Microvascular and arterial remodeling induced by chronic hypoperfusion

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Microvascular and arterial remodeling induced by chronic hypoperfusion

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The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

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December 2011

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December 2011

ACKNOWEDGEMENT

This dissertation would not have been possible without the guidance and the help of several individuals who contributed and extended their valuable assistance in the preparation and completion of this study.

First and foremost, my utmost gratitude to Professor Ji Hoe Heo, whose sincerity, encouragement, and careful guidance I will never forget. Prof. Heo has been my inspiration as I hurdle all the obstacles in completing this research work. I will always be grateful to professor Dong Goo Kim, Yang Soo Jang, Hye Yeon Lee, and Seo Hyun Kim for their kind concern and advice. My fieldwork could not be possible without the support of colleagues, especially Eun Hee Kim. And many thanks to colleagues of the Neurology department in Yonsei University and Kyung Hee University.

Last but not the least, I would like to thank my family for their love and encouragement.

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ABSTRACT

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Atherosclerotic stenosis may induce chronic hypoperfusion of the brain when it is severe. Extremely high degree of stenosis which could cause cerebral hypoperfusion was reported to have a paradoxically lower risk of stroke development and less severe stroke when it develops. In the previous study, vascular remodeling and ischemic tolerance induced by chronic hypoperfusion were suggested as the probable mechanisms. However, it remains uncertain which type of vascular remodeling process, that is arteriogenesis or angiogenesis, occurs and from when it is observed after induction of cerebral hypoperfusion if it presents. This

study was aimed to document temporal course of vascular remodeling and molecular expression related to arteriogenesis and angiogenesis after induction of sublethal cerebral hypoperfusion. Cerebral hypoperfusion was induced in wistar rats with the bilateral common carotid artery (CCA) ligation. Regional cerebral perfusion (rCP) was measured in the territory of the middle cerebral artery by laser Doppler flowmetry (LDF). Latex angiography was performed. Protein expression was examined by the western blot. The rCP was decreased by 40.2% immediately after bilateral CCA ligation, when compared with the baseline, and recovered to the baseline level 14 days later. Increased arterial diameter, arterial tortuosity, and increased vascular density were observed at 1 week after bilateral CCA ligation. Connexin 37 was also increased at 1 week and decreased to the baseline level at 3 weeks, after bilateral CCA ligation. The levels of vascular endothelial growth factor and fibroblast growth factor-2 were not changed. In the vascular remodeling induced by sublethal cerebral hypoperfusion, arteriogenesis appeared to play a key role and to start within 1 week.

Key words: cerebral hypoperfusion, vascular remodeling, arteriogenesis, angiogenesis

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I. INTRODUCTION

Atherosclerosis, which is the most common mechanism of ischemic stroke, may induce chronic hypoperfusion of the brain when it is severe. In general, the degree of atherosclerotic stenosis increases risk of ischemic stroke.[1,2] However, extremely high degree of stenosis, which could cause cerebral hypoperfusion, was reported to have a lower risk of stroke development and show fair outcome when stroke occurred.[3-6]

Chronic cerebral hypoperfusion, which was not fatal, could provide neuronal

protective effect against following ischemic insult. Kitagawa et al first described that brief ischemia renders hippocampal neurons tolerant to subsequent ischemia.[7] Ischemic tolerance could be induced by several sublethal stimuli, such as hypoperfusion, metabolic stress, and physical stress.[8-10] Underlying mechanism of ischemic tolerance is still not fully understood, however, cellular stress response was suggested as a major defense mechanism of ischemic tolerance induced by several conditions.[9] In the ischemic tolerance model induced by hypoperfusion, vascular remodeling was also suggested as a possible protective mechanism, besides cellular response.[11]

When ischemia occurs, collateral circulation may develop and influence degree of tissue damage. Stimulation or augmentation of collateral vessel growth might be one of the therapeutic options for the patients with severe arterial stenosis. There are three different forms of vessel growth: vasculogenesis, angiogenesis, and arteriogenesis. Vasculogenesis is defined as the formation of vessels by angioblasts during early ontogenesis. Therefore, vascular remodeling after birth includes angiogenesis and arteriogenesis. Angiogenesis denotes the formation of new capillaries by sprouting and intussusceptions from pre-existent capillaries. Arteriogenesis denotes the formation of mature arteries from pre-existent interconnecting arterioles by smooth muscle remodeling, after an arterial occlusion.[12-17] Angiogenesis accompanies tissue growth and repair, like in wound healing.[18] In ischemic vascular diseases, angiogenesis occurs around areas of tissue necrosis and in

ischemic tissue.[19,20] Hypoxia is a main driving force of angiogenesis.[17] Therefore, it has been a therapeutic target for recovery from ischemic stroke. On the other hand, arteriogenesis refers to the adaptive growth of preexisting arteries in response to an increase of intravascular shear forces. Hemodynamically relevant obstruction or complete occlusion of a large artery causes redistribution of blood flow due to the change of pressure gradients across interconnecting arterioles. As a consequence, preexisting collaterals are recruited and intravascular shear stress increases. Arteriogenesis is potentially able to fully replace an occluded artery whereas angiogenesis cannot.

In previous studies, stroke was less severe when ischemic insults were induced by the middle cerebral artery (MCA) occlusion in rats that were preconditioned by chronic cerebral hypoperfusion.[21,22] Both cellular and adaptive vascular responses were seen after induction of chronic cerebral hypoperfusion 4 weeks after bilateral common carotid artery (CCA) ligation. Apurinic/apyimidinic endonuclease/redox factor-1 that plays a role in cellular defense mechanism increased in the sections close to the occipital pole in the brains of rats after induction of chronic cerebral hypoperfusion.[21] Vascular remodeling occurred in the form of vessel enlargement and increased density of vessels in the brain after induction of cerebral hypoperfusion.[22]

In the previous study, the presence of protective cellular responses and vascular remodeling were examined 4 weeks after the bilateral CCA ligation.[22] Therefore, it is unknown when the vascular remodeling process begins in the

bilateral CCA ligation model. In addition, it remains uncertain which type of vascular remodeling process, that is arteriogenesis or angiogenesis, occurs. Although they concluded that arteriogenesis do a dominant role for restoration of reduced cerebral flow, increased vascular density in the cerebral parenchyma was observed both in the capillary sized vessels and arteriole-sized vessels in rat bilateral CCA ligation models. There was still a possibility that both arteriogenesis and angiogenesis could have a role in the cerebral flow restoration.

This study aimed to document temporal course of vascular remodeling after induction of cerebral hypoperfusion. The temporal courses of molecular expression related to arteriogenesis and angiogenesis were investigated to examine which vascular response is responsible for restoration of cerebral perfusion after bilateral CCA ligation. I also investigate the brain location where the vascular remodeling initiates and dominates.

II. MATERIALS AND METHODS

1. Experimental animals and preparation

Male wistar rats, weighing 250-350g were used for all experiments. Animals were housed in plastic cages with soft bedding under a 12/12 hour reversed light and dark cycle, and freely accessed to food and water. The environmental temperature was maintained at 22.0 ± 2.0°C, the humidity at 50 ± 10%, and the noise level below 40-50 phons. A barrier system that had regular pad change (twice a week), and monitoring of microorganism for specific pathogen free animal were used for operative procedure. 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).

2. Experimental groups

Experimental animals were grouped into 6 groups according to the period after bilateral CCA ligation; 1 day, 4 days, 1 week, 2 weeks, 3 weeks and 4 weeks group. Four of 77 rats died. Among survived 73 rats, immunofluorescent staining was performed in the 1 day, 4 days, and 7 days groups. Latex angiography and western blot were performed in the 1 week, 2 weeks, 3 weeks, and 4 weeks group. Number of animals in each group was shown Table 1. Thirteen rats were subjected to sham operation and were used for the control

group (Table 1).

Table 1. Number of rats in each experimental group.

Investigational methods	Groups						
	1 day	4 days	1 week	2 weeks	3 weeks	4 weeks	control
immunofluorescent staining	3	3	3				3
latex angiography			8	8	8	8	5
western blot			8	8	8	8	5

3. Operation and induction of sublethal cerebral hypoperfusion

Sublethal cerebral hypoperfusion was induced by means of bilateral CCA ligation as previously described.[21,23-25] Briefly, after making a midline cervical incision, the CCAs were exposed bilaterally. The right CCA was double-ligated with 5-0 black silk sutures, and then the left CCA was double-ligated 1 hour later. The sham controls underwent the same operation, except bilateral CCA ligation. Anesthesia was induced by 5% isoflurane delivered with mixed gas of 30% oxygen and 70% nitrous oxide and was maintained with 2% isoflurane. Body temperature was monitored continuously with a rectal probe and was

maintained at 37.0 ± 0.5°C by a heating pad (Havard Apparatus, Holliston, MA, USA).

4. Measurement of regional cerebral perfusion

Regional cerebral perfusion (rCP) was determined in the territory of the MCA by laser Doppler flowmetry (BLF 21 laser Doppler flowmeter, Transonic System Inc., Ithaca, New York, USA). The skull around the bregma was exposed and drilled 2 to 3 mm in diameter at the point 1 mm posterior to 3 mm left of the midline with a dental burr. Laser Doppler flowmetry probe with 1.2 mm diameter tip was placed on the dura mater within the hole.

Regional cerebral perfusion was measured for 30-60 minutes, before the bilateral CCA ligation, just after ligation, 1 day, 4 days, 1 week, 2 weeks, 3 weeks, and 4 weeks after bilateral CCA ligation.

5. Evaluation of arteriogenesis

Postmortem angiography was performed by a modification of the Coyle's method.[22,26,27] Animals of each group under anesthesia with 2% isoflurane, were treated with lethal dose of intravenous papaverine hydrochloride (40-50 mg/kg body weight). The descending thoracic aorta was clipped and white latex solution, mixed with carbon black ink and warmed to 37°C, was injected through the ascending aorta to visualize cerebral vessels. The rat was decapitated 30 minutes after latex injection. The brain was fixed with 4%

formalin solution, and photographed using a stereozoom microscope with a camera.

The presence of arteriogenesis was examined macroscopically and microscopically. Macroscopic examination was done by measurement of vascular diameters of Circle of Willis and tortuosity of the basilar artery. On the view of the ventral surface of the brain, diameters were measured at the most proximal portion of the MCA, the most proximal portion of the anterior cerebral artery, the middle portion of the posterior communicating artery, the precommunicating segment and the postcommunicating segment of the posterior cerebral artery, and the middle portion of the basilar artery (BA) by using the Scion image software (Scion corporation, Maryland, USA). Vascular tortuosity (VT) of the BA, which was based on the method of a previous report, was defined as the ratio of the arterial length to the straight distance between the top of the BA and the vertebrobasilar junction (Figure 1).[22,26] The VT of the BA was calculated using the Scion image software. Data of vascular diameter was adjusted with dividing by body weight.

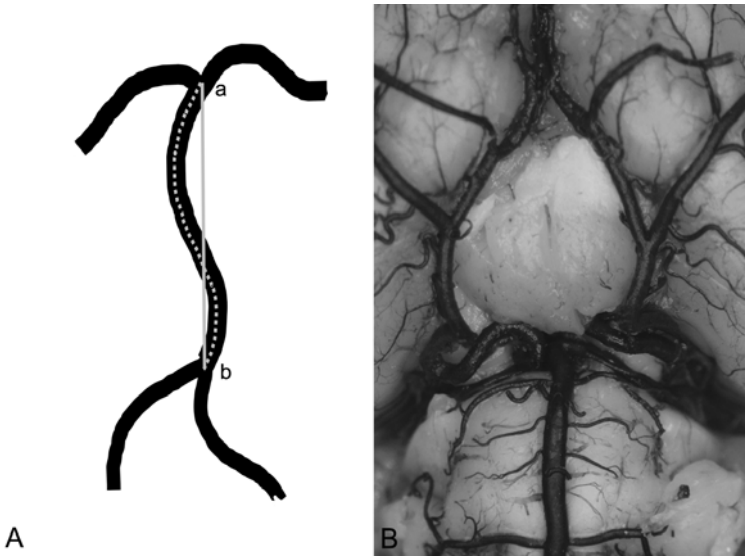


Figure 1. Measurement of vascular tortuosity of the basilar artery. Vascular tortuosity (VT) was defined as the ratio of the arterial length (a dotted curved line; a~b) to the straight distance (a solid line; a-b) between the two end points of the basilar artery (A and B). B. cerebral arteries on the ventral surface of a rat brain.

Microscopic examination of arteriogenesis was done by measuring vascular density in the coronal section of the brain. After measurement of vascular diameters and the BA tortuosity, each brain was sliced into coronal sections with 2.0 mm thickness, using a rat brain slicer matrix. From the frontal pole, five 2.0 mm-thick blocks were obtained from each rat brain. Each coronal blocks were embedded into cryomold with Tissue-Teck OCT compound (Miles Inc., Elkahart, IN) and frozen in 22-methylbutane cooled with dry ice and then stored at -80°C. Coronal cryostat sections with 40 μm-thickness were obtained at approximately 1, 3, 5, 7, and 9 mm from the frontal pole of the brain and mounted on a slide. Vascular diameters and numbers were measured in randomly selected 150-250 points of each section, which constituted a 25% fraction of the section, using Stereo Investigator (MBF bioscience, Vermont, USA). Vascular density was expressed as the number of vessels per unit area (mm^2).

6. Western blot analysis

Animals in each group were sacrificed by transcardiac perfusion using a peristaltic pump under deep anesthesia with intraperitoneal urethane injection. After sacrifice, the brains were immediately removed and were sliced serially into coronal blocks with 2.0 mm thickness using a rat brain slicer matrix. The 1^{st}, 3^{rd}, 5^{th} and 7^{th} fresh blocks from the frontal pole were embedded into cryomold with Tissue-Tek OCT compound (Miles Inc., Elkahart, IN, USA) and quick-frozen with 2-methylbutane cooled with dry ice and then stored at -80°C.

Protein extraction for connexin 37, vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), and desmin was performed as follows. The tissue was homogenized in ice-cold lysis buffer [10 mM Tris, pH 7.8, 10 mM KCl, 0.1 mM ethylene diamine tetra acetate (EDTA), 1.5 mM $MgCl_2$, 20% glycerol, 0.2% NP-40, 0.5 mM dithiothreitol (DTT), 1 mM Na_3VO_4, 0.4 mM phenyl methane sulphonyl fluoride (PMSF)]. They were centrifuged at 15000 rpm and 4°C for 5 min, and the supernatant was collected. Protein concentration of the supernatant was determined based on the Bradford method using bovine gamma globulin (BioRad Laboratories, Herculus, CA, USA). Total 60 µg of protein were resuspended in 2X sample buffer (4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH 6.8), boiled for 5 min, and was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Then, the gel was transferred for 35 min on to a poly-vinyli-dene-fluoride (PVDF) membrane. The nonspecific binding of antibodies was blocked with 5% skim milk Tris-buffered saline (TBS, 50 mM Tris-Cl, pH 7.5, and 150 mM NaCl) for 2 hours at room temperature. The membrane was then probed with polyclonal rabbit anti-connexin 37 (1:300, Abcam, UK), polyclonal mouse anti-FGF-2 (1:200, Millipore, MA, USA), polyclonal goat anti-VEGF (1:300, R&D systems, MN, USA), or polyclonal rabbit anti-desmin (1:500, Abcam, UK) overnight at 4°C. After three washes with 0.5% Tween 20 in Tris buffered saline (T-TBS), the blots were further incubated for 1 hour at room temperature with horseradish

peroxidase conjugated secondary antibodies (1:5000, Amersham Biosciences, UK). The immunoreactive bands detected by electrochemiluminescence (ECL) reagents were developed by Hyperfilm-ECL (Amersham Biosciences, Little Chalfont, UK). The relative density of bands was analyzed on Gene Tools (Pharmatech, Cambridge, UK). The densitometric values were normalized with respect to the values of α-tubulin (1:15000, Oncogene, CA, USA) immunoreactivity to correct for any loading and transfer differences between samples.

7. Immunofluorescent staining

Immunofluorescent staining was performed for detection of Ki 67. Brain tissue block preparation was performed using a same method for western blot. Coronal cryostat sections with 10 μm-thickness were obtained from the frozen 1st, 3rd, 5th and 7th fresh blocks from the frontal pole and mounted on a slide. The rabbit polyclonal Ki 67 (1:50, Abcam, Cambridge, UK) and the mouse monoclonal collagen type 4 (1:50, Santa cruze Biochnology, CA, USA) were used. The frozen sections were fixed with methanol for 10 minutes and then immersed in 100 mM glycine in phosphate buffered saline (PBS) (100 mM Na_2HPO_4, pH 7.4) for 10 minutes. This was followed by a rinsing in PBS solution and incubation with Blotto (5% hydrated nonfat dry milk with 1% BSA) for 1 hour to reduce nonspecific binding. Under humidified conditions, primary antibody was applied to each section followed by overnight incubation

period at 4°C. After washing each slide with PBS, Cy3-conjugated donkey anti-rabbit IgG (1:100, Jackson Immuno Research, PA, USA) and fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (1:100, Jackson Immuno Research, PA, USA) antibody were incubated for 1 hour at room temperature. After three washes with PBS, sections were cover slipped with a 4'-6-Diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). Tissues were examined using a Carl Zeiss LSM 510 confocal microscope equipped with argon and helium lasers.

8. Statistical analysis

Statistical analyses were performed using SPSS for Windows (version 15.0, SPSS Inc., Chicago, IL, USA). Quantitative data were expressed as mean ± standard deviation. The Kruskal-Wallis test, Mann-Whitney U test, and analysis of variance (ANOVA) were used as appropriate according to characteristics of variables. Statistical significance was set at $p<0.05$.

III. RESULTS

1. The temporal change of the regional cerebral perfusion following cerebral hypoperfusion

Regional cerebral perfusion (rCP) was decreased by 40.2% immediately after bilateral CCA ligation when compared with baseline. The rCP recovered to 54.2% at 1 day, 71.2% at 4 days, 76.9% at 1 week, 90.9% at 2 weeks, 94.5% at 3 weeks, and 97.8% at 4 weeks of the baseline level. Recovery of the rCP was similar to the baseline level from 14 days after bilateral CCA ligation ($p<0.01$) (Figure 2).

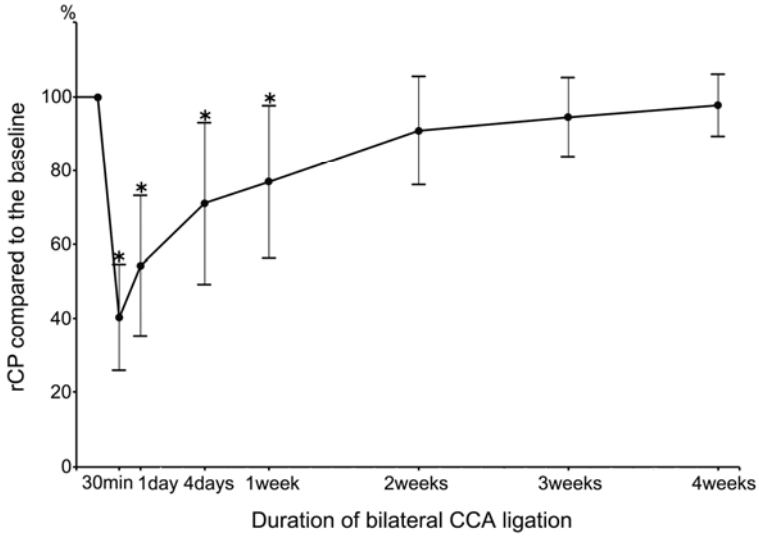


Figure 2. The temporal change of the regional cerebral perfusion (rCP). The rCP was decreased immediately after bilateral CCA ligation and recovered to the baseline level 2 weeks later.
CCA: common carotid artery
*$p<0.01$ by ANOVA

2. Presence of arteriogenesis

A. Macroscopic examination of arteriogenesis

Diameters of the arteries in the Circle of Willis and tortuosity of the basilar artery (BA) were assessed. Measured diameter was adjusted by dividing the diameter of the artery by the body weight. The adjusted diameters of the bilateral posterior cerebral arteries, right posterior communicating artery, and BA in the all bilateral CCA ligated groups were larger than those in the control group ($p<0.05$, Table 2). The adjusted diameters of the left posterior communicating artery in the 2 weeks, 3 weeks, and 4 weeks group were larger than those of the control group. The adjusted diameters of the bilateral MCAs in the 4 weeks group were larger than those in the control group. There was no significant difference of the adjusted diameters of the anterior cerebral arteries between the bilateral CCA ligation groups and control group.

Table 2. Diameters of the arteries in the circle of Willis.

	Control	Groups				p value*
		1 week	2 weeks	3 weeks	4 weeks	
Rt MCA	0.306±0.014	0.342±0.457	0.358±0.431	0.390±0.290†	0.403±0.065†	0.01
Lt MCA	0.317±0.020	0.315±0.027	0.344±0.045	0.367±0.049	0.383±0.033‡	0.007
Rt ACA	0.284±.028	0.307±.054	0.314±0.044	0.033±0.046	0.033±0.054	0.419
Lt ACA	0.284±0.035	0.303±0.056	0.308±0.030	0.355±.038	0.360±0.059	0.016
Rt Pcom	0.271±0.031	0.384±0.060†	0.417±.066†	0.465±0.049†	0.447±0.063†	0.001
Lt Pcom	0.281±0.033	0.406±0.069	0.435±0.143†	0.475±0.066†	0.492±0.068†	0.006
Rt P1	0.158±.052	0.412±0.059†	0.443±0.046†	0.434±0.069†	0.412±0.062†	0.001
Lt P1	0.190±0.024	0.358±0.151†	0.456±0.050†	0.421±0.056†	0.429±0.091†	0.001
BA	0.274±0.049	0.444±.087†	0.447±0.053†	0.510±0.082†	0.511±0.070†	0.001

Diameters are adjusted by body weight. Values are mean ± SD (m/g).

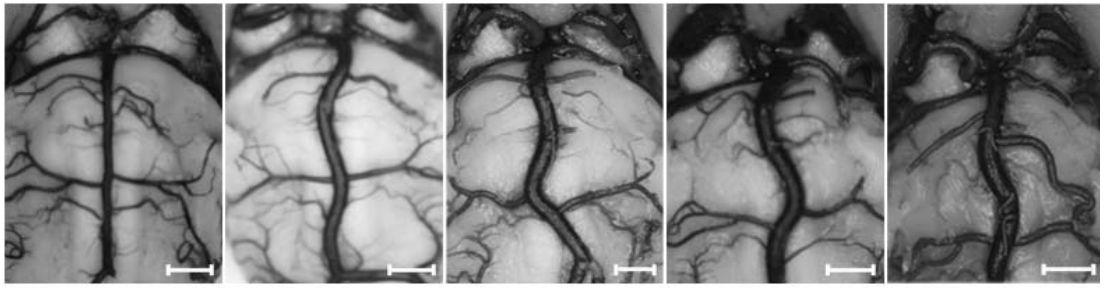
BA, basilar artery; MCA, middle cerebral artery; Pcom, posterior communicating artery; P1, precommunicating segment of the posterior cerebral artery.

* mean statistic significance between groups by ANOVA.

† $p<0.05$ different from the control group by post-hoc analysis, ‡ $p<0.05$ different from the 1 week group by post-hoc analysis.

Tortuosity of the BA was significantly increased in the all bilateral CCA ligation groups ($p<0.05$, Table 3) when compared with that of the control group. The tortuosity of the BA was not different among the bilateral CCA ligation groups.

Table 3. Tortuosity of the basilar artery.

	Group					p
	Control	1 week	2 weeks	3 weeks	4 weeks	value*
Representative photographs						
Mean±SD	1.011±0.004	1.089±0.032†	1.081±.054†	1.094±0.020†	1.111±0.031†	0.001
Range	1.01-1.02	1.06-1.12	1.05-1.11	1.08-1.11	1.09-1.13	

*means statistical significance by ANOVA
† $p<0.01$ by post-hoc analysis. Scale bar = 2 mm

B. Microscopic examination of arteriogenesis

The density of any size of the vessel was increased in the one week group, compared to the control group. The difference of the vascular density tended to be greater in the sections far from the frontal pole, that is, in the rear sections among the serially sliced coronal blocks from the frontal pole (Figure 3). Comparing to the control group, increase of the density was more obvious in larger vessels (larger than 70 μm) than was in the smaller vessels (smaller than 30 μm) in the all groups with bilateral CCA ligation (Figure 4, $p<0.01$).

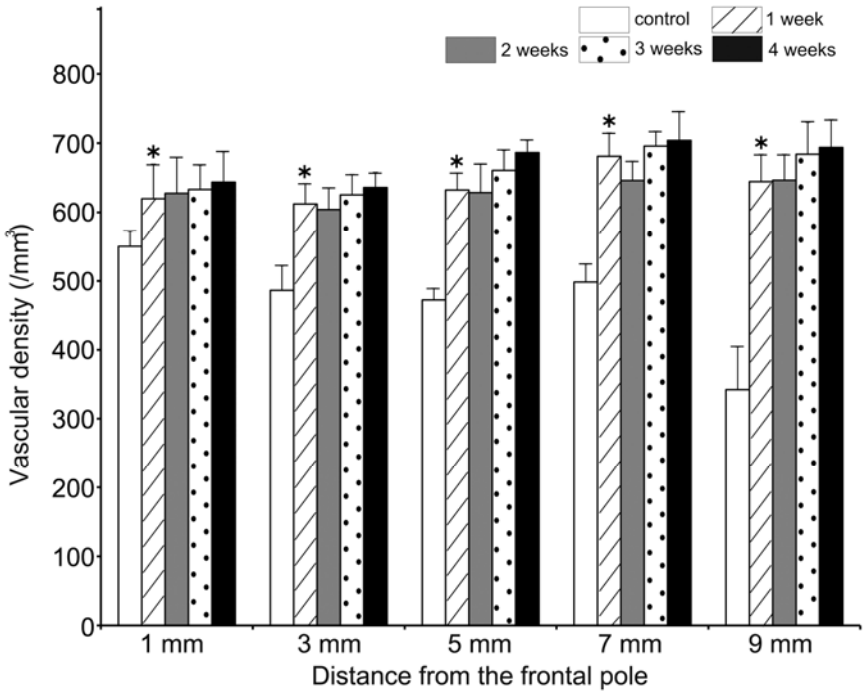


Figure 3. Density of any size of vessels (/mm^3). The vascular densities of the bilateral common carotid artery ligation groups were larger than those of the control group. The difference of the vessel density tended to be greater in the sections far from the frontal pole.

* $p<0.05$ compared to the sham control group.

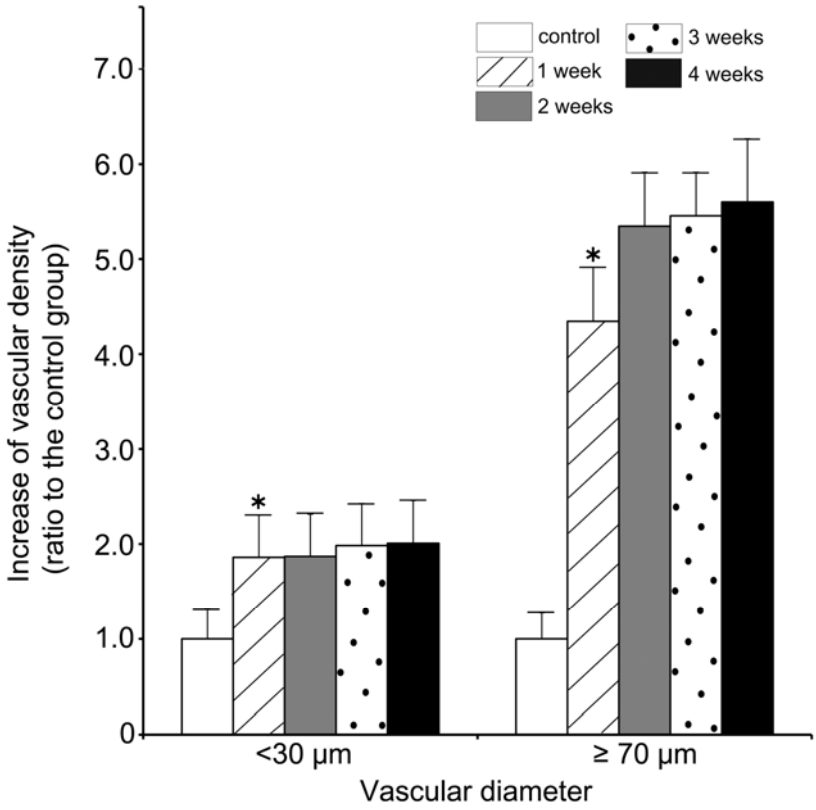


Figure 4. The vascular density according to the vascular diameter. Comparing to the control group, vascular density was significantly increased both in the small vessels (<30 μm) and large vessels (≥70 μm). However, increase of vascular density was greater in larger vessels (≥70 μm) than was in small vessels (<30 μm). Increasing tendency was shown until 2 week after bilateral CCA ligation in the larger vessels, but not in the small vessels.
*$p<0.05$ compared to the control group.

3. The temporal change of connexin 37, desmin, and Ki 67

The levels of connexin 37 and desmin, which are known to be associated with arteriogenesis, were measured using western blot. The level of connexin 37 in the whole brain was significantly higher in the one week group than that in the control group (optical density 3.12±0.07 in control group vs. 5.27±1.63 in the one week group, p=0.002, Figure 5). In the 3 weeks group, the level of connexin 37 tended to be decreased. Increase of connexin 37 was more obvious in the rear sections compared to those in the front sections, among the serially sliced coronal sections (Figure 6). The level of desmin showed a decreasing tendency after bilateral CCA ligation, however there was no significant difference among the groups, both in the whole brain and in each coronal section. Mean optical densities of desmin in the whole brain were 10.056 (control), 9.386 (1 week group), 6.436 (2 weeks group), 6.713 (3 weeks group), and 10.284 (4 weeks group).

Double immunofluorescent staining was performed using samples obtained 1 day, 4 days, and 1 week group. Ki 67 was co-localized with DAPI positive cells and collagen type IV positive vessels. Expression of Ki 67 was increased at 4 days group (Figure 7).

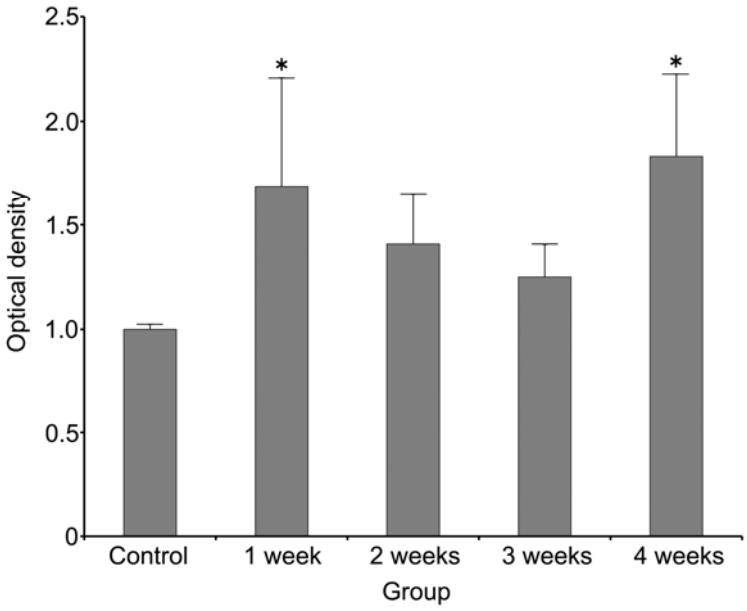
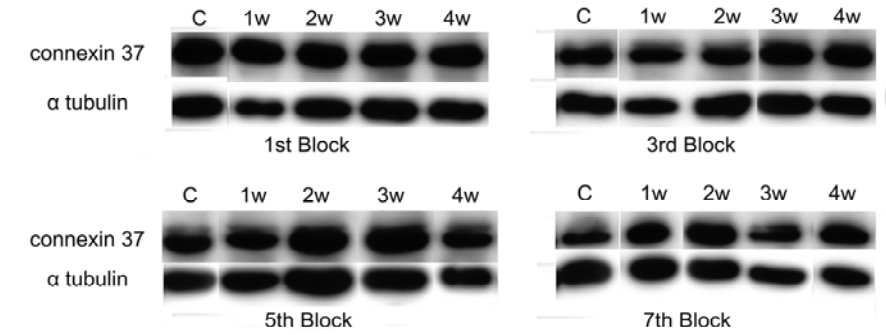


Figure 5. The temporal change of connexin 37 in the whole brain following bilateral common carotid artery ligation. The level of connexin 37 of the whole brain was significantly higher in the 1 week and 4 weeks groups (p=0.002).
CCA, common carotid artery
*p<0.05 compared to the control group.

A.



B.

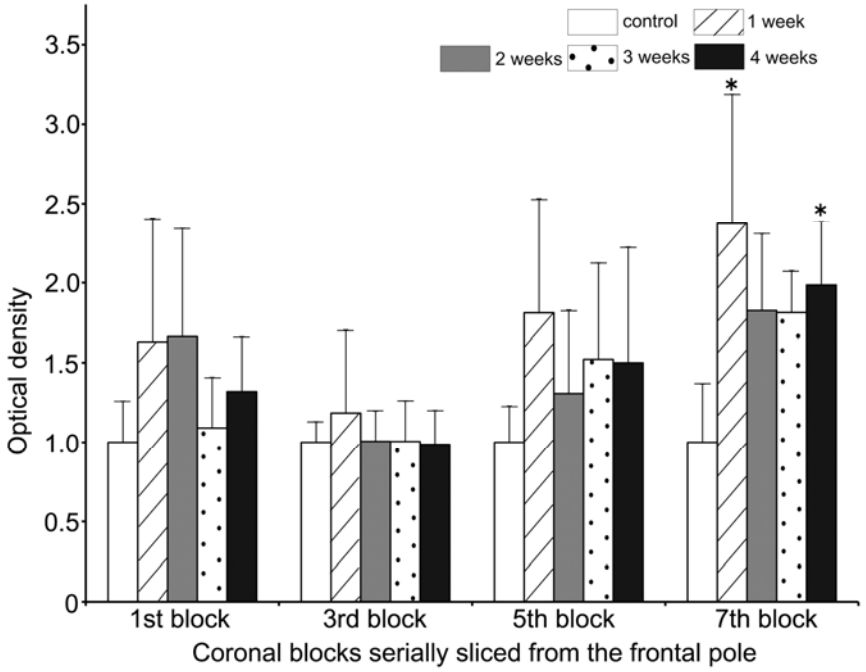


Figure 6. The temporal change of connexin 37 in the each coronal block following bilateral common carotid artery ligation ligation. A. Western blot of connexin 37 of 1st, 3rd, 5th, and 7th coronal sections after bilateral common carotid artery ligation. B. The level of connexin 37 was shown as a ratio of experimental group to control group in each coronal block which was serially sliced from the frontal pole, with 2.0 mm thickness. In the block near the

occipital pole, connexin 37 increased significantly at 1 week after bilateral CCA ligation, compared with the control group ($p=0.005$). 1st block, 0-2 mm from the frontal pole; 3rd block, 4-6 mm from the frontal pole, 5th block, 8-10 mm from the frontal pole; 7th block, 12-14 mm from the frontal pole.

C, control; w, week

* $p<0.05$ compared to the control group.

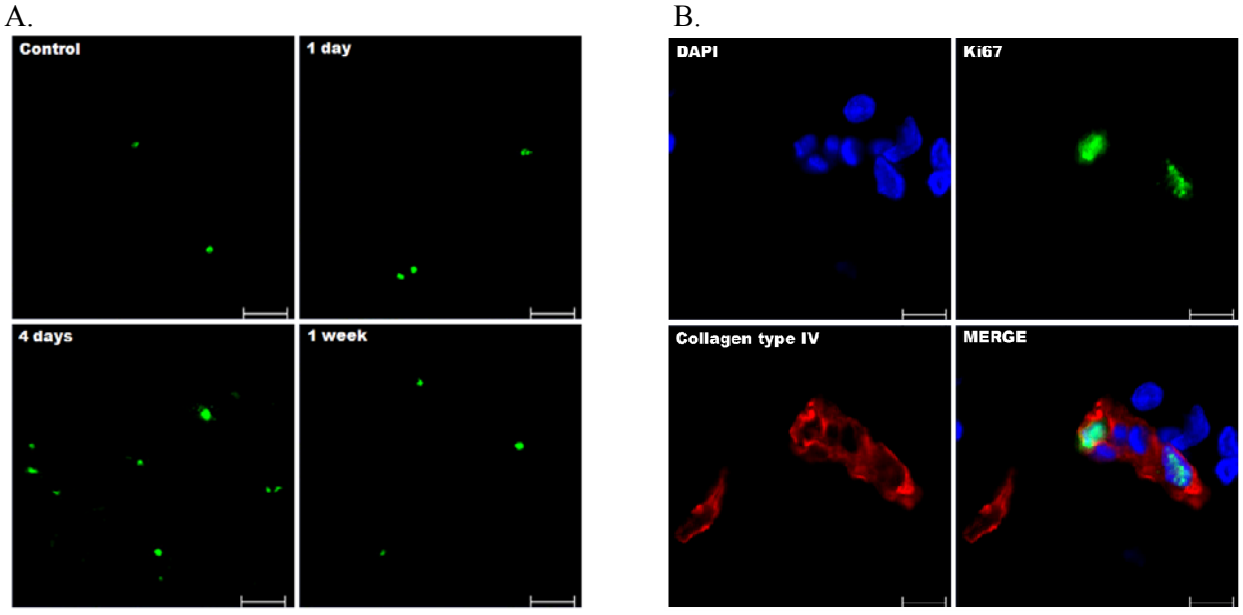


Figure 7. Expression of Ki 67 after bilateral common carotid artery ligation. A. Ki 67 expressed in the vascular wall increased in the four days group. B. Expression of Ki 67 in the nucleus of vascular endothelial cells. Ki 67 is co-localized with DAPI positive neurons and collagen type IV positive vessel walls.
DAPI; nucleus marker, collagen type IV; vascular marker
DAPI, 4'-6-Diamidino-2-phenylindole
Scale bar = 20 μm

4**. The temporal change of vascular endothelial growth factor and fibroblast growth factor-2**

The levels of vascular endothelial growth factor (VEGF), and fibroblast growth factor -2 (FGF-2), which are known to be associated with angiogenesis, were measured using western blot. There was no significant difference among the groups, both in the whole brain and in each coronal section (Figure 8 and Figure 9).

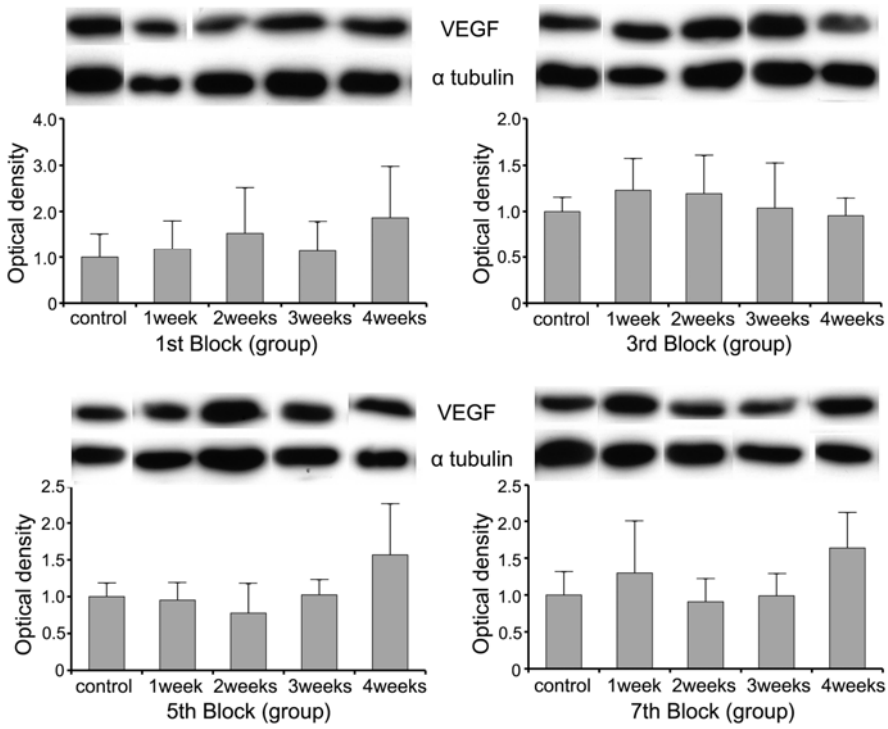


Figure 8. The temporal change of vascular endothelial growth factor after bilateral common carotid artery ligation. The levels of vascular endothelial growth factor (VEGF) were measured using western blot. There was no significant difference among the groups in each coronal block which was sliced from the frontal pole with 2.0 mm thickness. 1st block, 0-2 mm from the frontal pole; 3rd block, 4-6 mm from the frontal pole, 5th block, 8-10 mm from the frontal pole; 7th block, 12-14 mm from the frontal pole.

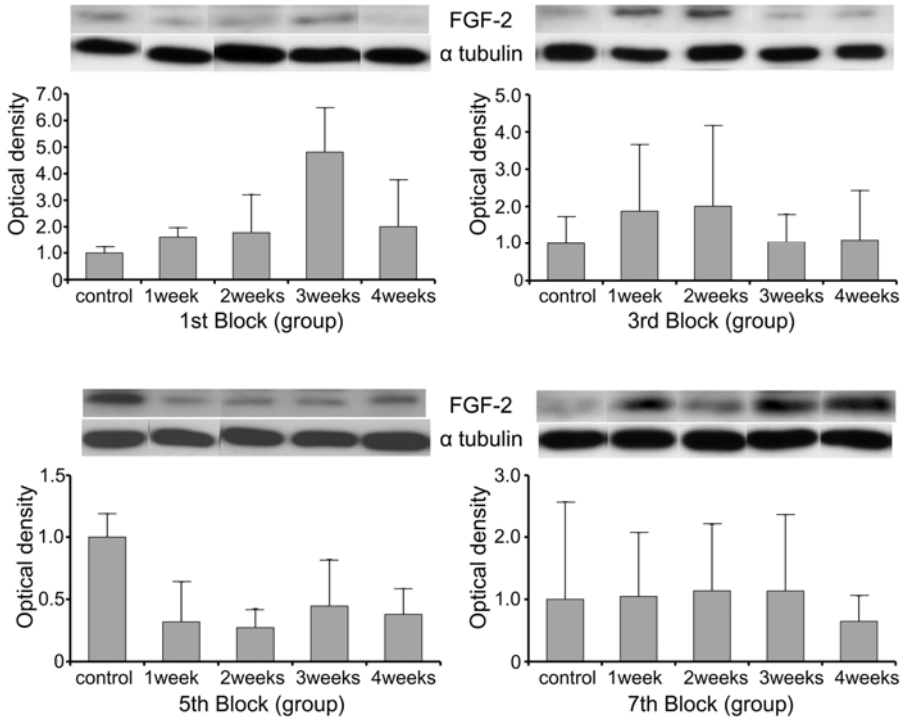


Figure 9. The temporal change of fibroblast growth factor-2 (FGF-2) after bilateral common carotid artery ligation. The levels of FGF-2 were measured using western blot. There was no significant difference among the groups in each coronal block which was serially sliced from the frontal pole with 2.0 mm thickness. 1st block, 0-2 mm from the frontal pole; 3rd block, 4-6 mm from the frontal pole, 5th block, 8-10 mm from the frontal pole; 7th block, 12-14 mm from the frontal pole.

IV. DISCUSSION

Patients with very severe stenosis of the carotid artery were thought to be at very high risk of stroke and those should be treated urgently. However, in the carotid endarterectomy trials, risks of stroke in those patients were low.[3-6] In addition, the New England Medical Center Posterior Circulation Registry study, stroke severity was better in patients with most severe stenosis of the vertebral artery.[28] Those clinical observations of less severe stroke in patients with very severe arterial stenosis were admitted as being unexpected among physicians. Although the growth of collateral circulations was suggested as a possible mechanism, this concept had not been proved in experimental studies.[11,29]

In the previous studies in our lab, the concept raised based on the clinical observations could be proved in rats that stroke was less severe when the brain was preconditioned by cerebral hypoperfusion using bilateral CCA ligation. Both cellular and vascular responses were suggested to play a role in the protective mechanisms. In the previous studies, the cellular and vascular responses were examined at one time point of 4 weeks after bilateral CCA ligation.[22] This study demonstrated that cerebral perfusion, which decreased by bilateral CCA ligation, began to improve 1 day after the bilateral CCA ligation, gradually improved and recovered to the baseline level at 2 weeks. Increased arterial diameter and arterial tortuosity were observed at 1 week. These findings suggest that adaptive vascular remodeling occur early after induction of cerebral

hypoperfusion.

In this study, I showed that the degree of vascular remodeling after bilateral CCA ligation was different between the cerebral arteries. Diameters were greater in the arteries of the posterior circulation than those of the anterior circulation. Vascular density was also greater in the brain area closer to the occipital pole. These findings indicate that vascular remodeling process occurs in the direction from the posterior circulation to the anterior circulation after bilateral CCA ligation, as time goes on. In rats, the posterior communicating artery is well developed and plays a role of conduit to deliver blood to the anterior circulation upon the bilateral CCA ligation.[27,30] When blood circulation was altered, the vessel wall would endure altered mechanical loads, including strain and shear stress. Both shear stress and pressure changes are most likely the initiative factors for collateral vessel growth.[13,14,31-33] Findings in this study suggest that arterial remodeling process occur more rapidly and greatly in the parent artery and its branch arteries, where the shear stress is directly conducted.

I further investigated which type of vascular remodeling process, which is arteriogenesis or angiogenesis, plays a major role in the severely occlusive arterial disease. Several molecules from the vascular endothelial cells play roles in inducing vascular remodeling. Connexin 37, the gap junction protein, has been expressed in the endothelial and the smooth muscle cells (SMC). The gap junctions have been reported to modulate vasomotor tone and to maintain the circulatory homeostasis. During collateral growth, Connexin 37 was

significantly induced in the SMCs from the small-large arteries to the precapillary arterioles.[34,35] In the mature vessels, Connexin 37 was down-regulated, similar to the normal vessels. In the present study, Connexin 37 was increased at 1 week after bilateral CCA ligation. The level of connexin 37 was greater in the area near the occipital pole than the area near the frontal pole. These finding was compatible with the profound increase of vascular density in the area near the occipital pole. These findings suggest that arteriogenesis play a role in vascular remodeling after bilateral CCA ligation and occurs very early, at least within 1 week after induction of cerebral sublethal hypoperfusion, especially in the area that is under shear stress and pressure changes. Furthermore, expression of arteriogenic molecules in that area might influence to the area under possible hypoxia and induce arteriogenesis.

Ki 67, which was known as a proliferative marker related to arteriogenesis,[36] was also expressed in the nucleus of vascular endothelial cells in this study. I evaluated expression of Ki 67 in vascular endothelial cells at 1 day, 4 days and 1 week after bilateral CCA ligation. Expression of Ki 67 was increased at 4 days after bilateral CCA ligation, which indicates that induction of arteriogenesis begins early after arterial occlusive state. Ki 67 may act earlier than arteriogenic morphological change. These findings are consistent with findings in the perfusion measurements that showed beginning of increase in perfusion even at 1 day after bilateral CCA ligation in this study.

The increased expression of Connexin 37 returned to the control level at 3

weeks after bilateral CCA ligation. After remodeling of the vascular wall results in enlargement of vascular diameter, shear stress will be decreased and then process of arteriogenesis will be terminated.[36] These natures may explain transient increase of Connexin 37 after bilateral CCA ligation in this study.

Unlike arteriogenesis markers, expression of known markers of angiogenesis such as VEGF and FGF-2 were not changed, which suggested that angiogenesis might not play a major role. However, angiogenesis also seemed to be a part of vascular remodeling after induced hypoperfusion because vascular density was increased in all sizes of vessels. Temporary hypoperfusion by bilateral CCA ligation might induce hypoxic state in the area supplied by the carotid artery. When considering hypoxia is a major stimulus for inducing angiogenesis,[17,37] angiogenesis might play a role, although it was much less than arteriogenesis, in flow restoration. Unlike angiogenesis, arteriogenesis is one of the main compensatory phenomena that occur in the conditions of the severe arterial stenosis or occlusion.[27] Arteriogenesis occurs in an environment of normoxia.[16] In conditions of reduced blood flow, angiogenesis may not be enough to restore blood flow, and arteriogenesis is required to compensate the physiologic demand. However, arteriogenesis ceased before perfusion defect was completely recovered.[32,36] Therefore, partial recovery of perfusion is expected by arteriogenesis. However, this study showed that perfusion was completely recovered in 2 weeks after bilateral CCA ligation. This finding also suggests that angiogenesis might have a supplemental role in restoring the blood flow or

perfusion.

There was a limitation in this study. Only a few molecular markers for determination of contributing roles of arteriogenesis and angiogenesis were examined. Therefore, the presence of angiogenesis, which was assumed based on results of vascular density and perfusion, was still speculative. Although we showed that arteriogenesis occurs at least within one week after bilateral CCA ligation, exact timing could not be determined because we performed time-course experiments on a weekly basis. Finally, the experimental design of this study may not exactly match with clinical conditions. Although the key question of this study was based on clinical observations in patients with chronic and severe atherosclerosis, arterial occlusion (bilateral CCA ligation) was induced in an abrupt way. Nevertheless, this study provided scientific evidence on the presence, type and temporal course of adaptive vascular remodeling that occur in the severe occlusive state of the large artery in the brain.

V. CONCLUSION

I investigated the presence and time course of vascular remodeling and expression of molecular markers of arteriogenesis and angiogenesis in a rat model of sublethal cerebral hypoperfusion, which was induced by bilateral carotid artery ligation. This study demonstrated that 1) cerebral perfusion was recovered to the baseline level at 2 weeks, 2) increased arterial diameter and arterial tortuosity were observed at 1 week, 3) the difference of the vessel density tended to be greater in the sections far from the frontal pole, 4) increase of vascular density was greater in larger vessels (≥70 μm) than was in smaller vessels (<30 μm), 5) the level of connexin 37 was increased at 1 week, 4) the level of VEGF and FGF-2 showed no change, 6) level of Ki 67 was increased at 4 days, after bilateral CCA ligation, and 7)increase of the level of connexin 37 was more obvious at the sections far from frontal pole.

I showed that arteriogenesis had a major role in blood flow restoration by demonstrating changes of the vessel tortuosity and diameter and increase of molecular markers. Arteriogenesis appeared to start at least within one week after induced cerebral hypoperfusion. Angiogenesis was also suggested to contribute to improve in perfusion. Findings in this study provide experimental evidence and mechanisms on clinical observations.

REFERENCES

1. Beneficial effect of carotid endarterectomy in symptomatic patients with high-grade carotid stenosis. North American Symptomatic Carotid Endarterectomy Trial Collaborators. N Engl J Med 1991;325:445-53.
2. MRC European Carotid Surgery Trial: interim results for symptomatic patients with severe (70-99%) or with mild (0-29%) carotid stenosis. European Carotid Surgery Trialists' Collaborative Group. Lancet 1991;337:1235-43.
3. Henderson RD, Eliasziw M, Fox AJ, Rothwell PM, Barnett HJ. Angiographically defined collateral circulation and risk of stroke in patients with severe carotid artery stenosis. North American Symptomatic Carotid Endarterectomy Trial (NASCET) Group. Stroke 2000;31:128-32.
4. Morgenstern LB, Fox AJ, Sharpe BL, Eliasziw M, Barnett HJ, Grotta JC. The risks and benefits of carotid endarterectomy in patients with near occlusion of the carotid artery. North American Symptomatic Carotid Endarterectomy Trial (NASCET) Group. Neurology 1997;48:911-5.
5. Rothwell PM, Gutnikov SA, Warlow CP. Reanalysis of the final results of the European Carotid Surgery Trial. Stroke 2003;34:514-23.
6. Rothwell PM, Warlow CP. Low risk of ischemic stroke in patients with reduced internal carotid artery lumen diameter distal to severe symptomatic carotid stenosis: cerebral protection due to low poststenotic flow? On

behalf of the European Carotid Surgery Trialists' Collaborative Group. Stroke 2000;31:622-30.

7. Kitagawa K, Matsumoto M, Tagaya M, Hata R, Ueda H, Niinobe M, et al. 'Ischemic tolerance' phenomenon found in the brain. Brain Res 1990;528:21-4.
8. Chopp M, Chen H, Ho KL, Dereski MO, Brown E, Hetzel FW, et al. Transient hyperthermia protects against subsequent forebrain ischemic cell damage in the rat. Neurology 1989;39:1396-8.
9. Kirino T. Ischemic tolerance. J Cereb Blood Flow Metab 2002;22:1283-96.
10. Kitagawa K, Matsumoto M, Tagaya M, Kuwabara K, Hata R, Handa N, et al. Hyperthermia-induced neuronal protection against ischemic injury in gerbils. J Cereb Blood Flow Metab 1991;11:449-52.
11. Busch HJ, Buschmann IR, Mies G, Bode C, Hossmann KA. Arteriogenesis in hypoperfused rat brain. J Cereb Blood Flow Metab 2003;23:621-8.
12. Buschmann I, Schaper W. The pathophysiology of the collateral circulation (arteriogenesis). J Pathol 2000;190:338-42.
13. Helisch A, Schaper W. Arteriogenesis: the development and growth of collateral arteries. Microcirculation 2003;10:83-97.
14. Schaper W, Scholz D. Factors regulating arteriogenesis. Arterioscler Thromb Vasc Biol 2003;23:1143-51.
15. Wahlberg E. Angiogenesis and arteriogenesis in limb ischemia. J Vasc Surg 2003;38:198-203.

16. Heil M, Schaper W. Insights into pathways of arteriogenesis. Curr Pharm Biotechnol 2007;8:35-42.
17. Semenza GL. Vasculogenesis, angiogenesis, and arteriogenesis: mechanisms of blood vessel formation and remodeling. J Cell Biochem 2007;102:840-7.
18. Klagsbrun M, D'Amore PA. Regulators of angiogenesis. Annu Rev Physiol 1991;53:217-39.
19. Beck H, Plate KH. Angiogenesis after cerebral ischemia. Acta Neuropathol 2009;117:481-96.
20. Navaratna D, Guo S, Arai K, Lo EH. Mechanisms and targets for angiogenic therapy after stroke. Cell Adh Migr 2009;3:216-23.
21. Choi SA, Kim EH, Lee JY, Nam HS, Kim SH, Kim GW, et al. Preconditioning with chronic cerebral hypoperfusion reduces a focal cerebral ischemic injury and increases apurinic/apyrimidinic endonuclease/redox factor-1 and matrix metalloproteinase-2 expression. Curr Neurovasc Res 2007;4:89-97.
22. Kim SH, Kim EH, Lee BI, Heo JH. Chronic cerebral hypoperfusion protects against acute focal ischemia, improves motor function, and results in vascular remodeling. Curr Neurovasc Res 2008;5:28-36.
23. Tanaka K, Ogawa N, Asanuma M, Kondo Y, Nomura M. Relationship between cholinergic dysfunction and discrimination learning disabilities in Wistar rats following chronic cerebral hypoperfusion. Brain Res

1996;729:55-65.

24. Wakita H, Tomimoto H, Akiguchi I, Kimura J. Glial activation and white matter changes in the rat brain induced by chronic cerebral hypoperfusion: an immunohistochemical study. Acta Neuropathol 1994;87:484-92.
25. Wakita H, Tomimoto H, Akiguchi I, Kimura J. Protective effect of cyclosporin A on white matter changes in the rat brain after chronic cerebral hypoperfusion. Stroke 1995;26:1415-22.
26. Coyle P. Diameter and length changes in cerebral collaterals after middle cerebral artery occlusion in the young rat. Anat Rec 1984;210:357-64.
27. Coyle P, Panzenbeck MJ. Collateral development after carotid artery occlusion in Fischer 344 rats. Stroke 1990;21:316-21.
28. Caplan LR, Wityk RJ, Glass TA, Tapia J, Pazdera L, Chang HM, et al. New England Medical Center Posterior Circulation registry. Ann Neurol 2004;56:389-98.
29. Buschmann IR, Busch HJ, Mies G, Hossmann KA. Therapeutic induction of arteriogenesis in hypoperfused rat brain via granulocyte-macrophage colony-stimulating factor. Circulation 2003;108:610-5.
30. Brown JO. The morphology of circulus arteriosus cerebri in rats. Anat Rec 1966;156:99-106.
31. Schierling W, Troidl K, Mueller C, Troidl C, Wustrack H, Bachmann G, et al. Increased intravascular flow rate triggers cerebral arteriogenesis. J Cereb Blood Flow Metab 2009;29:726-37.

32. Schaper W. Collateral circulation: past and present. Basic Res Cardiol 2009;104:5-21.
33. Cai W, Schaper W. Mechanisms of arteriogenesis. Acta Biochim Biophys Sin (Shanghai) 2008;40:681-92.
34. Cai WJ, Koltai S, Kocsis E, Scholz D, Schaper W, Schaper J. Connexin37, not Cx40 and Cx43, is induced in vascular smooth muscle cells during coronary arteriogenesis. J Mol Cell Cardiol 2001;33:957-67.
35. Cai WJ, Kocsis E, Scholz D, Luo X, Schaper W, Schaper J. Presence of Cx37 and lack of desmin in smooth muscle cells are early markers for arteriogenesis. Mol Cell Biochem 2004;262:17-23.
36. Scholz D, Cai WJ, Schaper W. Arteriogenesis, a new concept of vascular adaptation in occlusive disease. Angiogenesis 2001;4:247-57.
37. Moulton KS. Angiogenesis in atherosclerosis: gathering evidence beyond speculation. Curr Opin Lipidol 2006;17:548-55.

Abstract (in Korean)

만성 뇌혈류저하에 의해 유도되는 미세혈관 및 동맥 재형성

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최혜연

동맥경화증에 의한 뇌혈관의 협착은 그 정도가 심해지는 경우 뇌조직의 만성 저관류상태를 유도할 수 있다. 뇌관류저하를 유도할 만큼 극도로 심한 정도의 협착이 발생한 경우에는 오히려 뇌졸중발생의 위험도도 적고, 뇌졸중이 발생했을 경우에도 임상증상의 중증도나 뇌졸중의 크기도 더 적은 것으로 알려져 있으며 이는 서서히 진행되는 뇌혈류저하가 허혈자극에 대한 내성을 유도한다는 것을 암시한다. 본 연구에서는 이러한 내성이 혈관이 재구성되어 뇌혈류가

회복되는데 중요한 역할을 하는지를 알아보고, 이때 나타나는 뇌혈류 회복과 혈관 재형성의 시간적 관계를 알아보고자 했으며, 또한 혈관재형성, 동맥재형성과 관련된 인자의 발현 및 그 시간적 관계도 알아보고자 했다. 뇌혈류 저하는 Wistar 흰쥐에서 양측 경동맥 결찰을 시행함으로써 유도하였다. 양측 경동맥 결찰 이후 1, 4, 7, 14, 21, 28 일 후에 뇌 미세혈관 조직관류를 측정하였으며, 신생동맥형성여부와 혈관재형성과 관련된 인자를 측정하여 이를 대조군과 비교하였다. 뇌 미세혈관 조직관류는 레이저 도플러 유량계를 이용하여 중대뇌동맥 영역에서 측정하였고, 신생동맥형성여부는 라텍스 관류법을 이용한 사후 뇌혈관조영술을 이용하여 측정하였다. 관련인자의 발현은 western blot 과 면역형광염색을 이용하여 관찰하였다. 뇌 미세혈관 조직관류는 양측총경동맥 결찰 직후 기저치의 40.2 %까지 감소하였다가 2 주경과 후 기저치와 같은 정도로 회복되었다. 양측총경동맥 결찰 이후 1 주째 후방순환계의 혈관 (기저동맥, 후대뇌동맥, 후교통동맥)의 직경이 증가하였으며, 기저동맥의 길이가 더 길어졌다. 뇌관상면의 혈관밀도 역시 대조군에 비해 총경동맥결찰 군에서

1 주째부터 증가하는 것이 관찰되었다. 동맥재형성과 관련된 인자인 connexin 37 은 1 주째에 증가하는 것이 관찰되었으며, ki 67 은 4 일째에 혈관내피세포 핵에서의 발현이 증가한 것으로 관찰되었다. 신생혈관형성과 연관된 인자인 VEGF, FGF-2 는 총경동맥결찰 후 1,2,3,4 주째 대조군과 비교해 차이가 관찰되지 않았다. 그러나, 혈관밀도증가가 미세동맥보다 더 작은 직경의 혈관에서도 관찰되어 동맥재형성뿐 아니라 신생혈관형성도 유도되는 것으로 생각된다. 본 실험에서 만성뇌혈류저하가 유도되었을 때, 뇌미세혈관조직관류가 회복되기 이전에 혈관재형성이 유도되기 시작하였으므로, 동맥재형성이 뇌혈류량 회복에 주요한 역할을 하는 것으로 생각된다.

핵심단어: 만성뇌혈류저하, 혈관재형성, 동맥재형성, 신생혈관형성