Dually Supplied T-Junctions in Arteriolo-Arteriolar Anastomosis in Mice

Key to Local Hemodynamic Homeostasis in Normal and Ischemic States?

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Background and Purpose—The functional role of arteriolo-arteriolar anastomosis (AAA) between the middle cerebral artery (MCA) and anterior cerebral artery in local hemodynamics is unknown, and was investigated here.

Methods—Blood flow in AAAs was examined using fluorescein isothiocyanate–labeled red blood cells (RBCs) as a flow indicator in 16 anesthetized C57BL/6J mice before and after MCA occlusion up to 7 experimental days.

Results—We observed paradoxical flow in AAAs; labeled RBCs entered from both the MCA and anterior cerebral artery sides and the opposing flows met at a branching T-junction, where the flows combined and passed into a penetrating arteriole. The dually fed T-junction was not fixed in position, but functionally jumped to adjacent T-junctions in response to changing hemodynamic conditions. On MCA occlusion, RBC flow from the MCA side immediately stopped. After a period of “hesitation,” blood started to move retrogradely in one of the MCA branches toward the MCA stem. The retrograde blood flow was statistically significantly (*P*<0.05), serving to feed blood to other MCA branches after a lag period. In capillaries, MCA occlusion induced immediate RBC disappearance in the ischemic core and to a lesser extent in the marginal zone near AAAs. At day 3 after ischemia, we recognized the beginning of remodeling with angiogenesis centering on AAAs.

Conclusions—AAAs appear to play a key role in local hemodynamic homeostasis, both in the normal state and in the development of collateral channels and revascularization during ischemia. (Stroke. 2009;40:3378-3383.)

Key Words: arterio-arterial anastomosis ■ angiogenesis ■ cerebral ischemia ■ penumbra ■ red blood cell

Mchedlishvili1 reported abundant arteriolo-arteriolar anastomoses (AAAs) connecting cerebral arterioles, based on his extensive microscopic study of cerebral micro-circulation in the brains of many animals, including hens, rabbits, cats, dogs, and monkeys. By filling vessels with carbon black-labeled latex (Vultex), Coyle and Jokelainen2 investigated the vascular structure of major cerebral arteries in detail and found that there were about 29 anterior cerebral artery (ACA)/middle cerebral artery (MCA) junctions per hemisphere in normal Wistar rats. The supplying territories of the major cerebral arteries were interconnected by functional anastomoses forming boundary zones between the superficial pial arteries, constituting the so-called cortical watershed.3,4 However, little is known about the hemodynamic characteristics of AAAs involved in peripheral collateral pathways under normal and ischemic conditions. An unanswered question is whether or not flow exists in AAAs caught between 2 opposing arterial pressures, and if it does, what is the direction of flow in the connecting arterioles. Ganushkina et al5 reported that there was no flow, and that there was only plasma at the dead point of blood in AAAs. However, if no flow occurred in the channel, RBC aggregation would occur and the vessel would eventually become closed. Clinically, the issue seems relevant to hemodynamic compensation in watershed areas during ischemia and the development of collateral channels. If flow rescue fails, watershed infarction might theoretically ensue.

The main goal of this study was to investigate the functional role of AAA between MCA and ACA in mice before and after ischemia, by visualization of flow with fluorescein isothiocyanate (FITC)-labeled red blood cells (RBCs)6 and a high-speed camera laser scanning confocal fluorescence microscope system recently developed by us.7 We used a mouse model developed by Seylaz et al.8

Materials and Methods

Sixteen male C57BL/6J mice (10 weeks old) weighing 20 to 25 g, purchased from Animal Supply (Nihon Kurea International, Tokyo, Japan), were used under isoflurane (1.4 to 1.8%) anesthesia. Eight mice were used for 1-day experiments (acute), 8 mice for 2- to 7-day experiments (chronic), and 2 after acute experiments for Mercox
filling experiments. All experimental procedures were performed in accordance with the University’s guidelines for the care and use of laboratory animals, with the approval of the Animal Ethics Committee of Keio University. Preliminary surgical procedures were performed by using a surgical microscope (OPMI Pico, Carl Zeiss) as described by Tomita. Briefly, a 3-mm diameter hole was drilled above the left parieto-temporal cortex, 1 mm lateral from the sagittal suture and 1 mm posterior from the bregma. To avoid drying of the tissue and to keep the brain surface sterile for the chronic experiment, the trepanation was covered with a circular 140-μm thick quartz glass window, sealed onto the bone with dental cement (Ionosti, DMG). The area under the window corresponded to a supplying territory of distal branches of both the MCA and ACA. In 2 mice under deep anesthesia which were used for acute experiments, Mercox mixed with carbon black was injected into the heart to fill the cerebral microvasculature, including AAAs. After removal of the whole brain, the microvasculature on the brain surface was viewed and photographed from above to examine arteriolar connections between the MCA and MCA (Figure 1A).

To visualize blood flow changes in an AAA, we traced FITC-labeled RBCs and measured RBC velocities in the connecting branches of MCA and ACA before and after MCA occlusion (MCAO). A suspension of mouse RBCs labeled beforehand with FITC was injected into the systemic circulation through the tail vein. The mouse head was fixed to a special head holder with a metal bar securely cemented at the posterior edge of the skull bone. The bar was used to finely adjust the ROI in the brain surface stereotaxically to the same position in x-y-z coordinates so that the same site could be visualized consistently. A high-speed camera laser scanning confocal fluorescence microscope system recently developed by us was used for RBC velocity measurements. The microscope was equipped with a objective lens (Nikon LU Plan EPI SLWD 4×, 10×, and 20×) and a halogen lamp for obtaining a general view of the brain microcirculation with a video camera (30 fps, DCR-PC100, Sony); output was recorded on-line. Laser illumination (λ = 488 nm; Melles Griot) was used for observation of tracking and velocities of FITC-labeled RBCs with a high-speed camera (500 fps) in an optical slice of ~20 μm in thickness. Evaluation of the RBC tracking and velocities in single microvessels was performed by using the Matlab domain KEIO-IS2 software. Wall shear rates (gw) of blood flow in AAA were calculated from the RBC velocities thus obtained by application of the following equation

$$\gamma_w = 4v/r = 4Q/\pi r^2$$

where v is the velocity, r the radius (half of the diameter) of AAA, and Q the blood flow. After control observations of RBC flow the mouse was subjected to cerebral ischemia by occluding the proximal branch of the MCA (Figure 1A), using a slight modification of Tamura’s original method. Capillary network changes were examined with rhodamine isothiocyanate (RITC) dextran (λ=533 nm), which was injected into the tail vein and subsequently filled the capillaries in tissues, including the cerebral cortex.

In chronic experiments in 8 mice, after the observation of AAA and related microvasculature through the cranial window, which took approximately 1 hour, the mouse was returned to the animal center. Next day, the mouse was brought back to the laboratory and fixed in the holder, which was adjusted so that the same AAA and microvasculature as before could be observed, and FITC-labeled RBCs were injected into the tail vein. This procedure was repeated every day for a week. The AAA and microvascular changes were evaluated from the videotape recordings. Vascular structural changes were examined by the use of RITC dextran when required.

Results

Arterio-Arteriolar Anastomosis Before MCAO (Normal or Control Condition)

Three-Dimensional Structural Characteristics

Figure 1A is a dorsal overview of the epicortical pial arterial network filled with carbon black mixed in Mercox. Only cerebral arteries and arterioles were visualized, apparently because Mercox has such a high viscosity that it could not fill the veins. Dark dots in Figure 1A indicate the connecting points in the watershed area between peripheral branches of the MCA and ACA, representing arterio-arteriolar anastomoses (AAA). Figure 1B shows an enlarged microphotograph of an AAA in situ, running across the ROI under a large vein in the cerebral cortex. As indicated by arrows, there were 3 T-junctions of penetrating arterioles (this will be discussed later). The mean diameter of such connecting distal branches of MCA and ACA was 27±2.5 μm (mean±SD, n=8). However, the diameter of AAAs tended to be different depending on location along the channel: the average diameter was larger in the center of an anastomosis (28±2.5 μm, n=8) than at proximal sites (20±1.9 μm, n=8).

Direction of Flow in AAA

During continuous recorded observation of the cortical microvascular image of the watershed area with the fluorescence microscope through the skull window, a suspension of FITC-labeled RBCs was injected into the circulating blood through the tail vein so that its final concentration in the circulating blood became 0.4% of total RBCs. The labeled RBCs appeared in the capillary network of the microvasculature in the cortex like fireflies on a dark background. Interestingly, when the labeled RBCs arrived at an AAA, they
progressed into it from both the ACA and MCA sides (Figure 1B). The 2 opposing flows met at some point in the anastomosis and disappeared. Figure 2i shows a sketch of an AAA in which the directions of flow are indicated by arrows. At the meeting point, blood disappeared, apparently sinking into the origin of a penetrating arteriole, which, in most cases, could not be seen from above without the use of FITC-labeled RBCs; this may be the reason why such paradoxical flow has been missed in the past. When the passage of blood into the hole was closely examined, there was as if the two flows were separated by an imaginary membranous wall, which moved left or right depending on the relative flow strengths. In general, AAAs form a peripheral part of cerebral arteries, with penetrating arterioles in the form of T-junctions to supply blood to tissue. Some of these T-junctions are dually penetrating arterioles peripheral to the occluded site dramatically decreased until the flow momentarily came to a full stop (Figure 2ii). However, after a “hesitation” period of a minute or more, retrograde blood flow (Figure 2iv) appeared in the MCA branch (M1) (Figure 3A and 3B). The occurrence of the retrograde flow in this particular branch (M1) was statistically significant ($P<0.05$, in 7 cases of 8). At the same time, flow in another MCA branch (M2) started in the original direction. In general, the flow peripheral to the occlusion site slowed down and various degrees of flow decrease were observed. Increasing viscosity of blood with decreasing flow (non-Newtonian character)\(^9\) means that hemodynamic changes cannot be predicted from Poiseuille law,\(^8\) which assumes the blood viscosity is constant. In 2 cases, RBC aggregates (red) and clot formation (white) stalled blood flow on the MCA side of the AAA. In the capillary network, MCAO induced immediate disappearance of RBCs in the core of the ischemic region, probably caused by a sieving effect, and also to a lesser extent in the marginal zone near the AAA. On the other hand, blood flow near the ACA side of the AAA was rather well preserved. We next compared RBC velocity changes in penetrating branches connecting to the confluent T-junction and those in other penetrating branches of the proximal part of the MCA supplying the ischemic core. After MCAO, RBC velocity change in the T-junction was from $2.6\pm0.6$ mm/s to $2.4\pm0.9$ mm/s, whereas in the penetrating branches of proximal MCA, the RBC velocity decreased sharply from $2.6\pm1.2$ mm/s to $1.1\pm0.6$ mm/s (statistically significant; $P<0.005$, n=8).

**AAAs, Vascular Remodeling, and Angiogenesis**

Microvascular remodeling centering on AAAs in the ischemic tissue after MCAO was preliminarily examined by means of repeated observation every day for a week. Days 0 to 1 (Figure 4A): Capillaries became invisible within a few hours after MCAO, and thereafter there was no RBC passage in the ischemic core. Days 1 to 2 (Figure 4B): Fragments of microvessels and tissue debris were seen, accompanied with massive macrophage infiltration. The macrophages were accidentally made visible by phagocytosed RITC dye which had leaked into the tissue through the disrupted BBB. The dye had originally been injected to visualize the capillary architecture. Day 3 (Figure 4C): Development of new vessels,

**Wall Shear Rate of Blood in AAA**

Flow magnitudes were estimated from velocity values of FITC-labeled RBCs multiplied by the cross-sectional area of the vessel. When RBC flow velocity through a vessel with a radius of approximately 0.012 mm (see above) was approximately 2.6 mm/s (see below), the wall shear rate of blood in AAA calculated with Equation 1 was approximately 80/s. However, the shear rate value was found to vary depending on the location of anastomosis. Strangely, it tended to be lower in the proximal parts of connecting branches of the MCA and ACA than around the meeting point, whose diameter tended to be larger. A large variation in flow through connecting arterial branches along T-junctions was a common observation even under physiological conditions. In general, velocity changes in the microvasculature, especially in venules, seemed to be completely unpredictable from diametric changes of vessels.

**Hemodynamic and Microvascular Changes Before and After MCAO**

**Hemodynamic and Hemorheological Changes in AAA and Tissue**

When the mouse brain was subjected to ischemia by occluding the left MCA,\(^10\) the flow balance in AAAs was immediately disrupted. Both acute and chronic adaptive hemodynamic changes followed. Acutely, the blood flow velocity in arterioles peripheral to the occluded site dramatically decreased until the flow momentarily came to a full stop (Figure 2ii). However, after a “hesitation” period of a minute or more, retrograde blood flow (Figure 2iv) appeared in the MCA branch (M1) (Figure 3A and 3B). The occurrence of the retrograde flow in this particular branch (M1) was statistically significant ($P<0.05$, in 7 cases of 8). At the same time, flow in another MCA branch (M2) started in the original direction. In general, the flow peripheral to the occlusion site slowed down and various degrees of flow decrease were observed. Increasing viscosity of blood with decreasing flow (non-Newtonian character)\(^9\) means that hemodynamic changes cannot be predicted from Poiseuille law,\(^8\) which assumes the blood viscosity is constant. In 2 cases, RBC aggregates (red) and clot formation (white) stalled blood flow on the MCA side of the AAA. In the capillary network, MCAO induced immediate disappearance of RBCs in the core of the ischemic region, probably caused by a sieving effect, and also to a lesser extent in the marginal zone near the AAA. On the other hand, blood flow near the ACA side of the AAA was rather well preserved. We next compared RBC velocity changes in penetrating branches connecting to the confluent T-junction and those in other penetrating branches of the proximal part of the MCA supplying the ischemic core. After MCAO, RBC velocity change in the T-junction was from $2.6\pm0.6$ mm/s to $2.4\pm0.9$ mm/s, whereas in the penetrating branches of proximal MCA, the RBC velocity decreased sharply from $2.6\pm1.2$ mm/s to $1.1\pm0.6$ mm/s (statistically significant; $P<0.005$, n=8).
mostly immature capillaries and reconstructed veins, was seen around the marginal zone of the infarction. Figure 5 shows newly developed straight capillaries (angiogenesis), sprouted from the arteriolo-arteriolar cut end, that connected with dilated and tortuous vessels, remodeled veins and a reactive region of the veins with many modular protrusions. By this stage, labeled RBCs started to flow in capillaries only in the marginal area and remodeled veins, showing unsteady motions. Figure 5 presents a trace of one RBC traveling through new microvasculature in the sequence arteriole-new straight capillary-unidentified channel and reconstructed vein. Day 7: Revascularization similar to that reported by Tomita et al was observed in cerebral ischemic tissue at the 7th day after MCAO in mice. In our experiments, all 8 mice survived 7 days although the ischemic core appeared to be liquefied. In short, angiogenesis was already apparent in the marginal zone at day 3 after MCAO in mice.

Discussion

When blood flow decreases the blood tends to be viscous undergoing transformation to a non-Newtonian fluid, in which lower shear rate results in higher viscosity \( \eta \). Under these circumstances, the pressure-flow relationship becomes modified with an additional pressure drop attributable to viscous dissipation.\(^\text{13}\)

\[ \Delta P = A \eta Q + B \rho Q^2 \]

where \( A \) and \( B \) are geometric factors determined by the shape of the vessels, \( \Delta P \) is the perfusion pressure drop, and \( \rho \) the fluid density.\(^\text{13}\) The vascular diametric control of flow becomes less effective. This viscosity change has to be taken into consideration in flow changes in AAA. On MCAO, the blood flow in the vessels downstream from the occluded site and penetrating arteriole from AAA decreases greatly. Surface of endothelial cells in the peripheral vessels changes from anticoagulant to procoagulant in character,\(^\text{14,15}\) and water leaks from blood into the tissue,\(^\text{16}\) resulting in hemoconcentration and tissue edema. Capillary RBC flow is greatly disturbed in association with neuronal depolarization (see Tomita et al, available from Nature Preceedings http://precedings.nature.com/documents/3220/version/1). However, there are also some unknown positive factors derived...
The shear rate of blood flowing through the AAA was approximately $80^{-1}$ in the control state. This is the critical threshold below which the blood tends to behave as a non-Newtonian fluid. Because there was no significant diametric change of the channel at the time of occlusion, hemorheological factors of the blood per se in the vessels, such as the increased viscosity, may govern the hemodynamic flow changes. If zero flow or low flow persisted, it might cause severe infarction. However, Shih et al reported active dilation of penetrating arterioles leading to restoration of red blood cell flux to the penumbral neocortex after focal stroke. Maeda et al found that larger anastomoses in angiotensinogen-knockout mice attenuate early metabolic disturbances after MCAO. The penetrating arterioles presumably respond to peripheral requirement by providing blood to the area via a vis a fronte. Flow would restart if the pressure gradient exceeded the yield point of the RBC aggregates, and the patent AAA would supply blood through collateral vessels to the atelastically ischemic territories (so-called ischemic penumbra). The MCA branch would also supply blood to the MCA territories retrogradely from the ACA via small diameter to larger diameter arterioles. We observed in all cases studied that blood flow was redistributed to other MCA branches. For the efficient functioning of AAA, 2 points seem to be important: blood in the AAA channel must be kept flowing, and the channel wall must be strong enough preventing vessels from collapse. The latter could be the reason for the ampoule shape, whose diameter was larger than that of the orifice of the AAA. The T-junctions in AAA thus served as a center of collateral channels to rescue the ischemic area, and this occurred within minutes after MCAO, determining the fate of the ischemic tissue.

During successive daily observations of the same microvasculature of the mouse cortex, we noticed the appearance of new vessels, consisting mostly of immature capillaries and peculiarly shaped veins, remodeled around the marginal zone of the infarction, by day 3. RBCs were already flowing through the new capillaries and remodeled veins with unsteady motions, presumably providing oxygen to the penumbral region. On the other hand, in the ischemic core RBCs disappeared from the capillary network from the beginning of ischemia, provoking immediate, severe tissue anoxia, because RBCs carry much more oxygen than plasma. Capillaries were destroyed, and the ischemic core became liquefied.

**Sources of Funding**

This work was supported by JSPS Grant-in-Aid #17390255 and #19591008. We thank Otsuka Pharmaceutical Co Ltd, Pfizer Japan Inc, and Novartis Pharma K.K. for their financial support.
Disclosures
None.

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Stroke. 2009;40:3378-3383; originally published online August 6, 2009;
doi: 10.1161/STROKEAHA.109.558577

Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2009 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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