The Temporal Changes of Microvascular Basement Membrane and Astrocyte Contact Following Focal Cerebral Ischemia in the Rat

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ABSTRACT

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Cerebral microvasculature is a constituent of the blood-brain barrier (BBB), which impedes influx of most compounds from the blood to the brain. The BBB is composed of endothelial cells, basement membrane (BM), astrocyte endfeet, and pericytes. Although attention has been focused increasingly on the interendothelial tight junction, it is believed that all the components of the BBB are essential for normal function and stability. The BM is a scaffold which is composed of extracellular matrix (ECM) molecules and plays a critical role in maintaining the integrity of the BBB. Endfeet of astrocytes form an envelope around blood vessels and are attached to the BM tightly by their adhesion molecules. Matrix metalloproteinases (MMPs) are a group of zinc-dependent enzymes, which may play a major role in matrix alteration. Especially, MMP-2 and MMP-9 are key enzymes that degrade the ECM components of the BM.

Loss of microvascular integrity occurs in the ischemic area and contributes to brain edema, hemorrhagic transformation, and cell death. Detachment of the BM-astrocyte contact as well as degradation of the BM is responsible for the loss of

microvascular integrity, however, their temporal patterns and association with MMPs are not well known.

In the rat subjected to a permanent middle cerebral artery occlusion (MCAO), for 1, 4, 8, 12, 16, 20, and 48 hours, the temporal changes of the BM-astrocyte contacts and mean BM density were investigated with transmission electron microscopy (TEM). Their association with MMP activities, which were measured by using gelatin zymography, was also examined.

As a result, the loss of BM-astrocyte contacts was evident as early as 4 hours after MCAO and continued in a time-dependent manner. The mean BM density of the microvessels was decreased in the ischemic area, which was significant after 12 hours of ischemia. In addition, not only swollen astrocytes but also accumulated extracellular fluid was visible in the ischemic region. MMP-9 activities were elevated significantly in ischemic hemispheres, but those of MMP-2 were not different among all groups at time points examined. The relationships among BM-astrocyte contacts, BM density, and MMP-9 activities were analyzed. The intact BM-astrocyte contacts and the mean BM density showed significantly positive correlation ($r=0.784$, $P=0.000$). Negative correlation was observed between the proportion of intact BM-astrocyte contacts and the MMP-9 activity ($r=-0.711$, $P=0.000$) as well as between BM density and the MMP-9 activity ($r=-0.538$, $P=0.00159$). The temporally increasing pattern of MMP-9 activities showed strong association with decreasing pattern of BM density and BM-astrocyte contacts during cerebral ischemia.

This study investigated the temporal and ultrastructural changes occurring in the microvascular BM and astrocyte endfeet interface and also demonstrated the potentially contributing role of MMP-9 in degradation of the microvascular BM and focal loss of its contact to astrocyte endfeet following focal cerebral ischemia.

Key Words: Cerebral ischemia, Basement membrane, Astrocyte, Matrix metalloproteinase, Electron microscopy

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I. INTRODUCTION

Stroke is one of the leading causes of death and the most common cause of disability in Korea as well as in the world.[1] Ischemic stroke is developed by an interruption of blood supply, which can lead to cell and tissue injury. Consequences of cessation of blood flow include energy failure, free radical production, excitotoxicity, altered Ca^{2+} homeostasis, and activation of proteinases.[2]

The cerebral microvasculature serves as a conduit for the blood and is also functionally dynamic by delivering communicative signals between intravascular components and glial/neuronal cells, and by providing cellular nutritional support.[3-7] The cerebral microvessel is a constituent of the blood-brain barrier (BBB),[4, 8] which are composed of microvascular endothelial cells, the basement membrane (basal lamina, BM), astrocyte endfeet, and pericytes.[9] Astrocytes occupy 20-30% of the brain volume, and participate in both the microvascular BM and in communication with nearby neurons.[10] Astrocyte endfeet form an envelope around blood vessels and are attached to their BM tightly by adhesion molecules including integrin $\alpha_6\beta_4$. The BM is a scaffold that attaches endothelial cells to one side and endfeet of astrocytes to

the other side. The BM, which is composed of extracellular matrix (ECM) molecules such as type IV collagen, laminins, fibronectin, heparan sulfates, and proteoglycans, plays a critical role in maintaining the integrity of the BBB by providing structural support to the endothelial cell wall and astrocytes.[11-16]

Microvascular integrity is lost seriously during focal cerebral ischemia. ECM molecules are decreased in the ischemic area following middle cerebral artery occlusion (MCAO) in non-human primates.[17] The integrins that attaches the BM to the endothelium and astrocyte endfeet are attenuated in the ischemic area.[4, 18, 19] There is accumulating evidence that the breakdown of microvascular integrity is an important mechanism of brain edema and hemorrhagic transformation following ischemic injury. [8, 20, 21] The loss of microvascular contact with glial cells also contributes to cell death.[19]

The degradation of ECM molecules of the BM is mediated by uncontrolled activity of proteinases. Matrix metalloproteinases (MMPs) are a group of zinc-dependent enzymes, which may play a primary role in matrix alteration.[17] MMP-2 and MMP-9 are key enzymes that degrade the ECM components of the BM because their substrates include types IV and V collagen, fibronectin, elastin, and laminins, which are main components of the BM.[8, 17, 22-24] While MMP-2 is known to play a role in angiogenesis, increase of MMP-9 is associated brain edema, hemorrhagic transformation and ischemic injury after cerebral ischemia.[8, 17, 19-21, 25-27]

Although detachment of the BM-astrocyte contact as well as degradation of the BM in the microvessels has been suggested as being occurred in the ischemic area, their temporal patterns and association with MMPs are not well known.

In this study, the temporal changes of cerebral microvascular basement membrane and surrounding astrocyte contacts, and density of basement membrane were examined with transmission electron microscopy (TEM) in the rat subjected to a permanent MCAO using a nylon suture. Additionally, their correlation and with MMP activities which were assessed by using gelatin zymography, was investigated.

II. MATERIALS AND METHODS

1. Experimental Animals and Preparation

Male Sprague-Dawley (SD) rats weighing 250-320 g were used as the subjects in this study. Animals were housed in plastic cages with soft bedding under a 12/12 hr reversed light and dark cycle, and freely accessed to food and water. The care and use of laboratory animals in this experiment were performed according to the institutionally approved protocol in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC).

For operative procedures, the animals were anesthetized with inhalation of 5% isoflurane in a mixture of 70% nitrous oxide and 30% oxygen. Anesthesia was maintained with 2% isoflurane. During the operative procedures, body temperature was monitored continuously with a rectal probe and was maintained at 37.0 ± 0.5°C by means of a homeothermic blanket control unit and heating pad (Harvard Apparatus, Holliston, MA, USA). The left femoral artery was cannulated with PE-50 tubing for monitoring of mean arterial blood pressure with Pressure Transducer (Harvard Apparatus, Inc., Holliston, MA, USA), and for blood sampling for analysis of pH, PaO_2, $PaCO_2$ and hemoglobin concentration before and after MCAO (OPTI critical care analyze, AVL, AVL Scientific Corporation, Roswell, Georgia, USA). Regional cerebral blood flow (rCBF) was determined in the territory of the MCA by laser-Doppler flowmetry (LDF) (BLF 21 laser Doppler flowmeter, Transonic Systems Inc., Ithaca, NY, USA). For placement of a LDF probe, a burr hole of 2 to 3 mm in diameter was created in the left parietal bone 1 mm caudal to the bregma and 3 mm lateral to the midline.

2. Induction of Acute Focal Cerebral Ischemia

All animals were surgically prepared for MCAO according to the modified method from a known nylon suture model.[28, 29] Under an operating scope and general anesthesia, the left common carotid artery (CCA) was exposed via a midline pretracheal incision; a self-retaining retractor was positioned between the digastrics

and sternomastoid muscles, and the omohyoid muscle was divided. The vagus and sympathetic nerves were separated carefully from the artery.

The CCA was double-ligated permanently with 5-0 black silk proximal to its bifurcation. The external carotid artery (ECA) was ligated superior to the origin of occipital artery and inferior to the superior thyroid artery. The internal carotid artery (ICA) was dissected distally to expose the origin of the pterygopalatine artery (PPA) and carefully separated from the adjacent vagus nerve. The occipital artery was then ligated with PPA and coagulated. At this point, the ICA is the only remaining extracranial branch of the CCA.

The ICA was ligated loosely proximal to its origin and a microvascular clip was placed across the ICA. And then, 4-0 monofilament nylon suture (Ethicon, Edinburg, UK), its tip rounded by heating near a flame and coated with 0.1% poly-L-lysine (Sigma, St. Louis, MO, USA), was introduced into the ICA lumen through a puncture of the origin of ICA. The silk suture around the ICA stump was tightened around the intraluminal nylon suture to prevent bleeding, and the microvascular clip was removed. The filament was advanced approximately 23 mm from the ICA origin into the MCA or stopped when there was slight resistance. The nylon suture was tightly fixed at the final position with a silk suture.

3. Experimental Groups

The rats were subjected to permanent MCAO for 1 hr (n = 4), 4 hr (n = 5), 8 hr (n = 4), 12 hr (n = 4), 16 hr (n = 4), 20 hr (n = 4), and 48 hr (n = 3). Four rats were used for controls that were underwent the sham operation, which was performed in the same manner with MCAO, except that the MCA was not occluded.

4. Motor Disability Test

Neurologic evaluation was performed before and after MCAO, and before sacrifice. All surviving animals were graded on two types of scales, which were modified from the scales by Longa et al.[28] and Garcia et al.[30]

A. Modified Longa's method[28, 29]

Four items were evaluated and one point was given to each item when an animal had a deficit, then summed for a total score. They were (1) failure to grasp the edge of a table when an animal was hung by its tail, (2) failure to extend right forepaw fully, (3) a circling motion toward the paretic side when attempting to walk, and (4) falling to the lateral side when pushed gently. Thus, an animal without a neurologic deficit scored 0 while that with maximal deficits scored 4 points.

B. Modified Garcia's method[30]

Six items were evaluated. The 0-3 point was given to each item when an animal had a deficit and summed for a total score. The minimal neurologic score is 3 and the maximum is 18. The items evaluated were (1) ability to approach all four walls of the cage, (2) symmetry in the movement of four limbs, (3) forepaw outstretching, (4) climbing and gripping abilities on wire cage, (5) reaction to stimulus on both sides of body, (6) response to vibrissae touch.

5. Brain Tissue Preparation

All animals were sacrificed by transcardiac perfusion using a peristaltic pump under deep anesthesia with intraperitoneal urethane injection. The brains were removed immediately after transcardiac perfusion with cold heparinized normal saline, and sectioned into 2 mm-thick coronal blocks using a rat brain. The 4th coronal slices were pre-fixed with Karnovsky solution (2% glutaraldehyde, 2% paraformaldehyde, 0.5% $CaCl_2$) and used for TEM.

The 3rd blocks of the brain were divided into ischemic and non-ischemic hemispheres. The divided blocks were embedded into cryomold with Tissue-Tek OCT compound (Miles Inc., Elkahart, IN, USA) and quick-frozen with 2-methylbutan cooled with dryice and then stored at -80°C. These frozen blocks were used for gelatin zymography.

6. Extraction of Protein and Purification of MMPs

The 50 Cryostat 10 μm-thick frozen sections obtained from the frozen blocks were homogenized with 400 μl working buffer (50 mM Tris HCl [pH 7.5], 150 ml NaCl, 5 mM $CaCl_2$, 0.05% BRIJ-35, 0.02% NaN_3, 1% Triton X-100) containing 1 mM phenylmethyl sulfonyl fluoride and centrifuged at 4°C and 9000 rpm for 20 minutes.

The supernatant was stored at -80°C. The protein concentration was measured based on the Bradford method using bovine gamma globulin (Bio-Rad Laboratories, Herculus, CA, USA).

MMPs were purified based on a method using gelatin-sepharose 4B, as previously reported.[17, 29] Briefly, after rinsing 50 μl of gelatin-sepharose 4B (Pharmacia Biotech, Uppsala, Sweden) with 200 μl of working buffer 3 times, the protein extracts were mixed and incubated on a rocking plate for 30 minutes at 4°C. Then, the samples were centrifuged at 7000 rpm for 5 minutes, rinsed with 200 μl of working buffer, and centrifuged again. The precipitants were added to 50 μl of elusion buffer (working buffer to which 10% dimethylsulfoxide was added) and incubated for 30 minutes, rocking at 4°C. After centrifugation, the supernatant was taken and stored at -80°C until needed for gelatin zymography.

7. Gelatin Zymography

The purified protein extracts were mixed with an equal volume of sample buffer (80 mM Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate [SDS], 10% glycerol, 0.01% bromophenol blue) and were subjected to zymography as previously published. [17, 29]

The gelatinolytic activity of these samples was detected by 8% SDS-polyacrylamide gel containing 1% gelatin. Sample gels were rinsed in 150 ml of 2.5% Triton X-100 (15 minutes) and incubated with 250 ml of 50 mmol/L Tris-HCl buffer (pH 7.5, 10 mM $CaCl_2$, 0.02% NaN_3) for 43 hours at 37°C. After incubation, the gels were stained with 0.1% amido black containing acetic acid, methanol, and distilled water (volume ratio 1:3:6) for 1 hour, then destained by four washes with the same solution without amido black for 130 minutes. These gels were scanned using a flatbed scanner, and the gelatinolytic bands were analyzed and quantified by means of

a gel plotting macro using the Scion Image program

8. Transmission Electron Microscopy

For electron microscopic observations, the 4th blocks of the brain were fixed with glutaraldehyde in 2% paraformaldehyde for overnight at 4°C, washed in 0.1 M phosphate buffer, pH 7.4 and then post-fixed in 1% osmium tetroxide in the same buffer for 15 min. Then, the specimens were dehydrated through a graded series of ethanol, exchanged through propylene oxide, and embedded in a mixture of EPON.

Subsequently, ultrathin sections were obtained by ultramicrotome (ULTRACUT UCT, Leica, Australia) with a diamond knife. The sections (1 mm × 1 mm) were obtained from the basal ganglia in each animal, where an infarction is consistently found in this model (Figure 1). Ultrathin sections were double stained with uranyl acetate and lead citrate, and examined in a TEM (JEOL-1011, JEOL, Japan) at 80 kV.

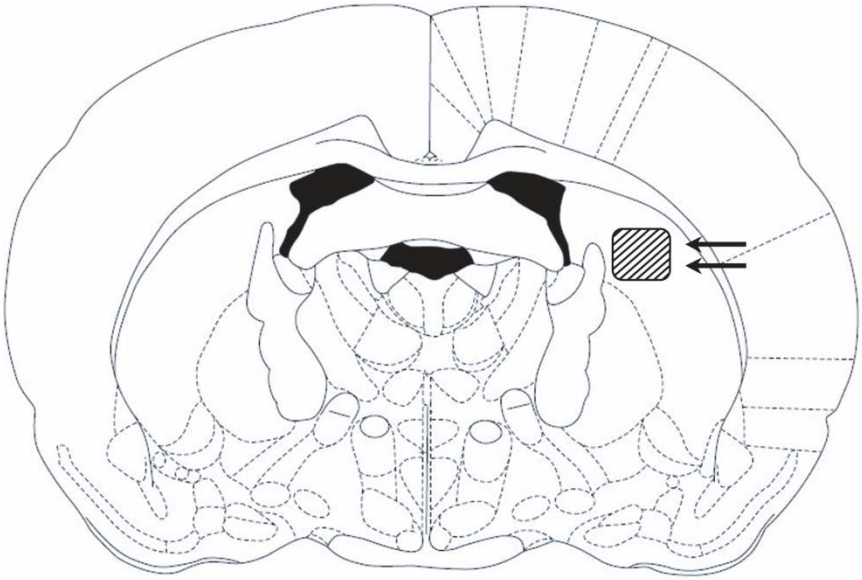

Figure 1. The sampling site of the rat brain for TEM. The diagram was adopted from the atlas for the rat brain[31]

9. Measurements of Microvascular Basement Membrane and Astrocyte Contacts and of Mean Basement Membrane Density

The proportion of intact BM-astrocyte contacts and mean BM density were measured from five randomly selected microvessels in the ischemic basal ganglia. The digital images of the microvessels were transferred to the PC. By using a Scion image program, the circumferential length of the microvessel and the length of intact astrocyte endfeet that contacts to the BM were measured. The percent of the intact contact (the length of intact BM-astrocyte contact/total circumferential length of the microvessel x 100) were calculated. The mean density of circumferential basement membrane was also measured by using a Scion image program. To correct photographic variation among each electron micrograph in measurement of mean BM density, the density of darkest regions of adjacent three myelin sheaths was measured and was used as the standard. The densities of the myelin sheaths were not different among the subjects measured (238.27 ± 15.21, $P = 0.151$).

10. Statistical Analysis

All statistical computations were performed using SAS version 8.2 (SAS Institute, Cary, NC, USA). The differences among all groups were compared by Kruskal-Wallis test followed by post hoc Dunn's method (MMPs activity and neurological outcome). The differences among all groups were compared by one way ANOVA test followed by post hoc Duncan's method (BM-astrocyte contacts, density of BM and myelin sheaths). The differences of physiological variables and regional cerebral blood flow between before and after MCAO were compared by means of paired t-test. Correlations among BM-astrocyte contact, BM density, MMP-9 activity were evaluated by using Spearman's test. The values were presented as a mean ± standard deviation. P values below 0.05 were considered significant.

III. RESULTS

1. Mortality and Neurological Outcome

Two of the 30 rats (6.7%) that underwent MCAO died, while all 4 rats that underwent sham operation survived. None of the animals following the sham operation exhibited motor disability. All rats that were subjected to MCAO showed some degree of neurologic deficits. The mean scores assessed by modified Longa's scale and Garcia's scale were 3.05 ± 0.52 and 8.79 ± 1.55, respectively. The scores among groups were not significantly different from one another (Figure 2).

No significant changes were noted in mean arterial blood pressure, pH, $PaCO_2$, PaO_2 and hemoglobin before and after the MCAO. The mean rCBF was decreased by about 74.4% of the baseline 30 minutes after MCAO (Table 1).

Table 1. Physiological variables and changes of regional cerebral blood flow

Group	MABP (mmHg)	pH	$PaCO_2$ (mmHg)	PaO_2 (mmHg)	Hb (g/dL)	rCBF (%)
Before MCAO	101.95 ± 5.93	7.42 ± 0.014	46.6 ± 2.46	123.8 ± 4.96	12.90 ± 0.81	100
After MCAO	103.22 ± 5.02	7.42 ± 0.017	46.0 ± 1.47	121.9 ± 4.49	12.73 ± 0.55	25.6 ± 4.1*

Values are means ± SD (n=3 to 5 per group).
MABP, mean arterial blood pressure; Hb, hemoglobin; rCBF, regional cerebral blood flow.
* before MCAO vs. after MCAO ($P<0.001$).

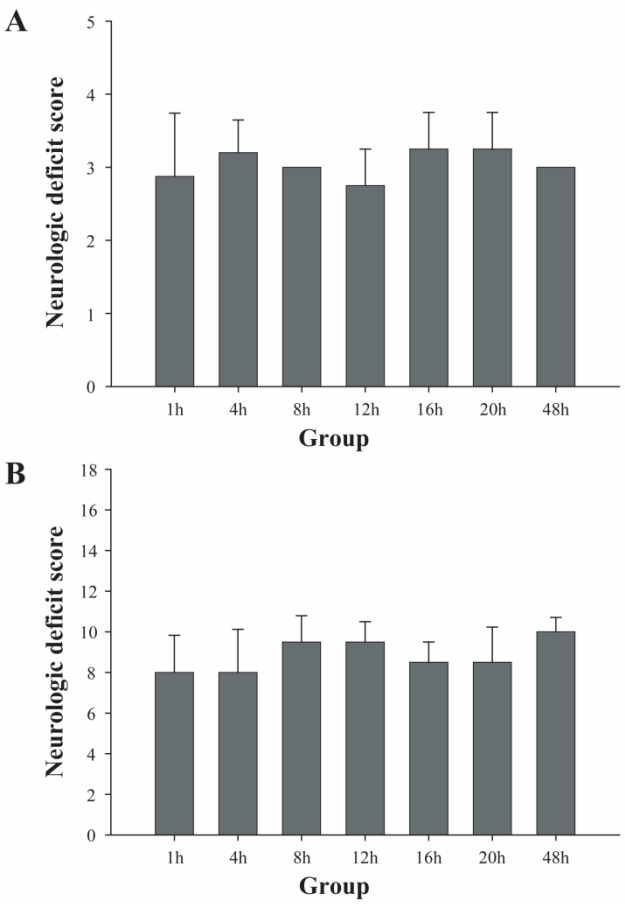

Figure 2. Neurologic deficit scores assessed based on a modified Longa's scale (A) and a Garcia's scale. (B). There is no significant difference among the groups.

2. MMPs Activity during Focal Cerebral Ischemia

MMP-2 and MMP-9 activities were measured by gelatin zymography. MMP-9 activity was detected at 1 hour after MCAO in the ischemic hemisphere and increased at 4 hours. The increase was significant at 12 hours after MCAO. However, MMP-2 activity was not increased in both ischemic and non-ischemic hemispheres at all time points examined until 48 hours after MCAO (Figure 3).

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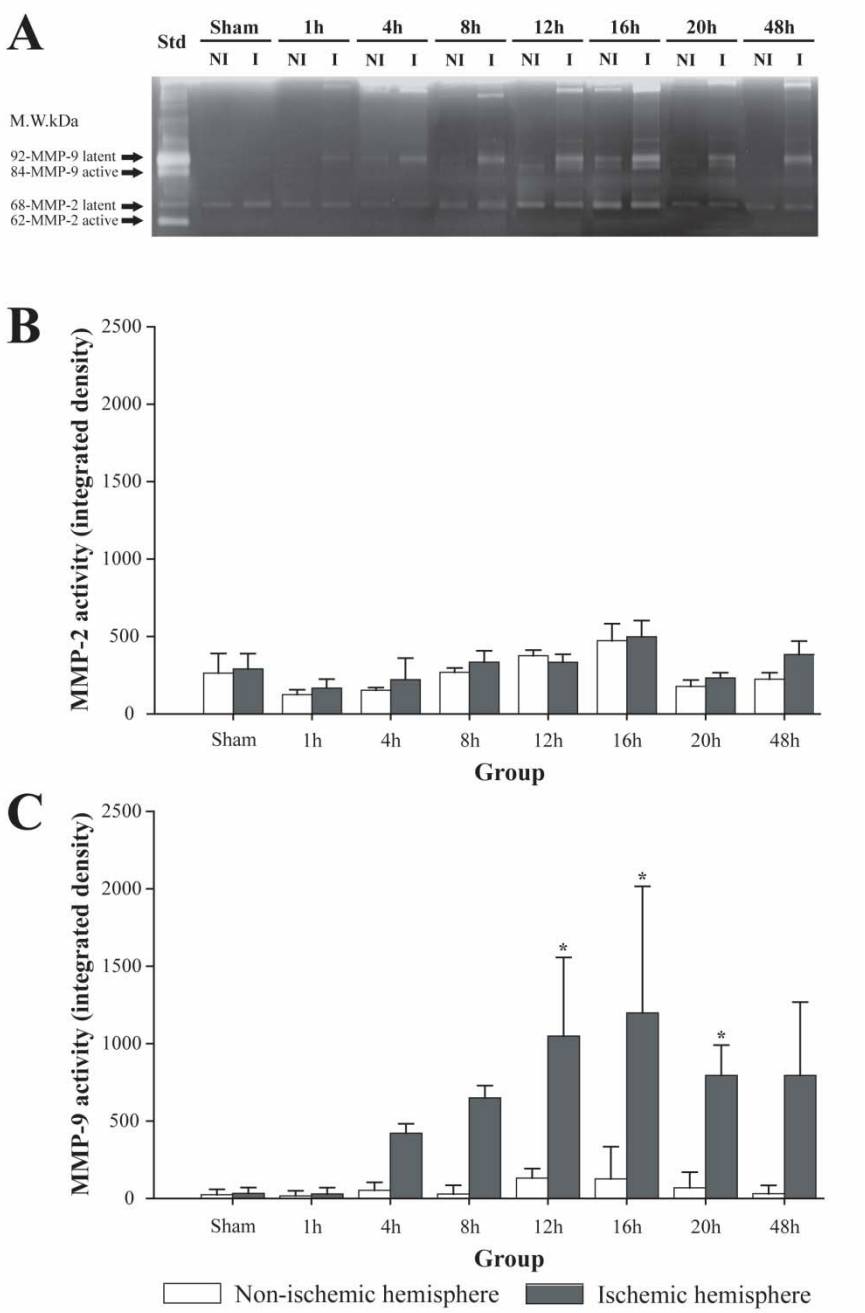

Figure 3. MMP-2 and MMP-9 activities measured by gelatin zymography at various intervals after middle cerebral artery occlusion. (A) Representative zymograms showing clear bands of MMP-2 and MMP-9. (B) There is no change in MMP-2 activities. (C) MMP-9 activity is significantly increased at 12, 18, and 20 hours after MCAO. (*P<0.05) NI, non-ischemic hemisphere; I, ischemic hemisphere; Std, mixture of recombinant MMP-2 and MMP-9 used as the standard.

3. The Temporal Changes on Neurovascular Units in Focal Cerebral Ischemia

In sham-operated rats, 94.08 ± 6.04 % of the microvascular surface was covered by astrocyte endfeet, and the basement membrane was intact (mean density: 174.78 ± 8.28). The cellular components and adjacent matrix were undamaged and compact (Figure 4A). The proportion of intact area of BM-astrocyte contacts was significantly decreased at 4 hours after MCAO and thereafter when compared with the sham-operated group. Mean values of the corrected BM density were decreased in a time-dependent manner. The decrease was significant at 12 hours after MCAO when compared with the sham-operated group (Figure 4 and 5).

At 1 hour after MCAO, astrocyte endfeet ensheathed 85.74 ± 9.45 % of the microvascular basement membrane (Figure 5). Mildly swollen astrocyte endfeet began to be shown occasionally in the ischemic core region (Figure 4B). And the mean density of the BM was 173.64 ± 20.99.

Severe swelling and simultaneous reduction of cytoplasmic density of astrocyte endfeet were observed at 4 hours after MCAO. Focal detachment of astrocyte endfeet from the abluminal surface of the BM was also visible (mean 74.08 ± 7.83 %)(Figure 4C). The mean density of the BM was 168.14 ± 15.02.

At 8 hours after onset of ischemia, the electron micrograph depicts seriously swollen and pale astrocyte endfeet abutting a microvessel. Separation of astrocyte endfeet from the degraded BM (mean density, 160.31 ± 9.14) was observed in average 40.28 ± 13.80 % of the microvascular surface. Also, noticeable tissue damages were seen (Figure 4D and 5).

More altered neurovascular units were demonstrated at 12 hours after MCAO. The astrocyte endfeet enveloped 42.11 ± 27.65 % of microvascular surface (Figure 5). Destruction of the BM was evident in that its margin appeared fuzzy and the decrease of its density was significant (142.61 ± 11.43)(Figure 4E).

At 16 hours after MCAO, damages of the BM and brain tissues were serious. The mean proportion of astrocyte endfeet which attached to the BM was reduced to 15.92 ± 17.54 % and the mean BM density was 128.95 ± 29.85 (Figure 5). Extravasation of water caused by rupture of swollen astrocytic plasma membrane and/or disruption of

the BBB was evident (Figure 4F).

The excessive accumulation of extracellular fluid was observed and only 13.20 ± 13.50 % of the contacts remained and the mean BM density was decreased to 124.48 ± 24.75 at 20 hours after onset of focal ischemia. Also, more astrocyte endfeet were ruptured at this point (Figure 4G and 5).

The contacts between BM and surrounding astrocyte endfeet were rarely observed at 48 hours after MCAO (mean 3.55 ± 10.10 %). The BM was faint and severely damaged (120.45 ± 27.98)(Figure 4H and 5). Collection of water molecules did not localized to the pericapillary space, but was found to be dispersed in the surrounding tissues.

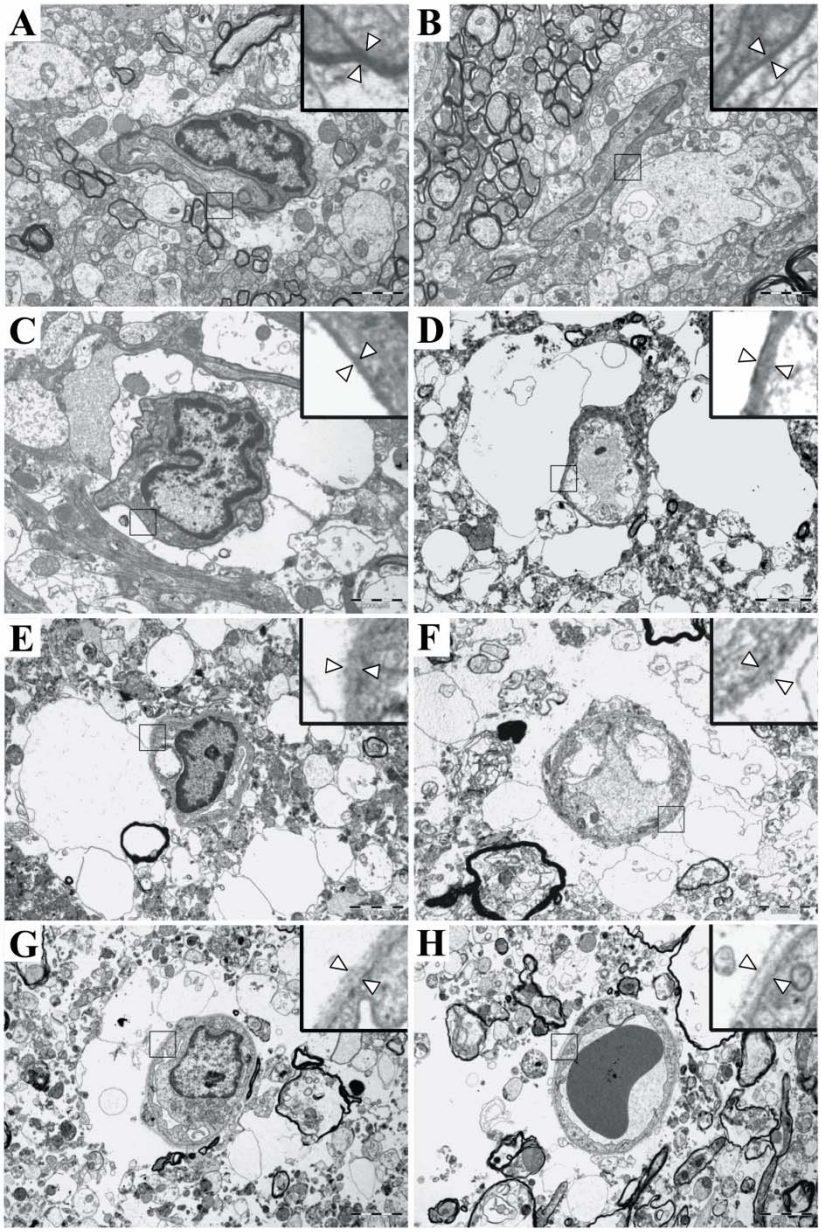

Figure 4. The electron microscopic photographs demonstrating impact of occlusion of the middle cerebral artery on the neurovascular unit. A number of ultrastructural alterations in the neurovascular unit occur during focal cerebral ischemia. (A) Sham-control. Almost all surface areas of the microvessel are covered by endfeet of astrocytes. (B) 1 hr after MCAO, (C) 4 hrs after MCAO. Swelling of astrocytes and focal detachment of astrocyte endfeet from the basement membrane is seen. (D) 8 hrs after MCAO. Marked astrocyte swelling is demonstrated. (E) 12 hrs after MCAO. The proportion of intact basement membrane-astrocyte contacts is markedly decreased. (F) 16 hrs after MCAO. Degradation of the basement membrane is evident. (G) 20 hrs after MCAO. There is excessive accumulation of water around the microvessels. (H) 48 hrs after MCAO. Astrocyte endfeet is no more visible.
(arrow heads = basement membrane)(Original magnification, x 8 000)

4. Temporal Patterns of Changes in the Basement Membrane (BM), BM-Astrocyte Contacts, and MMP Activities

The gross pattern of temporal changes in BM-astrocyte contacts appeared paralleled with that of changes in the BM density (Figure 5A). However, the loss of contacts was evident very early after MCAO and seemed to occur before the rise of MMP-9 values (Figure 5B) or decrease of the BM density (Figure 5A). The loss of contacts and increase of MMP-9 seemed paralleled in a time-dependent manner from 4 hours to 12 hours after MCAO. After 16 hours, while the increase of MMP-9 was ceased, the loss of contacts was further aggravated until 48 hours of observation period (Figure 5B).

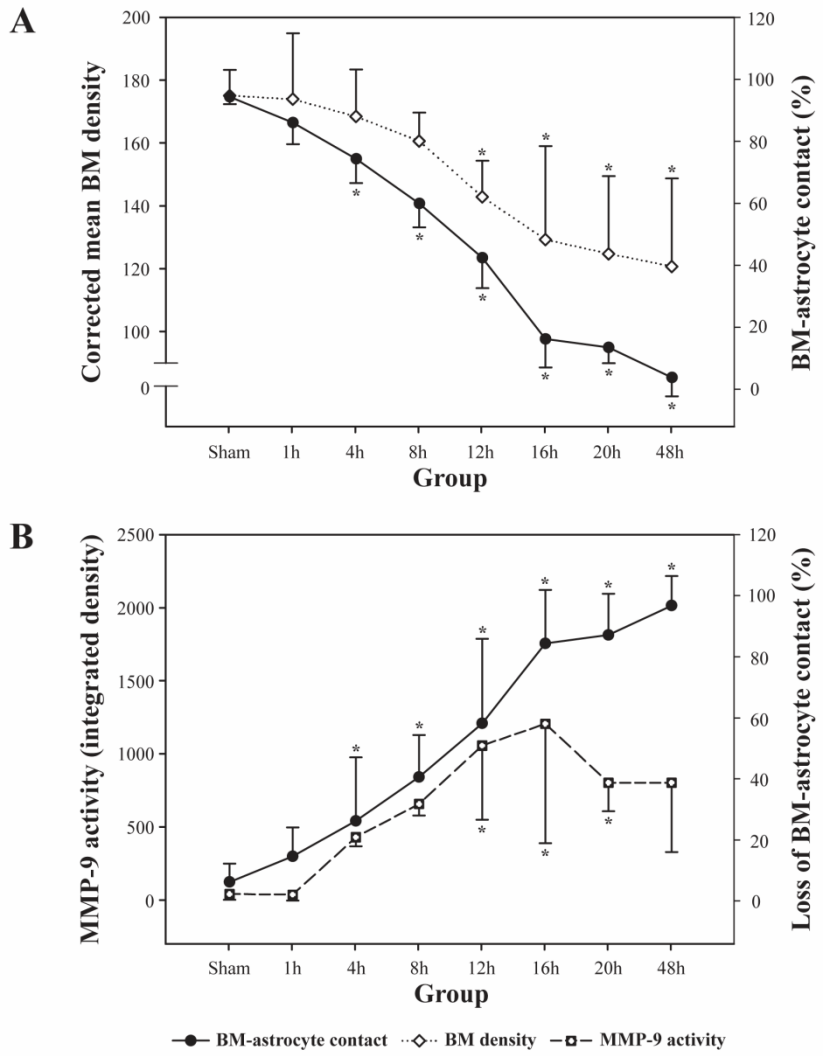

Figure 5. The graphs demonstrating mean values of basement membrane (BM)-astrocyte contacts, BM densities, and MMP-9 activities at each time point after middle cerebral artery occlusion (MCAO). Solid lines indicate the percentage of microvascular surface attached (A)/detached (B) to the enveloping astrocyte endfeet following focal cerebral ischemia. The intact contacts are reduced in a time-dependent manner and rarely remained at 48 hours after MCAO. The decrease is significant 4 hours after MCAO and thereafter when compared with sham-operated group; Dotted line indicates the mean corrected density of microvascular BM which was standardized by using data of mean density from adjacent myelin sheaths. The BM density is significantly decreased at 12 hours and is continuously decreased until 48 hours after MCAO; Long-dashed line indicates MMP-9 activity. The BM-astrocyte contact and BM density appear parallel. The loss of contacts and increase of MMP-9 activities also show a parallel pattern, particularly from 4 hours to 12 hours after MCAO. (*P<0.05, significantly different from the sham)

5. The Correlation among Loss of Microvascular Basement Membrane and Astrocyte contact, Mean Basement Membrane Density, and MMP-9 Activity during Focal Cerebral Ischemia

The relationship between an increase of proteinase (MMP-9) and damage of the BM (BM density and BM-astrocyte contacts) was assessed by means of Spearman's test (Figure 6). The intact BM-astrocyte contacts and the mean BM density showed strong association ($r = 0.784$, $P = 0.000$) (Figure 6A). The mean BM density and the MMP-9 activity exhibited negative correlation ($r = -0.538$, $P = 0.00159$) (Figure 6B). There was a strong negative correlation between the proportion of intact BM-astrocyte contacts and the MMP-9 activity ($r = -0.711$, $P = 0.000$) (Figure 6C)

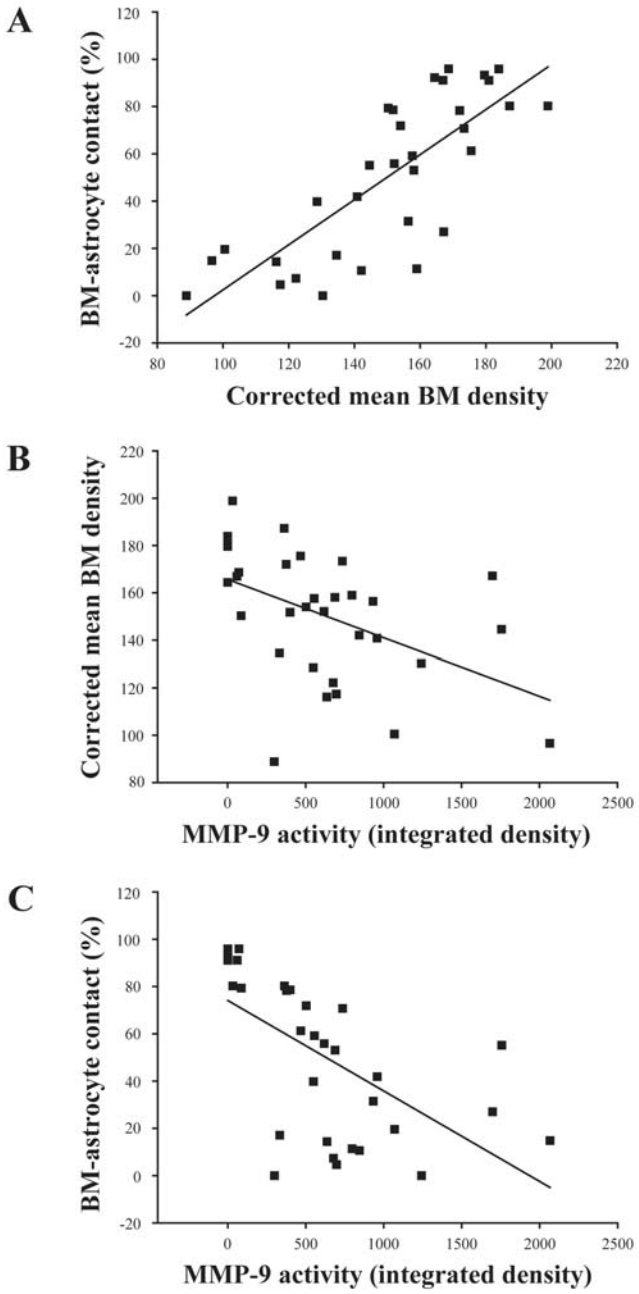

Figure 6. Correlations among percentage of microvascular surface attached to astrocyte endfeet, mean density of basement membrane, MMP-9 activity in focal cerebral ischemia. (A) BM-astrocyte contact and mean BM density shows positive correlation ($r = 0.784$, $P = 0.000$) (B) BM density and MMP-9 activity shows negative correlation ($r = -0.538$, $P = 0.00159$) (C) BM-astrocyte contact and MMP-9 activity shows negative correlation ($r = -0.711$, $P = 0.000$).

IV. DISCUSSION

This study demonstrated that 1) the proportion of the intact microvascular BM and astrocyte contacts was decreased 4 hours after permanent MCAO in rats and continued to decrease in a time-dependent manner thereafter, 2) that the density of microvascular BM was continuously decreased and was significantly decreased from 12 hours after MCAO, 3) that MMP-9 activity was increased at 4 hours and its increase was significant from 12 hours after MCAO, 4) that the BM-astrocyte contact and the mean BM density showed positive correlation, whereas they showed negative correlation with the MMP-9 activity, and 5) that the loss of the contacts, BM density, and increase of MMP-9 activities had close temporal relationships.

Severe ischemia affects not only on neuropils but also on microvessels.[10, 13, 14, 16-19, 32-38] Microvessels are seriously perturbated by ischemic insults. The consequences of the microvascular damage are brain edema[8, 20] and hemorrhagic transformation[21] that may occur following cerebral ischemia.[17, 39-41] In addition, there is accumulating evidence that the loss of cell-ECM contact may result in cell death.[4, 12, 19] Communications between cells and cell-ECM molecules are essential for cell survival and maintenance of cellular function. Thus, a functional concept of neurovascular unit, which emphasizes structural and functional linkage among microvessels, astrocytes and neurons, constitutes an important understanding of the ischemic injury mechanism as well as normal physiology of the brain.[42]

There have been studies that demonstrated indirect evidence of loss of microvascular integrity in the form of decrease in ECM molecules of the vascular BM, loss of integrins that attaches the BM to astrocyte endfeet, and increase of proteinases that degrade vessel wall ECM components.[8, 17, 32, 34, 36, 39, 43] The present study showed in a more direct way that contacts between the microvascular BM and astrocyte endfeet are lost after cerebral ischemia, and that density of BM is decreased in a time-dependent manner. Loss of the contacts occurred at least within 4 hours after ischemia, which suggests that loss of microvascular integrity develops very early after ischemia. This is consistent with findings in non-human primates that reported decrease of

microvascular ECM molecules in 2 hours,[32] that of vascular integrins within 1-2 hours,[4, 18, 19] and that of endothelial cell β_1-integrin expression by 2 hours after MCAO in the ischemic core.[4, 36] These losses are sustained and are not recovered in spite of restitution of blood flow. In this study, loss of the BM-astrocyte endfeet contacts was coincided with degradation of the BM. It also seemed to be accompanied by rupture and loss of astrocyte endfeet.

In this study, the hypothesis that up-regulation of MMP-9 and its detrimental role which degrade the BM are associated with loss of the BM-astrocyte contacts was investigated. Although numerous studies documented that up-regulation of MMP-9 contributes to degradation of most ECM molecules,[8, 17, 20, 39, 44] a detrimental role of MMP-9 in maintaining integral adhesions between cerebral microvessels and surrounding astrocytes is not fully defined. In this study, inverse relationships of MMP-9 activity with BM density and BM-astrocyte contacts were evident, which is an evidence of role of MMP-9 in loss of the microvascular integrity. Furthermore, BM density and BM-astrocyte contact showed positive correlation. In addition, temporal courses of their changes were coincided. In zymographic study, MMP-9 expression seems to be increased after 4 hours, was maximally expressed after 16 hours, and was decreased slightly but maintained higher levels than that in sham-operated group. Similarly, BM density and intact BM-astrocyte contacts were decreased from 4 hours and continuously to 48 hours in a time-dependent manner. The increase of MMP-9, decrease of BM density, and loss of BM-astrocyte contacts paralleled from 4 hours to 12 hours after MCAO. Damages of the basement membrane were significant on electron microscopy 12 hours after MCAO. After this time, loss of the contacts was worsened with more extensive damages of the BM. Although numerous factors may affect on loss of microvascular integrity, the up-regulation of MMP-9 may play a critical role during cerebral ischemia. It may degrade ECM molecules resulting in disruption of normal attachment of the BM to astrocyte endfeet.

Of note, detachment occurred before the increase of MMP-9 or decrease of BM density. In addition to degradation of the microvascular BM, loss of vascular integrins that has normally glued microvascular BM to astrocytes may play a role in the

detachment. Considering that decrease of the integrins occurs very early after ischemia[4, 18, 19] and that destruction of the BM was seen relatively late, early focal detachment of the contacts may be mediated by loss of integrins.

In addition to the BM, several alterations could be observed in electron microscopic findings. Swelling of astrocytes, which is a major response in ischemia,[8, 19, 45-47] is associated with eventual cell death, swelling of astrocyte endfeet around microvessels was already documented to occur within 5 minutes of energy crises.[47] Expectedly, mild astrocytic swelling appeared as early as 1 hour after MCAO in this study. Recent studies suggest that aquaporin-4 (AQP4), a water transporting protein which is mostly found in astrocytes, contributes to swelling of astrocytes.[48, 49] Swelling of astrocytes occur primarily in endfeet adjacent to microvessels, and morphologically, the cytoplasmic density of astrocytes decreases simultaneously in this region.[50, 51] After 4 hours of ischemia, severe swelling and notably decreased density of the astrocytic processes were observed in the present study. Astrocytes that were swollen and detached from the microvascular BM rendered rupture of their membranes, which highlights the importance of cell-ECM contact in cell survival. Extracellular collection of fluid was at first confined to around the microvessels. However, at the time when astrocyte endfeet is no more visible and cellular death is evident, extravasated fluid was dispersed.

V. CONCLUSION

Detachment of the BM-astrocyte contact as well as degradation of the BM in the microvessels has been suggested as being occurred in the ischemic area. MMP-9 plays a key role in the degradation of microvascular BM in cerebral ischemia. However, their temporal patterns and association with MMP-9s are not well known. This study showed the temporal and ultrastructural changes occurring in the BM of microvessels and astrocyte endfeet interface and also demonstrated the potentially contributing role of MMP-9 in degradation of the BM and focal loss of its contact to astrocyte endfeet.

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ABSTRACT (IN KOREAN)

흰 쥐에서 실험적 국소 뇌허혈 유도 후 미세혈관 기저막과 성상세포의 연접의 시간적 변화

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권 일

뇌혈관계는 특징적으로 내피세포, 기저막, 성상세포 말단돌기 및 혈관 주위세포로 구성되는 혈뇌장벽을 갖는다. 그 동안의 혈뇌장벽에 대한 많은 연구들이 내피세포 사이의 치밀이음부에 그 초점이 맞추어져 왔지만, 최근에는 혈뇌장벽을 이루는 모든 구성요소가 뇌혈관의 정상적인 기능 유지에 필수적이라는 관점이 대두되고 있다. 다양한 세포외기질로 구성된 기저막은 혈뇌장벽의 골격을 이루어 뇌혈관의 구조 유지에 중요한 역할을 하며, 성상세포 말단돌기는 유착분자에 의해 기저막에 강하게 부착하여 혈관을 감싸고 있다. 단백분해효소인 Matrix metalloproteinases (MMPs) 중, MMP-2와 MMP-9은 특히 뇌혈관 기저막의 세포외기질을 분해하는 핵심적인 효소로 잘 알려져 있다.

뇌허혈이 발생하면 기저막이 분해될 뿐만 아니라 기저막으로부터 성상세포 말단돌기가 떨어져나가지만, 이 사건들의 시간에 따른 변화 양상과 MMP와의 상관관계는 아직 잘 알려져 있지 않다.

본 연구에서는 흰 쥐의 영구적 중뇌동맥폐색 모델을 이용하여 기저막 세포외기질의 밀도변화와 기저막으로부터 성상세포가 분리되는 과정을 투과현미경을 통해 관찰하였다. 더불어 gelatin zymography를 이용하여 같은 시간대의 MMP 활성도 변화를 알아보고, 기저막-성상세포 연접의 분리, 기저막의 밀도감소가 MMP 활성도와 어떠한 상관관계가 있는지 규명하고자 하였다.

기저막-성상세포 연접은 중뇌동맥폐색 4시간 이후부터, 기저막 세포외기질의 밀도는 12시간 이후부터 의미 있게 감소하여 시간의 흐름에 따라 지속적으로 감소하였다. 허혈손상 부위에서 성상세포의 부종과 세포외액의 축적 또한 관찰되었다. MMP-2의 활성도는 모든 군에서 유의적인 차이를 보이지 않았지만, MMP-9은 허혈반구에서 의미 있게 증가하였다. 이 실험들에서 얻은 결과를 바탕으로 기저막-성상세포 연접, 기저막 세포외기질의 밀도와 MMP-9 활성도의 상관관계를 분석하였다. 기저막-성상세포 연접관계의 변화는 세포외기질의 밀도 감소와 양의 상관관계를 나타낸 반면 ($r = 0.784$, $P = 0.000$), MMP-9 활성도와 기저막의 밀도 변화 ($r = -0.538$, $P = 0.00159$), MMP-9 활성도와 기저막-성상세포 연접의 변화($r = -0.711$, $P = 0.000$)는 음의 상관관계를 보였다. 이처럼 국소 뇌허혈에서 시간에 따른 MMP-9 활성도의 변화는 기저막 세포외기질의 밀도 감소와 기저막으로부터 성상세포가 분리되는 양상과 밀접한 관계가 있는 것으로 사료된다.

본 연구를 통해 국소 뇌허혈 발생 시, 시간의 흐름에 따라 달라지는 뇌혈관의 미세구조와, MMP-9이 뇌혈관 기저막의 분해와 기저막-성상세포 연접 파괴에 미치는 영향을 확인할 수 있었다.

핵심 되는 말: 뇌허혈, 기저막, 성상세포, 투과전자현미경