Polymorphonuclear Leukocytes Occlude Capillaries Following Middle Cerebral Artery Occlusion and Reperfusion in Baboons

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Background and Purpose: Microvascular perfusion defects may accompany sustained occlusion and subsequent reperfusion of the middle cerebral artery; however, the nature of such "no-reflow" defects remains unclear.

Methods: In the absence of antithrombotic pretreatment, we documented lenticulostriatal microvascular flow integrity following 3-hour middle cerebral artery occlusion and 1-hour reperfusion in a baboon occlusion/reperfusion model by two methods identifying 1) microvascular occlusion and 2) microvascular patency.

Results: Microvascular "no-reflow" involved capillaries (vessels of 4.0-7.5 μm diameter) of the lenticulostriatal territory. Capillary reflow included 27-39% of all capillaries in two subjects, indicating a significant reduction of perfusion from normal (2p = 0.045). In identical experimental preparations, single polymorphonuclear leukocytes completely occluded 4.7% of microvessels of capillary diameter in randomly selected fields, partially occluded 33% of postcapillary venules, and occluded 40% (four of 10) of capillaries in linear reconstruction along a 110 μm length. Circumferential contact between polymorphonuclear leukocytes and the luminal endothelial cell membranes was documented, with an intercellular gap of, at most, 160 nm. Fibrin was found with degranulated platelets when the latter were associated with granulocytes, but not with polymorphonuclear leukocytes alone.

Conclusions: The finding of capillary-obstructing polymorphonuclear leukocytes in the microvascular bed following middle cerebral artery reperfusion in focal ischemia in this model satisfies an essential requirement for postulating their role in early microvascular injury and the "no-reflow" phenomenon. (Stroke 1991;22:1276-1283)
brovascular ischemia and reperfusion has not, to date, been directly demonstrated, although experience in some global cerebral ischemia models and in other organ systems suggests the hypothesis that the “no-reflow” phenomenon may involve leukocyte plugging of selected capillaries.

Del Zoppo and colleagues15 observed obstructions in microvessels in the peri-ischemic region following a short period of middle cerebral artery (MCA) occlusion and reperfusion in a nonhuman primate model. We report here the finding of PMN leukocytes exclusively in capillaries and postcapillary venules very early during reperfusion following focal ischemia in this model.

Materials and Methods

Four adolescent male baboons (Papio anubis/cynocephalus) weighing 8.5-10.7 kg were used for the present studies. All animals were conditioned and observed to be disease-free during a mandated quarantine period prior to entry into this study. All procedures were approved by the institutional Animal Research Committee and were performed in accordance with standards published by the National Research Council (the Guide for the Care and Use of Laboratory Animals), the National Institutes of Health policy on humane Care and Use of Laboratory Animals, and the USDA Animal Welfare Act. The surgical preparation of the nonhuman primate model of right middle cerebral artery (MCA) occlusion and reperfusion has been previously described in detail15,16 and was used here with minor modification. Anesthesia was undertaken with halothane by inhalation (5% induction) and maintained with the same agent (1.5-2.0%). Full recovery was routinely achieved 1-2 hours after completion of implantation of the MCA occlusion device (Mentor Corporation, Goleta, Calif.). Subsequently, all subjects were allowed a 7-day, procedure-free interval prior to entry into the experimental protocol and displayed normal neurological function (score=100) during the inter-

val.16 At study onset, the subjects had average hematocrits of 39.4±4.2%, platelet counts of 482±88x103/μl, and total white cell counts of 12.5±0.3x103/μl (fraction of PMN leukocytes, 0.38±0.16).

In each experiment, occlusion of the right MCA was accomplished by inflation of the extrinsic MCA balloon to 100 μl in the awake subject. Following a 3-hour period of MCA occlusion, reperfusion of the MCA territory for 1 hour was achieved by balloon deflation. A substantial reduction in neurological score was observed within 10 minutes of MCA compression in all subjects, which was sustained throughout the occlusion period (Table 1). The experiments were terminated by pressure-perfusion fixation via left ventricular puncture under pentothal Na+ (15 mg/kg infusion) anesthesia and assisted ventilation 60 minutes following MCA balloon deflation. Perfusion pressures and flows as well as neurological scores are noted in Table 1.

Perfusion-fixation consisted of an initial perfusion flush phase to wash out all blood elements under antithrombotic and isosmotic conditions, and a subsequent isosmotic fixation phase. Glutaraldehyde and paraformaldehyde in the proportions used allowed rapid, adequate fixation of adherent cellular elements and vascular structures as confirmed by electron microscopy. All solutions were chilled to 4°C prior to delivery. Two perfusion-fixation protocols were designed to evaluate different features of microvascular flow integrity: Microvascular occlusion, (scheme 1, n=2) involved perfusion with 3.0 1 flush solution consisting of colloidal gold (18-20 nm diameter) stabilized in 0.002% polyethylene glycol (20,000 d) suspended in Plasmalyte (Baxter Healthcare, Deerfield, Ill.), bovine serum albumin (BSA, 25 g/l; Sigma Chemical Co., St. Louis, Mo.), heparin (2,000 IU/l), 6.7 μM Na+ nitroprusside (Fisher Scientific, Fair Lawn, N.J.) adjusted to 340 mosmol/l with NaCl, and to pH 7.35, followed by fixation with 7.0 1 colloidal gold suspended in 2% paraformaldehyde/0.5% glutaraldehyde in 10 mM phosphate-buffered saline solution (adjusted to 340 mosmol/l, pH 7.40) delivered at 160-200 mm Hg. Colloidal gold was used as a tracer of endothelial cell permeability and adequacy of perfusion by electron microscopy. Microvascular patency (scheme 2, n=2) involved perfusion with 3.0 1 flush solution containing 25 g/l BSA, 2,000 IU/l heparin, and 6.7 μM Na+ nitroprusside in Plasmalyte adjusted to 340 mosmol/l with NaCl and to pH 7.4, followed by fixation with 7.0 1 tracer solution consisting of india ink (1:1, vol/vol) in Plasmalyte/paraformaldehyde (2% final concentration)/glutaraldehyde (0.5% final concentration) delivered at 160-180 mm Hg, and flows of 600-850 ml/min. India ink (Pelikan AG, Hannover, FRG) was filtered through...
FIGURE 1. Reconstruction of occluded capillary in post-ischemia/reperfusion bed of middle cerebral artery from serial transverse sections (light microscopy). The location of a capillary-occluding polymorphonuclear leukocyte is noted by a reference (0, left margin, and arrow), and the relative distance of successive sections (in microns) is also shown. Sections negative to the reference (0) contain distorted erythrocytes within the vessel and sections positive to the reference contain plasma, a portion of the leukocyte, and colloidal gold. Minimum diameter at the single leukocyte occlusion, 5.8 \( \mu \)m.

Fluted filter paper (grade 371, Baxter/Scientific Products, McGraw Park, Ill.) before it was mixed into solution. Here india ink, a standard tracer of vascular patency, was combined with glutaraldehyde/paraformaldehyde to provide consistent high-quality fixation of patent vessels for light microscopy video imaging. In both schemes 1 and 2, high arterial perfusion pressures were chosen to identify adherent cellular elements in occluded microvessels.

Following scheme 1, the brain was removed in toto and sectioned coronally. Stereoanatomically identical portions of the right and left (normal) temporal cortical gray and white matter, and stereoanatomically identical portions of the peripheral zone of the left (normal) basal ganglia and of the right (post-ischemia/reperfusion) basal ganglia were postfixed in osmium tetroxide, dehydrated in ethanol, and embedded in TAAB-812 epoxy resin (TAAB Laboratory Equipment, Ltd., Reading, UK). Sections were cut serially at 1 \( \mu \)m intervals, stained with toluidine blue, and examined at high magnification by light microscopy (\( \times 400 \) and \( \times 1,000 \); Axioskop 20, Carl Zeiss, Munich, FRG) for the presence of vascular occlusions and PMN leukocytes. In addition, sections from the right basal ganglia were reembedded in epoxy resin and recut. Ultrathin sections (silver to light gold), were mounted on 100-mesh copper grids, stained with uranyl acetate and lead citrate, and examined by transmission electron microscopy (Hi-
With scheme 2, the exposed brain was immersed in alcohol-formaldehyde-acetic acid solution (87% ethanol, 10% formaldehyde, 3% glacial acetic acid, vol/vol)\textsuperscript{12} for 4–6 days. The brain was subsequently removed, sectioned coronally at 1-cm intervals, and immersed for a further 7 days in alcohol-formaldehyde-acetic acid solution to achieve complete intravascular gelation of the carbon tracer. Regions of decreased perfusion in the right basal ganglia were apparent by visual inspection in both subjects. Tissue blocks (1.0 cm×1.0 cm×0.2 cm) from the left and right temporal cortex and blocks (1.0 cm×1.0 cm×0.2 cm) from stereanatomically identical sites of the right and left basal ganglia were embedded in glycol methacrylate (Polysciences, Inc., Warrington, Pa.), cut at 5 μm and 10 μm thickness, stained with basic fuchsin/methylene blue, and examined by light microscopy for the presence of india ink-filled vascular structures (microvascular patency).

Sections prepared for light microscopy were analyzed with the aid of a video-imaging system consisting of an image processing unit connected in-line with a Hamamatsu C2400-07 Newvicon NTSC video camera staged vertically on the light microscope (VIDAS; Kontron and Carl Zeiss, Munich, FRG).

For evaluation of microvascular occlusions (scheme 1), the minimum transverse diameter of all patent, occluded, and PMN leukocyte-containing vessels in six random nonoverlapping 0.5 cm×0.5 cm sections chosen at >50 μm intervals from one another at three separate sites were counted. Additionally, serial reconstruction of 10 random occluded capillaries at 1 μm intervals along a length of 110 μm was undertaken to identify the presence and location of PMN leukocytes. For evaluation of microvascular flow patency (scheme 2), the minimum transverse diameter of india ink-containing (i.e., patent) microvessels from four to seven nonoverlapping sections (0.5 cm×0.5 cm) with an individual size of 526 μm×491 μm (at ×200 optical magnification) were computed by video-imaging microscopy. The sections were taken >30 μm interval from one another from stereanatomically identical sites of both ischemic (right) and control (left) basal ganglia. An identical number of fields from each of the paired basal ganglia were analyzed (total number of fields, 364–637). With perfusion scheme 1, no occlusions were observed in the normal cortex and normal basal ganglia, and single intact erythrocytes were rarely noted (<0.1% of microvessels).

For the purposes of this and subsequent studies microvessels are those vessels ≤100 μm minimum...
FIGURE 4. Transverse section of lenticulostriatal capillary following middle cerebral artery occlusion/reperfusion with occluding polymorphonuclear leukocyte. Capillary diameter, 6.7 μm. No leukocyte-endothelial gap apparent. Magnification bar, 1 μm.

diameter. As capillaries constitute microvessels of 4.0–7.5 μm transverse diameter,18 percent capillary reflow was computed as the ratio of the number of microvessels, 4.0–7.5 μm minimum diameter, in the ischemic to the control basal ganglia expressed per 100 vessel cross sections.

Two imaging controls were performed with specimens obtained with scheme 2 (india ink) to evaluate the reproducibility and reliability of data acquired with the video-imaging system. Reproducibility of the observations was assessed by 20 iterations of eight separate microvessels 4.2–23.9 μm minimum diameter, independently calibrated against a 10-μm reticule. Coefficients of variance were 0.0, 0.18, 0.47, 0.54, 0.89, 5.46, 5.74, and 5.93% and were independent of specific vessel minimum diameter. Reliability of the measurement of the biological event (microvascular patency) was assessed by the measurement of minimum diameters of microvessels in aligned 0.5 cm × 0.5 cm regions of interest in 20 successive 10-μm sections of the left (normal) basal ganglia of two baboons. Comparison of the distribution of minimum diameters from 4.0–100.0 μm in serial sections 1–10, 15, and 20 demonstrated no significant difference between sections up to 50 μm apart (e.g., sections 1 versus 5, 2 versus 6, 10 versus 15). In successive patency experiments with india ink perfusion-fixation scheme 2, when the ratio of microvessels in the left basal ganglion to the left temporal cortex (layers I–VI) was 0.51 ± 0.08 (n = 6; 0.5 cm × 0.5 cm regions of interest; data not shown), the distribution and morphology of patent microvessels in the left basal ganglia was consistent and judged excellent.

Data are presented in the literal form and as mean ± SD. Statistical analyses were performed with the Student’s t test (two-tailed) or the Kruskal-Wallis one-way nonparametric test, where appropriate.

Results

No occlusions were observed in lenticulostriatal microvessels in the nonischemic (left) basal ganglia or temporal cortex. Occlusions containing PMN leukocytes and/or erythrocytes were documented in microvessels of 4.0–7.5 μm diameter in the peripheral zone of the ischemic basal ganglia following 3 hours of occlusion and subsequent reperfusion of the ipsilateral MCA. Polymorphonuclear leukocytes were found in 8.2% of 425 microvessels ≤ 10 μm transverse diameter examined in 68 nonoverlapping fields (scheme 1, × 400). Complete occlusion by a single PMN leukocyte was observed in 20 microvessels (4.7%) 6.9 ± 1.3 μm diameter consistent with capillaries. Capillary-occluding PMN leukocytes appeared as single cells in transverse section, but were positioned between packed erythrocytes on both sides or,
ininfrequently, by plasma on one side and erythrocytes on the other (Figure 1). Partial occlusion by one or more PMN leukocytes was observed in substantially larger microvessels (3.5%, mean 10.2±2.0 μm diameter). The latter vessels each lacked a muscularis media typical of meta-arterioles and were morphologically similar to postcapillary venules. When 10 occluded capillaries were followed over a length of 110 μm, a completely obstructing PMN leukocyte was found in 40% (four of 10) of vessels of 6.4±0.8 μm diameter. Among the remaining vessels, no obstructing PMN leukocyte was observed within the sections examined (see “Discussion”).

Vascular patency studies confirmed a significant reduction in the number of microvessels of all size classes ≤60 μm diameter, but particularly of capillary diameter (4.0–7.5 μm) following ischemia/reperfusion (Figure 2). Capillary reflow was only 38.5% and 26.5% (n=2; 2p=0.045 against respective nonischemic basal ganglia).

Isolated obstructing PMN leukocytes were found to have an extended endothelial cell contact surface with an intercellular gap range of 0–160 nm (Figure 3). While endothelial cell swelling was not observed, increased permeability was implied by the presence of 20 nm gold particles in the endothelial cell cytoplasm and perivascular space. Transverse sections of isolated, completely occluded capillaries have demonstrated close circumferential contact of leukocyte and endothelial cell surfaces (Figure 4). Fibrin was not observed in association with any PMN leukocyte-endothelial cell contact. Rarely, degranulated platelets with associated fibrin were observed in juxtaposition to PMN leukocytes in obstructed capillaries (Figure 5).

**Discussion**

A role for nondeformable (relatively stiff) circulating cellular elements in the development of the “no-reflow” phenomenon has been suggested in several model settings. However, the presence of vascular obstructing PMN leukocytes has not been unequivocally demonstrated in a focal cerebral ischemic bed following cerebral arterial reperfusion. It is possible that intravascular trapping of PMN leukocytes could explain the regional microvascular low flow state observed in novel models of global ischemia. Here we document the presence of PMN leukocytes in occluded microvessels of capillary diameter in the post–focal ischemia/reperfusion lenticulostriate arterial bed within 60 minutes after MCA reperfusion. The presence of PMN leukocytes in microvessels is an essential requirement for postulat-
ing their role in very early microvascular injury following focal ischemia.

These findings suggest that PMN leukocytes may also play a role in occlusion formation following focal ischemia/reperfusion injury by delaying flow in the capillary bed. Review of the original sections by light microscopy from previous experiments have indicated the presence of PMN leukocytes in many occlusions. A more systematic examination of the vascular characteristics of PMN leukocyte-containing obstructions indicates that PMN leukocytes were present only within microvessels <10 μm inner lumen diameter, completely obstructing vessels of capillary diameter. Granulocytes also were present in postcapillary venules, and frequently, several cells in groups could be identified in a position traversing the lumen. Whether such cellular aggregates could produce obstruction could not be determined from this study. In patency (india ink) experiments, the proportionate loss of microvessels of all size classes <60 μm is consistent with the view that "no-reflow" is almost exclusively a capillary phenomenon in the lenticulostriate territory in this model.

No leukocytes in the perivascular parenchyma could be identified at this early time following MCA occlusion and reperfusion, suggesting that the event was intravascular only. It is possible that the participation of intravascular PMN leukocytes in capillary "no-reflow" provides triggers for the invasion of granulocytes observed after 24 hours and for their role in maturation of the infarct.

In a limited number of obstructed microvessels, 40% had PMN leukocytes completely occluding the capillary portion along a 110-μm length. In some studies involving strategies that induce leukopenia (e.g., antineutrophil serum), the failure to completely abrogate the "no-reflow" phenomena may have been due to residual circulating PMN leukocytes, which must traverse the capillary bed on reperfusion. Because of the rheologic and adhesive properties of these cells, it is possible that only few granulocytes might obstruct a significant portion of the postischemic microcirculation. This may also explain the limited number of capillaries containing PMN leukocytes among those reconstructed. However, the precise role of PMN leukocyte capillary plugging in focal ischemia/reperfusion requires some information about the three-dimensional lattice of the microvessels in the lenticulostriate arterial territory, specifically the distance between branch points. A formal three-dimensional reconstruction of this microvascular territory has never been performed in the nonhuman primate and has not been possible in humans.

The contact region between an obstructing PMN leukocyte and the endothelium has nonuniform gap width and may involve several sites of contact. Loss of membrane continuity in regions of narrow gap width was noted among several cells examined (Figures 3 and 4). The large contact surface together with the fact that the PMN leukocytes could not be removed, even after restoration of arterial pressure, suggest that these cells may develop significant attachment energies to the endothelium, such as seen in other organs.

Receptor-mediated attachment and activation of the PMN leukocytes may be involved. The most likely candidates for such attachments are the endothelial attachment protein GMP-140, expressed very early upon endothelial cell activation, other endothelial cell adherence proteins (e.g., ICAM-1), and the PMN leukocyte adherence complex CD11/CD18, for instance. This observation is also consistent with the relative nondeformable nature of PMN leukocytes traversing the normal microvasculature. More significantly, PMN leukocytes found obstructing capillaries in the posts ischemic lenticulostriatal bed had the appearance and configuration of those documented in the posts ischemic cardiac and skeletal muscle beds. Unfortunately, from the present study, it is not possible to determine whether the PMN population involved in capillary obstruction may have been previously activated while still in free circulation. The degree of leukocyte degranulation and therefore activation was not assessed quantitatively.

We found no evidence for "endothelial tags," sometimes observed in nonobstructed vessels in other model systems. It is possible that the perfusion and fixation conditions required for their appearance were not met in the current study or that they appear only in selected vascular beds. Such "tags" have been observed by transmission electron microscopy in the rat skeletal musculature by altering perfusion conditions, and in a global ischemia model in which scanning electron microscopy was used. Importantly, endothelial cell swelling was not observed in the microvascular territory under the perfusion conditions used. However, endothelial cell permeability was altered as seen by the extravasation of 18–20 nm gold particles into endothelial cytoplasm subjacent to the obstructing leukocyte and into surrounding ischemic parenchyma (after only 4 minutes of exposure to the gold tracer). Finally, in the acute ischemic state erythrocytes probably do not participate in the capillary obstruction because intravital studies have shown that upon exit of granulocytes from capillaries, flow is instantaneously and completely restored.

In an earlier report the preischemia use of a potent antithrombotic combination (ticlopidine/heparin) resulted in a significant reduction in occlusion formation, suggesting a thrombogenic component to the occlusions. The reduction of microvascular occlusion by preischemia intervention with a novel antithrombin in experimental global cerebral ischemia also supports the impression that thrombotic microvascular occlusions might be involved in post–cerebral ischemia/reperfusion injury. In this study, evidence of fibrin deposition and associated platelet aggregate formation was found in juxtaposition to PMN leukocytes. The association of degranulated platelets with PMN leukocytes may represent platelet activation upon reperfusion, or be a part of a complex set of events occurring during microvascular ischemia and
stasis. Although this study has not provided direct evidence, platelet activation and subsequent fibrin formation in this setting may result from elaboration of platelet-activating factor (PAF) by trapped granulocytes. The release of PAF, and the possible activation of some granulocytes, offers mechanisms for fibrin formation and microvascular occlusion, which may be inhibited by antithrombins and other approaches. To this end, the precise location and conditions of fibrin generation and the relationship to granulocyte activation and post-ischemia/reperfusion injury will require further study.

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References


KEY WORDS • neutrophils • reperfusion • baboons
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