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

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 quartz glass window, sealed onto the bone with dental cement (Ionosit, DMG). The area under the window corresponded to a region of interest (ROI) in the cortex like fireflies on a dark background.

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 tissues, including the cerebral cortex, became 0.4% of total RBCs. The labeled RBCs appeared in the capillary network of the microvasculature as before could be observed, and FITC-labeled RBCs through the tail vein so that its final concentration in the tissues, including the cerebral cortex, became 0.4% of total RBCs. The labeled RBCs appeared in the capillary network of the microvasculature as before could be observed, and FITC-labeled RBCs were visualized when required.

### Results

#### Arteriolo-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 arteriolo-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
lower in the proximal parts of connecting branches of the
AAA, 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,9 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±0.6 mm/s to 2.4±0.9 mm/s, whereas in the penetrating branches of proximal MCA, the RBC velocity decreased sharply from 2.6±1.2 mm/s to 1.1±0.6 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,
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.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.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, and water leaks from blood into the tissue, 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

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**Figure 3.** A, Direction of blood flow in M1 was from bottom (MCA stem) to top (periphery) in the control state (before MCAO). B, After MCAO the blood flow reversed from top to bottom; apparently blood was supplied from the ACA via the AAA. The blood changed direction at the branching site of M2 and was supplied to MCA territory.

**Figure 4.** Changes in microvascular geometry in the ROI after MCA occlusion. A, On day 0, small vessels and capillaries peripheral to the MCA became blurred, with destruction of vessel structure. The ischemic core (bottom area) appeared as if it was covered with a dark curtain. Top arrows indicate branches of ACA and bottom MCA. B, On day 2, the “curtain” was lifted and an amorphous white infarct area could be seen. The surrounding area was infiltrated with macrophages, apparently scavenging debris. Fragments of vessels were seen. C, On day 3, revascularization developed in the area surrounding the infarction. New straight capillaries formed by angiogenesis were seen to sprout from the cut end of the MCA branch (arrow). The MCA stem appeared to melt away in B and C.
The pressure was supposed to be lowest, and multiple occlusions were largely interpreted as thrombi secondary to microemboli. 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 force. Flow would restart if the pressure gradient exceeded the yield point of the RBC aggregates, and the patent AAA would supply blood through collateral channels to the acutely 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, 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.

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None.

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