Acute alterations in microvascular basal lamina after subarachnoid hemorrhage

FATIMA A. SEHBA, PH.D., M.D., GULAM MOSTAFA, M.B., B.S., JARED KNOPMAN, B.S., VICTOR FRIEDRICH JR., PH.D., AND JOSHUA B. BEDERSON, M.D.

Departments of Neurosurgery and Neurobiology, Mount Sinai School of Medicine, New York, New York

S ubarachnoid hemorrhage is accompanied by acute ischemic injury at the time of the initial hemorrhage (≤ 48 hours), followed by delayed vasospasm and ischemia that develop 72 hours to 7 days after bleeding.16 Acute ischemic brain injury after SAH accompanies reduced CBF and is the most important contributor to poor outcome post-SAH.10,19,54 Note, however, that the mechanisms underlying acute ischemic injury following SAH are poorly understood.

A marked increase in the permeability of cerebral capillary microvessels occurs during the acute phase of SAH in humans and in experimental models1,19,24,67 and has been correlated with both the development of delayed cerebral ischemia and a poor clinical outcome.16,17,23,24,47 and has been noted. The integrity of cerebral microvessels is maintained in part by components of basal lamina: collagen IV, elastin, lamina, and so forth. Destruction of basal lamina components by collagensases and matrix metalloproteinases (MMPs), especially MMP-9, has been known to occur in other ischemic models. The authors assessed the integrity of cerebral microvasculature after acute SAH by examining collagen IV and MMP-9 levels and collagenase activity in the microvessels.

Results of this study demonstrated an acute loss of collagen IV from the cerebral microvasculature after SAH and indicated that MMP-9 contributes to this event. The loss of collagen IV might contribute to the known failure of the blood–brain barrier after SAH.

Key words • microvessel • subarachnoid hemorrhage • collagen IV • matrix metalloproteinase

Abbreviations used in this paper: ANOVA = analysis of variance; BBB = blood–brain barrier; CBF = cerebral blood flow; FITC = fluorescein isothiocyanate; ICP = intracranial pressure; MMP = matrix metalloproteinase; SAH = subarachnoid hemorrhage.

after SAH have not been evaluated previously. Cerebral capillary microvessels consist of endothelial cells, basal lamina, a constituent of the vascular extracellular matrix, and astrocyte end-feet. Collagen IV constitutes up to 90% of the total protein of the basal lamina and confers structural integrity to the vessel wall.164 Authors of recent studies have established that basal lamina is degraded during other types of ischemic brain injuries.22,28,29,42,67 This degradation is associated with alterations in vascular tone, increased intravascular pressure, and increased microvascular permeability, allowing the extravasation of fluid (edema), fibrin, and erythrocytes.28

The precise mechanism through which the dissolution of vascular matrix in cerebral ischemia occurs is not known, but metalloproteinases, plasminogen activators, urokinase, and serine proteases have been implicated.20,58 In the brain, MMPs and serine proteases are secreted by microglia, astrocytes, and endothelial cells.22 Among the MMPs, gelatinase A (MMP-2) and gelatinase B (MMP-9) can digest the vascular basal lamina.13 Known substrates of MMP-2 and -9 include gelatin, Type IV collagen, fibronectin, and elastin.12,35 In other models of cerebral ischemia, increases in MMP-9, -2, and -3 accompany ischemic injury.49,57,58

In the present study we report on acute changes—those occurring at or before 48 hours after surgically induced SAH—in microvascular basal lamina in rats based on anal-
SAH would likely occur during second measurements and could alter results. 2) The ICP cannula cannot be left in place during a 48-hour survival time because it would clog and cause infection. 3) Recording of CBF is performed through a temporal bone window with the probe touching the dura mater; this area becomes progressively inflamed, altering the signals received and possibly affecting underlying tissue.

Data Acquisition and Statistical Analysis

Physiological Factors. Cerebral blood flow, ICP, and mean arterial blood pressure data were continuously recorded starting 20 minutes before until 10 to 30 minutes after SAH by using PolyView software (Grass-Telefactor, West Warwick, RI). Cerebral blood flow data were normalized to a baseline value averaged over 20 minutes prior to SAH and expressed as a percentage of the baseline. Statistical analysis was performed using a two-way ANOVA followed by multiple Fisher protected least-significant-difference post hoc t-tests, setting experimental significance at a probability value less than 0.05.

Rat Brain Preparation for Immunohistochemical Analysis. Rats were transcardially perfused with saline, and the brains were rapidly removed, embedded in Tissue-Tek OCT compound (Bayer AG, Elkhart, IN), and frozen in 2-methylbutane cooled in dry ice. Serial coronal brain tissue sections 8 μm thick were cut on a cryostat and thaw-mounted onto gelatin-coated slides. Tissue sections located at the bregma coordinates −8, −0.2, and +1.2 were used for immunohistochemical staining. Adjacent sections were used for each stain.

For collagen IV/hamster axial staining, animals killed at 10 minutes and 1, 3, or 6 hours after SAH or sham operation were used. For dual labeling and zymography, animals were killed at 10 minutes to 48 hours after SAH or sham operation.

Histological Studies. For collagen IV staining, monoclonal mouse anti-rabbit Type IV collagen (M3F7; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) was used. For MMP-9 staining, MMP-9 rabbit polyclonal immunoglobulin (sc-10737; Santa Cruz Biotechnology, Santa Cruz, CA) was used.

Collagen IV/Hematoxylin Staining. Unfixed brain sections were thawed briefly and fixed in acetone at −20°C. Following rehydration, sections were incubated with the collagen IV antibody (M3F7) and then incubated with a biotinylated goat anti-mouse antibody (Vector Laboratories, Burlingame, CA). For the detection process, tissue sections were incubated with avidin/horseradish peroxidase complex (Ready To Use VECTASTAIN Elite ABC Kit, PK-7100; Vector Laboratories), and immunoreactivity was visualized with diaminobenzidine. Finally, brain sections were counterstained with hematoxylin. Matching sections from sham-operated controls were processed identically.

In Situ Zymography. The procedure used is adapted from Rivera, et al. The technique uses DQ gelatin, a fluorescein-conjugated gelatin so heavily labeled that its fluorescence is quenched. On digestion by a gelatinase/collagenase, the gel releases a brightly fluorescent peptide that can be visualized by epifluorescence microscopy. Using this method, bright fluorescence is generated at sites with strong proteolytic activity.

Frozen cryostat sections of unfixed brain tissue were thawed and coated with a thin layer of FITC-labeled DQ-gelatin solution (Enz-Check collagenase kit; Molecular Probes, Eugene, OR). The coated sections were incubated overnight at 37°C in a humidified chamber. Once the sections were fixed with chilled 4% paraformaldehyde-fuchsin, coverslips were placed over them and they were examined and photographed using a standard epifluorescence microscope. Each experiment included similar sections from time-matched sham-operated control animals.

Immunofluorescence. Frozen cryostat brain sections (8 μm) were thawed briefly and fixed in acetone at −20°C for 10 minutes. Sections were incubated overnight at 4°C with mouse monoclonal anti-collagen IV (M3F7) and then with species-specific goat anti-mouse Texas red-conjugated secondary antibody (Vector Laboratories) for 1 hour at room temperature. Next, these sections were incubated overnight at 4°C with rabbit anti-MMP-9 (H-29; Santa Cruz Biotechnology, Inc.) followed by incubation with species-specific FITC-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology, Inc.) for
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1 hour at room temperature. Similar sections from time-matched sham-operated control animals were included in each experiment.

Morphometric Studies. Four brain regions (basal and frontal cerebral cortex, striatum, and hippocampus) were analyzed. For counting purposes, two to three fields per region and hemisphere were photographed under constant conditions: brightfield, 10× objective, field area 4 × 10^4 μm² (Nikon Labphot-2; Nikon Company, Tokyo, Japan) for fluorescence, 10× objective, field area 3 × 10^4 μm² (in situ zymography) or 20× objective, field area 8 × 10^4 μm² (Axioskop; Carl Zeiss, Inc., Thornwood, NY). For immunofluorescence, Texas red-labeled specimens were viewed through a rhodamine filter and photographed. After switching to the fluorescein filter, the same field was again photographed.

Quantification was performed with the micrographs by an observer blinded to specimen identity. For all tissue specimens, we counted the number of immuno- or zymographically reactive microvascular profiles per micrograph and expressed results as numbers per unit area of the image. For immunofluorescence, rhodamine- and fluorescein-filtered images were superimposed, and the number of vascular profiles positive for collagen IV only, MMP-9 only, or both was determined. Data obtained were analyzed and plotted in several different ways before concluding that they were best presented as a ratio of total blood vessels. In addition, in 3-hour-survival specimens we determined the aggregate length of vascular profiles per micrograph and expressed results as numbers per unit area of the image. For immunofluorescence, rhodamine- and fluorescein-filtered images were superimposed, and the number of immunofluorescent microvessels and the microvasculature. Following SAH, we found time-dependent decreases in staining (10 minutes–1 hour). At the later time intervals (> 3 hours after SAH), blood vessels appeared as interrupted sequences of segments rather than continuous profiles (Fig. 2). To confirm that the magnitude of the segmentation of microvessels depends on treatment, we analyzed segment lengths 3 hours after SAH or sham operation. A significant reduction occurred in the length of collagen IV–positive vascular profiles after SAH compared with that in sham-operated animals (SAH 0.260 ± 0.027; sham operation 0.438 ± 0.043; p < 0.001). In addition, we counted the number of microvascular profiles showing collagen IV immunoreactivity anywhere along the lengths. A significant decrease occurred in the number of collagen IV–positive microvessels in all areas 3 hours after SAH (Fig. 3).

Histological Analysis

Collagen IV Immunoreactivity. In sham-operated animals, collagen IV immunostaining clearly delineated both large vessels and the microvasculature. Following SAH, we found time-dependent decreases in staining (10 minutes–1 hour). At the later time intervals (> 3 hours after SAH), blood vessels appeared as interrupted sequences of segments rather than continuous profiles (Fig. 2). To further characterize these changes we counted fluorescent microvessels in all areas 3 hours after SAH (Fig. 3).

In Situ Zymography. Some fluorescent structures with vascular morphological features were displayed on zymograms obtained in animals that had undergone sham operation or SAH and had been killed 10 minutes after either procedure. Fluorescent microvessels subsequently increased in number in SAH-induced animals and decreased in number in sham-operated animals. Furthermore, fluorescent vascular profiles appeared to be increasingly segmented with time after SAH, as demonstrated by a noncontinuous distribution of fluorescence along a microvessel (Fig. 4).

To further characterize these changes we counted fluorescent vascular profiles on the zymograms. Results of an ANOVA showed a significant effect of time (F = 37, p < 0.0001) and treatment (SAH compared with sham surgery, F = 105, p < 0.0001) on the number of fluorescent profiles per area of tissue section. In addition, we found a significant interaction between time and treatment in SAH-induced compared with sham-operated animals (F = 37, p < 0.0001), indicating that the treatment groups differ signifi-
In sham-operated animals the number of fluorescent vessel profiles decreased with time, reaching a minimal value at 24 hours (Fig. 5A). In animals in which SAH had been induced, the number of fluorescent vascular segments increased significantly at 3 and 6 hours and decreased thereafter, reaching a minimum at 24 hours (Fig. 5). A small interhemispheric difference was found in SAH-induced animals, but not in sham-operated ones (data not shown).

To determine the nature of the microvascular collagenases activated after SAH, we used ethylenediaminetetraacetic acid, a divalent cation chelator that inhibits the activity of divalent cation-requiring enzymes. At 3 hours after SAH, a concentration-dependent (0.1, 1, and 10 mM) decrease in the number of fluorescent microvessels was observed, indicating that microvascular collagenases activated after SAH require divalent cation for their proteolytic activity (data not shown). Because MMPs require zinc for their catalytic activity, this result is consistent with their possible contribution to collagen degradation after SAH. Matrix metalloproteinase is a major gelatinase/collagenase involved in collagen IV degradation. We investigated whether MMP-9 is expressed during the period of collagen IV denudation following SAH.

Collagen IV and MMP-9 Dual Labeling. Qualitative observation showed a dramatic increase in MMP-9 immunofluorescence following SAH with a marked reduction in microvascular collagen IV staining. These changes were reversed by 48 hours after SAH. Three kinds of vascular segments could be identified in our dual-labeled tissue sections: 1) those immunoreactive for both collagen IV and MMP-9; 2) those immunoreactive for collagen IV but not MMP-9; and 3) those immunoreactive for MMP-9 but not collagen IV (Fig. 6). Quantitative analysis of profile numbers revealed significant effects of time (F = 9, p < 0.0001) and treatment (F = 28, p < 0.0001) on the number and proportion of the different types of segments. A significant interaction effect (treatment compared with time, F = 7, p < 0.0001) was also found, indicating that temporal changes in the proportion of segments differ between the SAH-induced and sham-operated groups. Analysis of our results indicates a small but statistically significant change over time in sham-operated animals, with much larger changes following SAH.

The number of microvascular segments immunoreactive for collagen IV (with or without MMP-9) decreased initially at 3 hours, reached a minimal value at 6 hours, and returned toward baseline values 24 to 48 hours after SAH (Fig. 7A). The number of microvascular segments immunoreactive for MMP-9 (with or without collagen IV) increased...
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at 3 hours, peaked at 6 hours, and returned to a value not significantly different from that in sham-operated controls at 48 hours after SAH (Fig. 7B). Similar changes of a smaller magnitude occurred in microvascular collagen IV and MMP-9 immunoreactivity in sham-operated animals (Fig. 7). The number of microvascular segments immunoreactive for both MMP-9 and collagen IV was maximal at 3 hours after SAH and decreased at 6 hours, when the majority of segments with MMP-9 lacked collagen IV (data not shown).

The number of vascular segments immunoreactive for collagen IV or MMP-9 following SAH was not significantly different in comparison between the left and right hemispheres.

Discussion

Our results demonstrate that microvessels are denuded of collagen IV after SAH in a reversible time-dependent fashion and that MMP-9 contributes to this event. The greatest loss in microvascular collagen IV occurred 3 to 6 hours after SAH, the time at which MMP-9 levels and collagenase activity were maximally increased. Microvascular collagen IV contents, MMP-9 levels, and zymographically visual-
Depletion of Microvascular Collagen IV Following SAH

Microvascular staining for collagen IV appeared fragmented after SAH, yielding shorter vascular profiles than those after sham operation, indicating a segmental loss of collagen IV post-SAH. A similar time-dependent loss in microvascular collagen IV and other components of basal lamina has been observed in other models involving cerebral ischemia. Note that our results also document a recovery of collagen IV at long (48 hours) survival times. To eliminate the confounding effect of nonspecific surgical trauma, wherever necessary we have expressed our results as a ratio of SAH values to sham-operated control values (Fig. 5B).

Changes in Cerebral Microvasculature Following Sham Operation

Sham-operated animals displayed small but significant changes in the microvascular basal lamina. A likely cause of these changes is surgical trauma due to placement of both the laser Doppler probe on the brain surface for CBF measurements and the ICP catheter into the cisterna magna for ICP recording. A general inflammatory response is known to follow surgical trauma in humans and animals; however, the much greater intensity and faster onset of microvascular changes after SAH compared with that following sham operation clearly differentiates the two injuries. To eliminate the confounding effect of nonspecific surgical trauma, wherever necessary we have expressed our results as a ratio of SAH values to sham-operated control values (Fig. 5B).

Immunoreactivity After SAH

Asterisks denote a significant difference (p < 0.01) between time-matched sham-operated animals.

Microvascular collagenase activity and MMP-9 immunoreactivity increased initially (3–6 hours) and recovered at 48 hours after SAH. Like collagen IV loss, MMP-9 immunoreactivity and collagenase activity were distributed segmentally along the microvasculature. The mechanism underlying this segmental pattern of activation/expression is not known. Analysis of our data, although largely correlative, indicates that MMP-9 causes or directly contributes to collagen IV degradation after SAH. 1) Ethylenediaminetetraacetic acid inhibits post-SAH collagenase activity; MMP-9 requires divalent cations for activation. 2) Collagen IV loss and MMP-9 immunoreactivity covaried; they were both maximal at 6 hours after SAH. 3) Dual immunofluorescence revealed that collagen IV is present in most segments positive for MMP-9 at 3 hours and missing from most of the segments at 6 hours after SAH, thus indicating that MMP-9 signals the location of collagen IV destruction. 4) Immunoreactivity for MMP-9 disappeared as that for collagen IV was restored (48 hours after SAH). In this regard, we note that collagenases other than MMP-9 may also be activated following SAH. Analysis of our data demonstrate that perivascular collagenases are activated segmentally following SAH and that MMP-9 is one of those collagenases.

Increased MMP-9 has been associated with decreased collagen IV after other forms of cerebral ischemia, and inhibition of MMP-9 activation using recombinant tissue plasminogen activator (low doses), MMP-9–neutralizing monoclonal antibody, or pharmacological agents such as BB-94 and KB-R7785 has been shown to decrease the extent of ischemia injury. These factors might indicate that similar treatments ameliorate ischemic injury after SAH.

The significance of collagen IV loss to cerebral microvascularity and microcirculatory function after SAH is not known. Because collagen IV is an integral part of the extracellular matrix and provides stability to the microvasculature, however, its loss might be expected to increase microvascular permeability and to compromise vascular wall integrity in SAH as observed in other cerebral ischemia. In this regard it is notable that increased capillary permeability and BBB opening are observed as early as 2 to 3 hours after SAH in other experimental SAH models. A clearer understanding of the physiological implications of our observations will require analysis of BBB function in our endovascular model of SAH.
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post-SAH (Bederson, et al., unpublished data). Intracranial pressure remains at the 1-hour value (above baseline) at 3 hours after SAH. Moreover, Jackowski, et al., in an injection model of SAH, demonstrated a full recovery of CBF 48 hours after SAH.

Structural alterations in cerebral microvessels are well studied in ischemic stroke. The loss of basal lamina collagen IV begins at least 4 hours after ischemia, reaching significance 24 hours later.65 Based on data from colocalization studies, there is an association between a decrease in microvascular collagen IV and the role of MMPs (especially MMP-9) in the degradation of basal lamina.2,4,48,56,65 We and other investigators have previously documented the presence of an acute ischemic cerebral environment after SAH.7,8,25,36–38,52 Hence, it could be argued that the changes in collagen IV and MMP-9 in our study were caused by ischemia and not specific to SAH. Note, however, that the temporal differences in microvascular collagen IV loss after ischemia and SAH and its unique later recovery post-SAH indicate that although ischemic microvascular injury after SAH and ischemic stroke may share common pathophysiological changes, these changes develop much faster after SAH and may reflect the actions of different signaling mechanisms.

Conclusions

In the present study, we examined the acute alterations in microvascular basal lamina after SAH and found an immediate loss of collagen IV together with the activation of MMP-9. The loss of collagen IV would compromise microvascular integrity, leading to increased vascular permeability and contributing to the opening of the BBB after SAH.

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