used as secondary antibodies. The slides were covered with Prolong Gold reagent with DAPI (Morecular Probes), and were observed underneath a fluorescence microscope (Olympus BX51; Olympus, Tokyo, Japan).

Quantification of white matter injury

Ten-μm-thick coronal sections (bregma -1.5 mm to -2.1 mm) were used for each analysis. For quantitative measurements of albumin staining, 4 brain sections were digitized under a 10x magnification using Olympus BX-51 microscope. Albumin positive areas in the white matter tract (corpus callosum, external capsule, and fimbria) were marked and calculated using Image-J. For quantification of LFB staining, 4 brain sections which contained 3 regions of interest (ROI) including the white matter tract were digitized under a 40x magnification. The white matter tract was marked in each ROI, and areas not stained by LFB were determined as the areas of myelin loss. Unmyelinated Schaffer collaterals obtained from same settings for each section served as an internal control.