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Brain Microvessels: Factors Altering Their Patency after the Occlusion of a Middle Cerebral Artery (Wistar Rat)

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The progression from ischemic injury to pannecrosis that occurs in the rat brain several hours after occluding a large artery may be partly attributable to a worsening of the circulation through the microvessels. The objective of this study was to quantitate selected structural changes involving astrocytes and endothelial cells within an area of focal brain ischemia created by the occlusion of a middle cerebral artery. The magnitude of these structural changes was correlated with alterations in the patency to a circulating macromolecule through the microvessels (≤15 μ in diameter) located within the territory of the occluded artery. One hundred eighty-five adult male Wistar rats had the right middle cerebral artery occluded after threading a nylon monofilament through the external carotid artery. Experiments were terminated by either cardiovascular perfusion or decapitation and immersion fixation at intervals ranging between 30 minutes and 7 days after the arterial occlusion. Randomly selected animals from each experimental subgroup were injected intravenously with horseradish peroxidase (molecular weight 44 kd) approximately 20 minutes before death. The progressive decline in the area fraction comprised by the vessels filled with horseradish peroxidase was preceded at 30 to 60 minutes by an increase in the surface area occupied (on a cross-section of a microvessel) by endothelial cells (both nucleus and cytoplasm). This was followed by an increase of 23.7% in the mean diameter of astrocytes nuclei and a decrease of approximately 35% in lumenal surface of the microvessels. These observations suggest that the occlusion of a large cerebral artery causes prompt swelling of endothelial cells and astrocytes; both of these early biological responses may interfere with erythrocyte circulation and oxygen delivery, which (after the arterial occlusion) are entirely dependent on the circulation provided by the collateral arterial connections. Through its interference with microvascular patency and oxygen delivery, cell swelling may influence the rate at which neurons become necrotic. In this model of brain infarct the number of necrotic neurons peaks approximately 72 hours after middle cerebral artery occlusion. If swelling affecting endothelial cells and astrocytes during the first hour after an arterial occlusion is a significant cause of neuronal necrosis, through their interference with erythrocyte circulation, therapeutic measures aimed at preventing the swelling of these cell types could have beneficial effects on the outcome of focal ischemic brain lesions. (Am J Pathol 1994, 145:728–740)

The progression of neuronal injury from potentially reversible ischemic damage to necrosis, after the occlusion of an intracerebral artery, may be influenced by biological changes that affect the patency of microvessels (ie, those with a cross-diameter ≤15 μ). Del Zoppo et al[1] documented lumenal obstructions in the microvessels of the basal ganglia among non-human primates subjected to transient occlusion of a middle cerebral artery (MCA). In addition, within 30 to 60 minutes after occluding a MCA in rats, the

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lumina of many dependent microvessels fill with various circulating cells that appear closely apposed to the endothelial lining of these microvessels.[2] Such cellular interactions may be partly responsible for the potential worsening of the cerebral perfusion in the territory of an occluded brain artery and in this manner may contribute, after a certain time, to make the process irreversible.

In this study, we asked the question: Are there structural changes involving either endothelial cells or astrocytes that may impair microvascular patency? Using brain tissues derived from adult Wistar rats that had the origin of the right MCA occluded for periods ranging between 30 minutes and 7 days,[3] we tested this hypothesis. The magnitude of time-dependent swelling of endothelial cells and astrocytes inversely correlates with the extent to which a freely circulating macromolecule fills the microvessels' lumina. For this purpose we calculated in the territory of the right MCA the area surface of all microvessels filled with a macromolecule (horseradish peroxidase, HRP) intravenously injected 20 minutes before death and compared those data with appropriate controls. In addition, we measured the diameter of astrocytes' nuclei (as an indicator of cell size) and estimated the severity of the narrowing affecting the lumenal surface of microvessels. The results are expressed in terms of magnitude differences between experimental animals and the controls.[4]

Materials and Methods

All experiments were conducted according to the guidelines issued by the institutional Animal Care Committee and in compliance with regulations formulated by the U.S. Department of Agriculture concerning the care of laboratory animals.[5]

One hundred eighty-five outbred male Wistar rats (body weight 270 to 290 g) purchased from Charles River Laboratories (Wilmington, MA) were used in this study. One hundred seventy rats were randomly divided into 16 separate experimental subgroups each having an arterial occlusion of different duration. The remaining group of 15 animals was divided into two control subgroups; those in control group A (n = 5) were not subjected to any surgical intervention. Rats in control group B (n = 10) were subjected to the same surgical procedure as those in the experimental group; however, the occluding monofilament was withdrawn less than 60 seconds after it was inserted into the artery and each rat was allowed to survive between 1 and 96 hours (Table 1).

Table 1. *Permanent MCA Occlusion Protocol for Study of Microvessels*

Hours After MCA Occlusion	Number of Rats	Number of Animals Used*				
		H & E	Toluidine Blue	EM†	GFAP	HRP
Experimental group						
0.5	12	12	9 (2)	2 (2)	3	3 (1)
1	10	10	7 (2)	2 (1)	3	4 (1)
1.5	11	11	6	1	2	3 (1)
2	11	11	7 (1)	2 (1)	4	3 (1)
3	12	12	8 (1)	3	4	3 (1)
4	12	12	7 (1)	3 (1)	7	2 (1)
6	13	13	7 (1)	1 (1)	5	4 (1)
12	6	6	3 (1)	1 (1)	3	2 (1)
18	5	5	2 (1)	1	2	1 (1)
24	13	13	9 (1)	2 (2)	3	2 (1)
48	12	12	5 (1)	1	3	3 (1)
72	7	7	4 (1)	1 (1)	2	1 (1)
96	13	13	7 (1)	1	3	2 (1)
120	11	11	7 (1)	1 (1)	3	2 (1)
144	12	12	11 (1)	2	2	1 (1)
168	10	10	6 (1)	2 (2)	3	2 (1)
Total	170	170	105 (17)	26 (13)	52	38 (16)
Control groups						
Control A	5	5	2 (1)		1	1 (1)
Control B						
Sham 1 h	1	1	1 (1)	1 (1)	1	
Sham 2 h	2	2				
Sham 4 h	2	2	1			
Sham 24 h	3	3	1		1	1 (1)
Sham 48 h	1	1	1		1	
Sham 96 h	1	1	1	1	1	
Total	15	15	7 (2)	2 (1)	5	2 (2)

* The number in parentheses indicates the number of subjects used for quantitation by image analysis methods.
† Em indicates electron microscopy.

Each experimental subject (n = 165) had the right MCA occluded (under general anesthesia with halothane and N_2O_2) after inserting a segment of a nylon monofilament (4-0) into the right external carotid artery, according to methods previously described in detail.[3, 6] The length of the intravascular filament varied between 17.5 and 20 mm, depending on the body weight of the individual animal. Body core temperature was maintained at 37 C during the surgical procedure and the immediate postoperative period with a recirculating pad and K module. The duration of the MCA occlusion ranged from 30 minutes to 7 days and each experimental subgroup included 5 to 13 animals (Table 1). At the completion of the surgical procedure, each animal's body temperature and general condition were monitored for approximately 1 hour; thereafter, animals were allowed free access to food and water.

Experiments were terminated at predetermined time intervals (Table 1). All brains were fixed by transcardiac perfusion, except those used in the HRP evaluation, which were fixed by immersion in a fixative of the same composition. As shown in Table 1, 40 brains (38 experimental and 2 controls) were fixed by immersion, leaving a total of 145 (or 132 experimental and 13 control) in the perfusion fixation category. Under analepsis (ketamine/xylazine) cardiovascular perfusion was started with 250 ml of 0.9% saline (USP Baxter, McGaw Park, IL) containing 1000 U of heparin (Upjohn, Kalamazoo, MI), followed by 250 ml of 4% paraformaldehyde (PF) in 0.1 M phosphate buffer; the buffer osmolality was 340 mOsm. A constant pressure (100 mmHg) equal to the rat's mean arterial blood pressure was maintained during perfusion fixation; immediately after completing the cardiovascular perfusion, the brain was removed and immersed overnight in 4% PF at 4 C. At the completion of the fixation period, each brain was sliced into five coronal slabs (each 3-mm thick) and labeled A (frontal) to E (occipital). Slabs A, B, D, and E were embedded in paraffin then cut into 6-μ sections and stained with hematoxylin and eosin. Slab C, which corresponds to the level of the anterior commissure and is located 0.30-mm caudal to Bregma, was retained for electron microscopy studies.

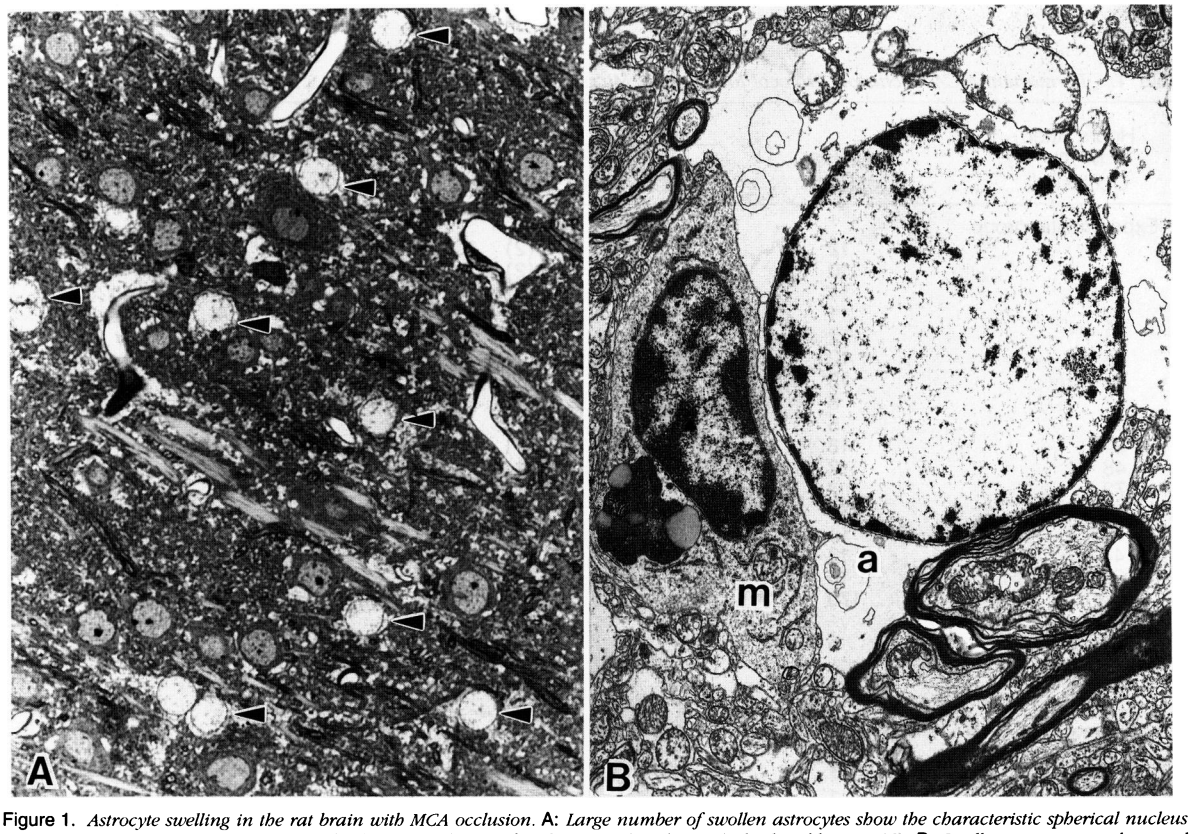

Figure 1. *Astrocyte swelling in the rat brain with MCA occlusion.* **A:** *Large number of swollen astrocytes show the characteristic spherical nucleus and clear cytoplasm (arrowheads). Cerebral cortex, 3 hours after the arterial occlusion (toluidine blue, ×132).* **B:** *Swollen astrocyte nucleus and cytoplasm (a) adjacent to a nonswollen microglial cell (m); the photograph illustrates the selective nature of this phenomenon. Cerebral cortex, 1 hour after MCA occlusion (×8500).*

Electron Microscopy Preparation

Brain samples from randomly selected animals (n = 28) were processed for electron microscopy. Brain slab C was cut into eight sections that included the entire surface of each hemisphere; these tissues were postfixed in 3% glutaraldehyde and osmium tetroxide and embedded in araldite. One-micron-thick sections were stained with toluidine blue and after identifying areas of interest, ultrathin sections were stained with lead citrate and uranyl acetate for examination with a Phillips electron microscope. Staining with lead uranyl was not applied to some of the samples processed for HRP evaluation; otherwise, the preparation of the samples was the same in all groups. A total of 1892 electron micrographs (approximately 67.5/animal) were evaluated in the ultrastructural analysis.

Immunohistochemistry

Multiple 6-μ-thick sections of paraffin-embedded tissues were obtained from brain samples of one randomly selected subject in each experimental group to demonstrate glial fibrillary acidic protein (GFAP). Antiserum used in these reactions was obtained from Dako Corporation (Carpenteria, CA). After diluting these antisera to 1:2000, the avidin-biotin peroxidase complex method (ABC kit; Vector Laboratories, Inc., Burlingame, CA) was used for demonstration of the antigen.

Quantitation of Light and Electron Microscopic Abnormalities

An image analysis system (IMAGIST-2, PGT; Princeton, NJ) was used to quantitate abnormalities involving astrocytes and microvessels. From each experimental subgroup, four 1-μ-thick sections (from the coronal section C), representing the entire hemisphere ipsilateral to the side of the MCA occlusion, were stained with toluidine blue and examined at a magnification of ×1000. Images from 30 nonoverlapping microscopic fields were collected from each specimen with the aid of a Sony CCD video camera interfaced with an Olympus microscopic system. Nuclei of astrocytes were identified on the basis of their intrinsic morphological features (Figure 1), and the mean diameter of each nucleus was calculated from a program based on 14 separate measurements of each nucleus' diameter.

Negative films of electron micrographs, each containing a cross-sectional view of a microvessel (≤15 μ in diameter), were exposed at a magnification of ×7000 after proper calibration of the electron microscope (Figure 2). The outer circumference of each microvessel or the area encircled by the outer line of the basal lamina (exclusive of pericytes) was traced with the aid of the Sony CCD video camera equipped with a 16-mm, 1:1.4 lens. Six hundred nine electron micrographs were collected from 10 different experimental subgroups and 2 control groups; a total of 609 images of microvessels were obtained and 238 of these had visible endothelial cell nuclei. For each microvessel we calculated the following: circumference, lumenal area surface, lumen diameters (longest, shortest, average of 14 different measurements), and percentage of the cross-surface of the microvessel that was occupied by the endothelial cell nucleus and cytoplasm (Figure 2).

Study of Microvascular Patency

Forty randomly selected animals from the experimental subgroups, including two controls, were injected with a macromolecular tracer (Table 1). Under anesthesia (N_2O_2 and halothane), 15 mg of HRP (molecular weight 44 kd, type VI; Sigma Chemical Co., St. Louis, MO) was dissolved in 1 ml of a normal saline

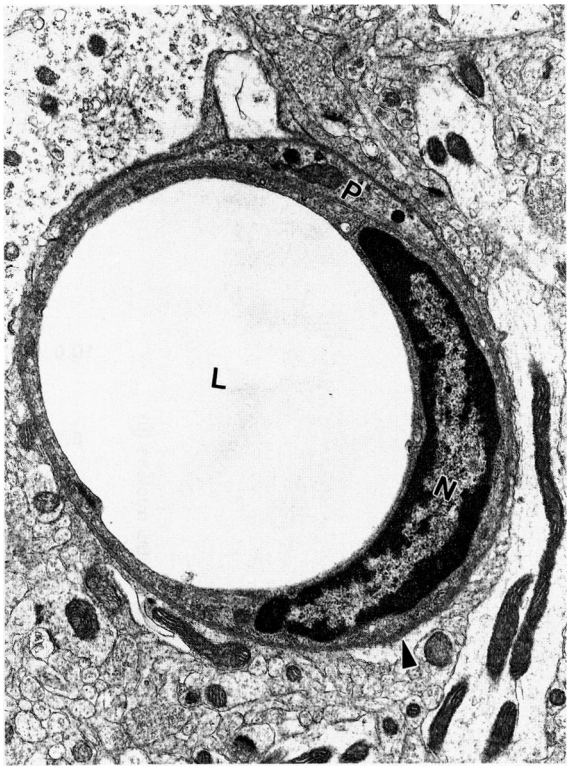

Figure 2. *Normal capillary from the nonischemic left hemisphere 12 hours after the right MCA was occluded. Capillary surface was calculated to include nucleus (N), endothelial cytoplasm, and lumen (L) but excluded the pericyte (P) (×13,000).*

solution and the mixture was slowly injected into the femoral vein. Twenty minutes later, and while still under the effects of anesthesia, the animal was decapitated. The brain was immediately removed then cut into five coronal sections (each 3-mm thick) and fixed in 4% PF overnight. The next day, coronal sections B, C, and D were placed in 2% purified agar (Sigma Co.) and maintained at 4 C for 3 to 4 hours; 100μ-thick sections were cut with a vibratome. These sections were reacted with 3–3′diaminobenzidine-H_2O_2 (DAB-H_2O_2) and examined with the light microscope. For electron microscopy, samples from slab C (100 μ) were reacted with DAB-H_2O_2 and postfixed in 3% glutaraldehyde. Ultrathin sections for this portion of the study included samples with and without staining with uranyl acetate and lead citrate.

Measurement of HRP Distribution

Brain coronal slice C of animals injected with HRP (n = 18) was used to measure area fraction applying the image analysis system. The area fraction of HRP in the ipsilateral hemisphere was calculated in three separate regions: cortex, striatum, and preoptic areas. Images from homotopic areas in the nonischemic hemisphere provided a total of six nonoverlapping microscopic fields. The extent of the HRP distribution was expressed as the ratio of the total area selected for analysis that contained vessels filled with HRP. The ratio was obtained after dividing the ischemic side value by the comparable measurement obtained from the contralateral homotopic area.

Statistical Analysis

Data used in the analysis include those derived from light and electron microscopy measurements of microvessels and astrocytes. Results of individual measurements are expressed as mean values ± standard deviation. Analysis of variance (ANOVA) followed by Bonferroni corrected *t*-test was used to determine significant differences between control groups and experimental subgroups. A $P < 0.05$ was considered statistically significant.

Results

Astrocytes

Astrocyte swelling involving both the nucleus and cytoplasm became especially noticeable in the basal ganglia and cerebral cortex. Within the first 60 minutes, the mean diameter of the ischemic astrocytes' nucleus increased by 23.4% compared with control values (Figures 1 and 3); astrocyte swelling extended to the pericapillary astrocytic processes (Figure 4). Swelling of astrocytes nuclei was most pronounced during the period between 3 and 24 hours after MCA occlusion, and the swelling of these cells persisted until the time when many began to undergo necrosis (24 hours after MCA occlusion) (Figure 3). In the preoptic area and during the first 60 minutes, scattered astrocytes disintegrated, and the intensity of the GFAP immunoreaction decreased. Necrosis involving large numbers of astrocytes was particularly noticeable during the period from 48 to 72 hours when

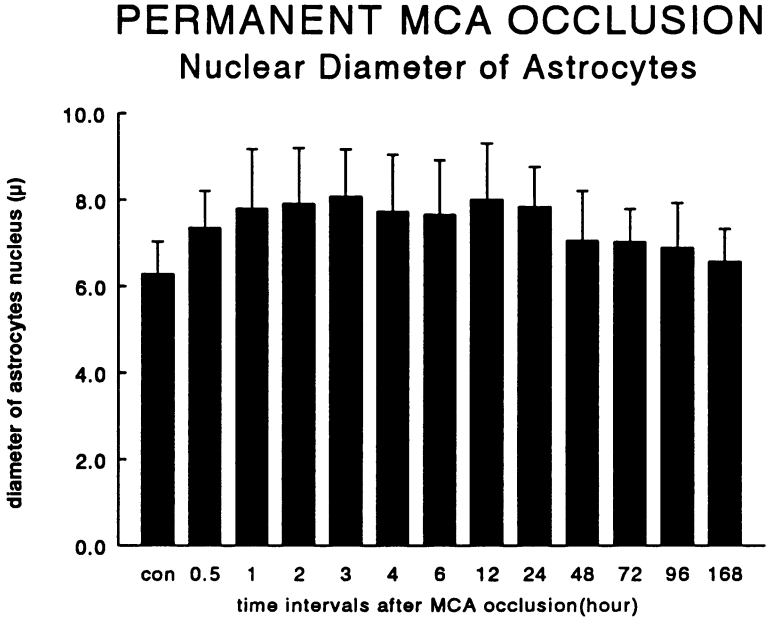

Figure 3. *Mean diameter of astrocyte's nucleus including control (con) and data obtained from animals with MCA occlusion of varying duration; each bar represents mean values derived from 30 nonoverlapping fields for each animal.*

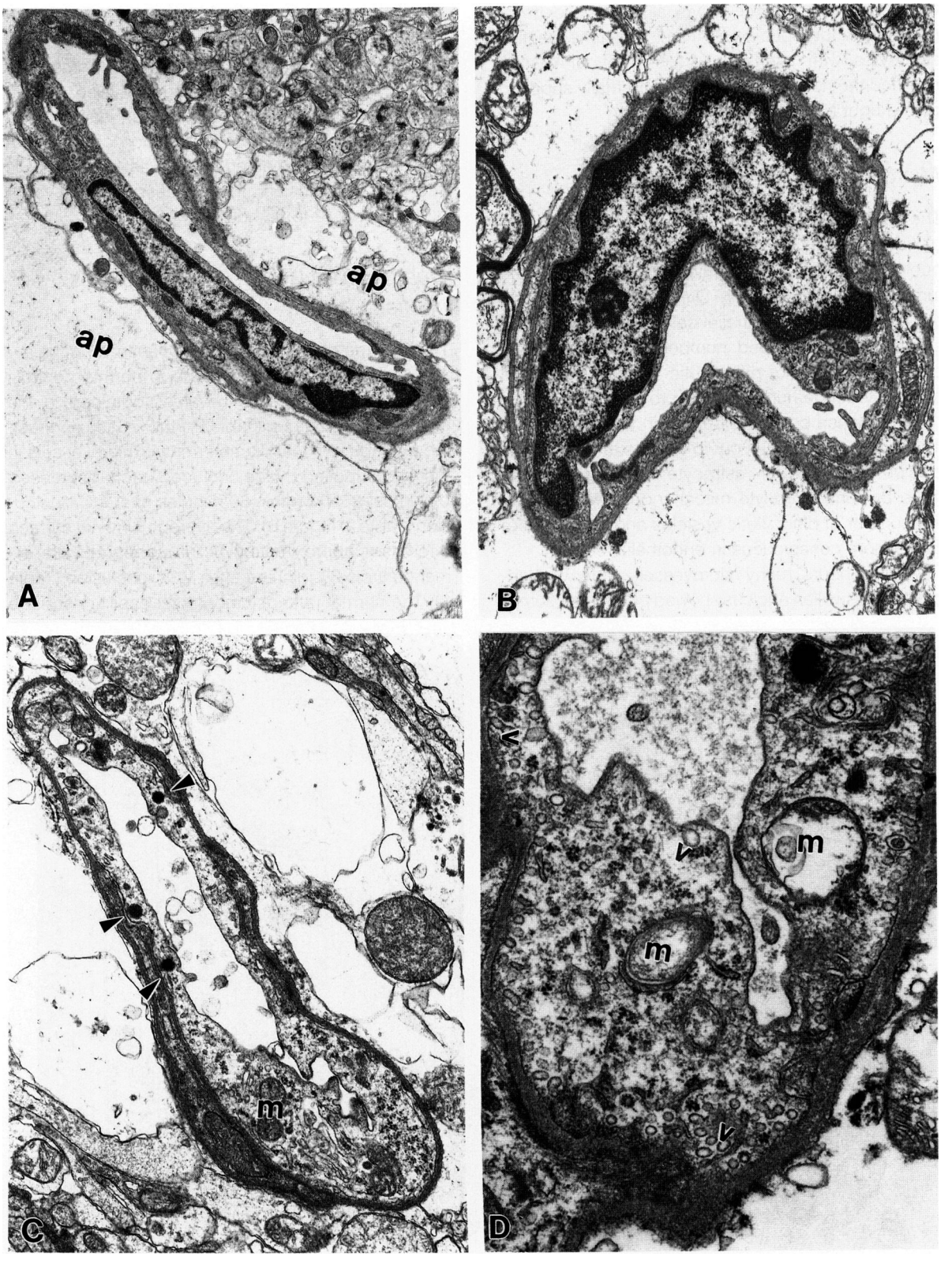

Figure 4. *Microvessels in rats with MCA occlusion of short duration (≤6 hours).* A: *Narrowed lumen in an area where astrocytes processes (ap) are markedly swollen and endothelial cells show prominent microvilli. Striatum, 6 hours after arterial occlusion (×8550).* B: *Enlargement of the endothelial cell nucleus may contribute to the narrowing of the capillary lumen. Cerebral cortex, 1 hour after arterial occlusion (×14,000).* C: *Prominent cytoplasmic organelles include dense bodies (arrowheads) and increased number of mitochondria (m). Preoptic area, 2 hours after arterial occlusion (×9450).* D: *Enlarged, swollen mitochondria (m) and increasing number of pinocytotic vesicles (v) are visible in the endothelial cytoplasm. Cerebral cortex, 1 hour after arterial occlusion (×36,000).*

GFAP-positive cells were not visualized in either basal ganglia or supraoptic area. At the same time reactive astrocytes with intense expression of GFAP surrounded the now necrotic territory normally supplied by the right MCA (Figure 5).

Microvessels

The earliest change observed in microvessels (≤15 μ) involved enlargement of the endothelial cell nucleus; this was accompanied by a decrease in the lumenal surface (Figure 4). During the initial 2 hours, the cytoplasm of endothelial cells showed moderate swelling and increased numbers of mitochondria; also, the number of pinocytotic vesicles increased mildly. Moderate numbers of microvilli and intracytoplasmic dense bodies appeared in scattered endothelial cells (Figure 4). Four to 6 hours after MCA occlusion, perivascular astrocyte swelling became more evident, astrocyte mitochondria swelled, and the number of pinocytotic vesicles and microvilli became more conspicuous in endothelial cells.

After 12 hours, many microvessels underwent necrosis: the swollen nucleus showed peripheral clumping of heterochromatin, cytoplasmic organelles disappeared and electron lucency increased, and fragments of necrotic cells (presumably fragments of sloughed endothelial cells) frequently filled the microvessel's lumen. A widened basal lamina remained identifiable. Regenerative proliferation of microvessels could be documented as early as 12 to 24 hours after MCA occlusion; this process became very prominent at 72 to 96 hours.

Quantitative Ultrastructural Changes in Microvessels

The average diameter of capillary lumina and the average capillary lumenal surface in normal controls were almost identical with values obtained in the sham-operated group (Table 2, Figure 6). In contrast, the average lumenal diameter of the microvessels in the area supplied by the occluded artery decreased by almost 23.7% within 60 minutes after MCA occlusion (Table 2, Figure 6). The average lumenal surface decreased approximately 49.2% compared with normal controls 30 minutes after MCA occlusion (Table 2). The normal ratio of lumen: total capillary surface

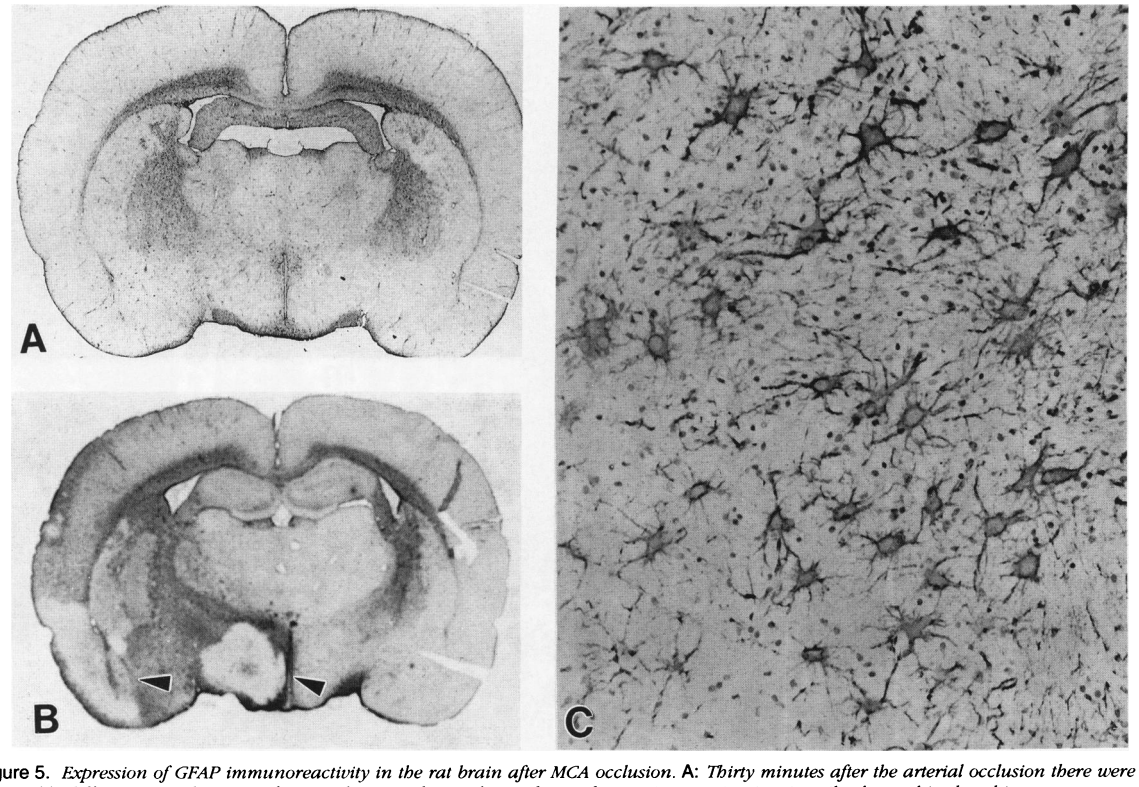

Figure 5. *Expression of GFAP immunoreactivity in the rat brain after MCA occlusion.* A: *Thirty minutes after the arterial occlusion there were no noticeable differences, at this magnification, between the two hemispheres; the positive reaction is primarily observed in the white matter astrocytes (GFAP, ×2.5).* B: *Forty-eight hours after the arterial occlusion, protein expression is absent from cells in the preoptic area and from selected patches of tissue in the basal ganglia and cerebral cortex (arrowheads). The intensity of the reaction is increased in cells located at the periphery of the necrotic sites (GFAP, ×2.5).* C: *Forty-eight hours after MCA occlusion there is strong reactivity of GFAP in astrocytes from the zones surrounding the necrotic areas (GFAP, ×160).*

Table 2. *Microvessels in the Ischemic Hemisphere After MCA Occlusion*

Hours After Occlusion	Number of Blood Vessels (<15 μ)	Lumen Surface (μ^2 ± SD)	Mean Diameter (μ ± SD)
0.5	63	7.19 ± 4.6*	3.74 ± 1.0
1	72	7.27 ± 4.3*	3.55 ± 0.8
2	62	7.20 ± 5.7*	3.58 ± 1.0
4	82	8.07 ± 5.4*	3.83 ± 1.0
6	73	6.81 ± 5.3*	3.63 ± 1.1
12	35	8.43 ± 5.2*	3.75 ± 1.0
24	32	9.85 ± 6.3	4.02 ± 1.1
72	23	12.66 ± 6.0*	4.51 ± 1.1
120	33	8.89 ± 6.4	3.90 ± 1.3
168	32	12.34 ± 7.2	4.29 ± 1.4
Sham	36	10.92 ± 8.1	4.23 ± 1.3
Control	50	14.13 ± 6.6	4.65 ± 1.2

ANOVA with Bonferroni corrected *t*-test. There were significant differences ($P < 0.05$) between control group and experimental subgroup.

* There were no significant differences between the control and the sham-operated groups.

area decreased by approximately 35% during the first 30 minutes (Table 3, Figure 6). The percentage of microvessels having a lumenal surface of ≤2.5 μm^2 was: control, <0.5%; before 6 hours, 11.3%; after 12 hours, 3.2%.

To estimate the contribution that endothelial cell swelling may have on the impairment of the microvascular patency, we calculated the ratio of endothelial nucleus: total capillary area surface. During the first 30 minutes the average diameter of endothelial cell nucleus increased by almost 54.4% compared with the control groups (Table 3, Figures 4, 6, and 7). Enlargement of the nucleus in the microvessels persisted in the experimental groups until 7 days after the occlusion of the MCA (Table 3).

Changes in Blood-Brain Barrier Permeability

Extravasation of HRP in the ischemic brain territory was seen only in experiments in which the MCA occlusion lasted between 3 to 24 hours and in every instance the leaked HRP remained almost entirely confined to the preoptic area (Figure 8). After numerous microvessels became necrotic (at 24 hours or later), diapedesis of a few red blood cells was also noted at several foci; this became especially noticeable in the preoptic area and cerebral cortex. All these hemorrhages were detectable only after careful microscopic evaluation; none were grossly visible. HRP was visible in the pinocytotic vesicles of endothelial cells and occasionally in the cytosol of pericytes (Figure 8). HRP was visible next to necrotic microvessels in the basal lamina and in adjacent

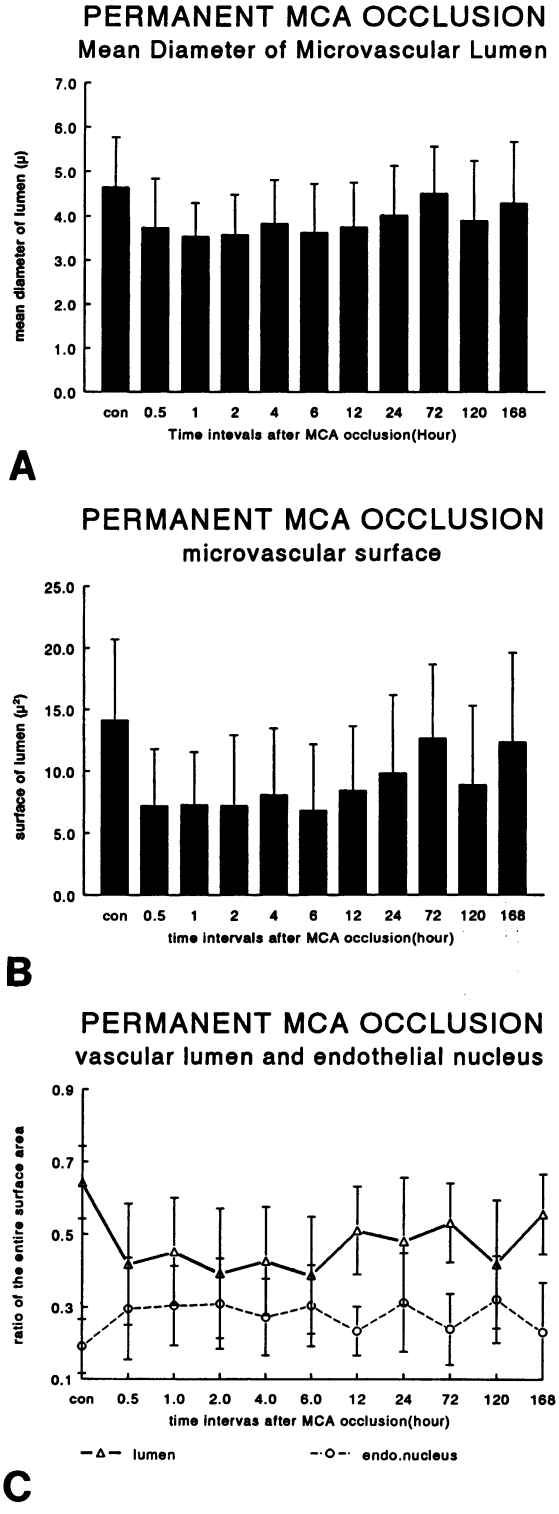

Figure 6. *Cerebral microvessels after MCA occlusion.* A: *Mean diameter of the lumen in microvessels (≤15 μ).* B: *Mean surface area of the lumen in microvessels (≤15 μ).* C: *Chronological relationship between changes in surface area of endothelial cell nucleus and surface area of capillary lumen.*

Table 3. *Surface Area of Microvessels in the Ischemic Hemisphere After MCA Occlusion (EM)*

Hours After Occlusion	Lumen Surface (%)	Endothelial Nucleus* (%)	Endothelial Cytoplasm (%)
0.5	41.8 ± 16.7	29.5 ± 0.14	28.7
1	45.3 ± 14.8	30.4 ± 0.11	24.3
2	39.3 ± 17.6	30.9 ± 0.12	29.8
4	42.7 ± 15.0	27.2 ± 0.10	30.1
6	38.8 ± 16.1	30.4 ± 0.11	30.8
12	51.0 ± 12.1	23.4 ± 0.06	25.6
24	48.4 ± 17.5	31.3 ± 0.13	20.3
72	53.3 ± 10.9	24.0 ± 0.09	22.7
120	41.8 ± 17.7	32.2 ± 0.12	26.0
168	55.7 ± 10.8	23.2 ± 0.13	21.1
Control	64.3 ± 10.0	19.1 ± 0.07	16.6

* ANOVA with Bonferroni corrected *t*-test. There were significant differences between the control group and each experimental subgroup ($P < 0.05$).

extracellular spaces; in the pannecrotic regions some macrophages also contained HRP.

The area fraction occupied by HRP-filled vessels began decreasing in the ischemic hemisphere 1.5 hours after MCA occlusion; a subsequent sharp decrease was especially marked after 24 to 48 hours (Figure 9). After the second day, the irregular, newly formed microvessels did not fill with circulating HRP and as a result an avascular area persisted in what now may be called the lesion's core, ie, the supraoptic area.

Discussion

Global cerebral ischemia of short duration (5 minutes) induced in rabbits by the simultaneous compression of all the cervical arteries and veins resulted in incomplete filling of the brain microvessels when reperfusion was attempted with a fixative solution containing carbon black particles. This incomplete vascular filling was called the no-reflow phenomenon and was attributed by the authors to the possible extrinsic compression of the microvessels by swollen perivascular astrocytes processes.[7] A subsequent failure to verify this finding in comparable experiments led the same authors to suggest that perhaps vasoconstriction or intraluminal thrombi formation would better explain the inability to evenly reperfuse a rabbit brain that had been made ischemic 5 minutes earlier by strangulation.[8] The topic of postischemic hypoperfusion, which is commonly observed in experimental models of global brain ischemia, was reviewed by Hossmann[9] who suggested that the phenomenon may have multiple putative causes including cell swelling, intravascular thrombi formation, vasoconstriction, and perivascular bleeding.

Because in experimental models of arterial brain infarct there is a lag of several hours between the time when the artery is occluded and the time when widespread coagulation necrosis becomes demonstrable,[3] some authors have suggested that after occluding a large artery, the circulation through the dependent microvessels may become progressively impaired and that this may contribute to the process ultimately responsible for neuronal necrosis.[10] Accordingly, reflow (after reopening the artery) could be impeded in areas where lumenal changes affect the microvessels.

Working with two slightly different paradigms of arterial brain infarct, del Zoppo et al[1] and Garcia et al[2] have shown lumenal obstruction in microvessels shortly after the parent artery is occluded. This lumenal obstruction of microvessels involves the apparent interaction between leukocytes and endothelial cells and this results in many microvessels being occluded by erythrocytes that appear closely apposed to the endothelial cells'

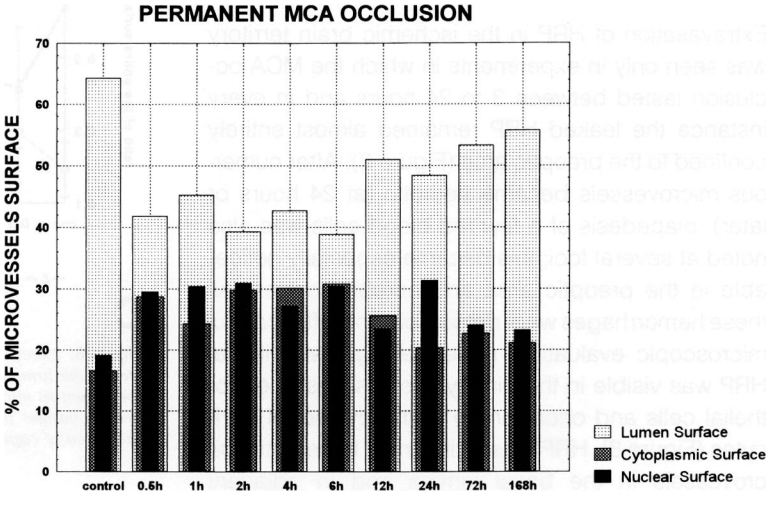

Figure 7. *Progressive changes in the percentage that each area fraction contributes to the capillary cross surface; cytoplasmic refers to the surface occupied by the endothelial cell cytoplasm and nuclear refers to the surface of the nucleus of the same cell type.*

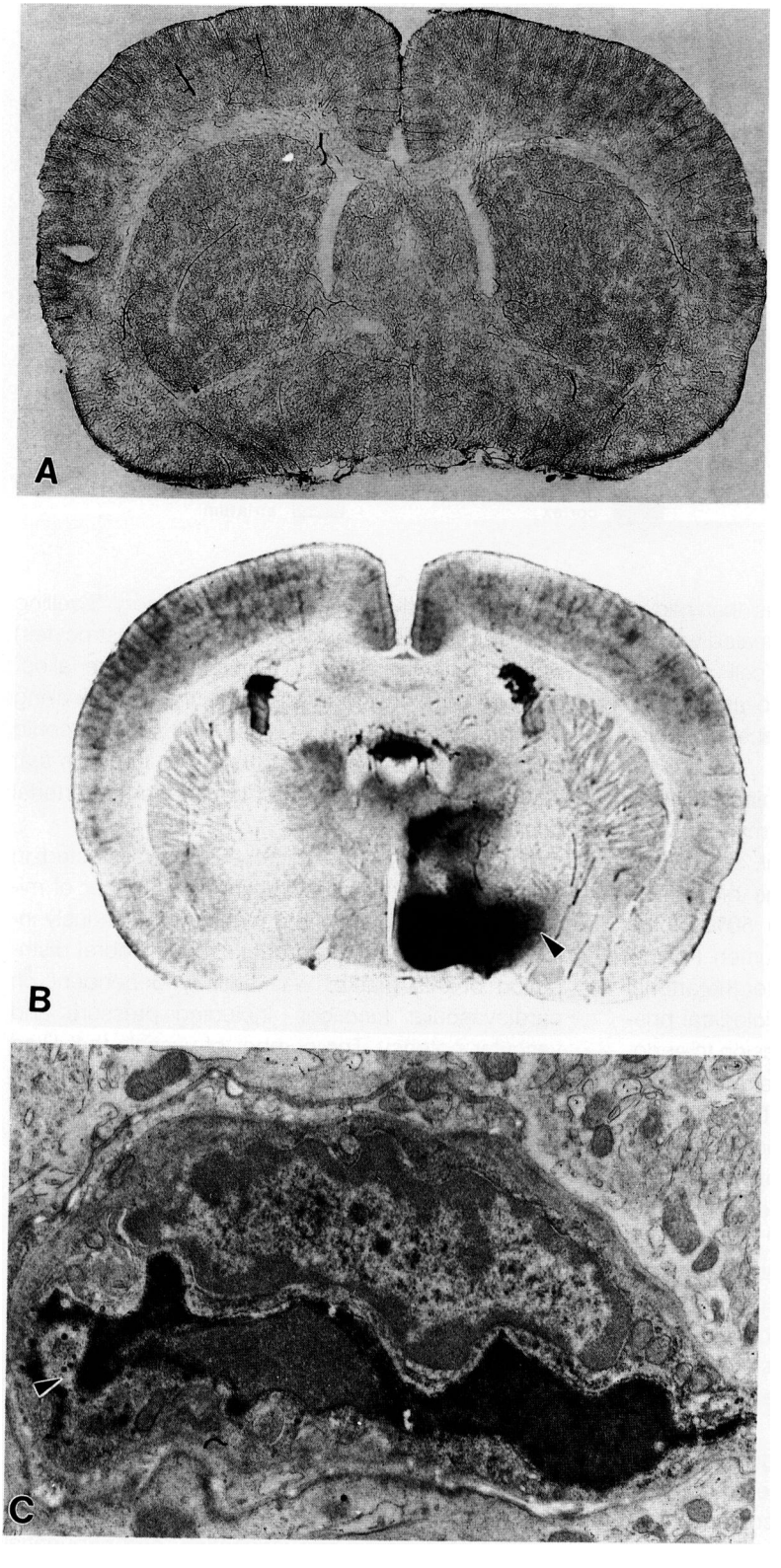

Figure 8. *Distribution of circulating HRP after MCA occlusion.* A: *Coronal section of cerebral hemispheres showing the distribution of HRP in a control animal. The same pattern of HRP distribution could be observed 30 minutes after the arterial occlusion (HRP/DAB, ×2.5).* B: *Leakage of HRP in the preoptic area (arrowhead) 3 hours after the arterial occlusion (HRP/DAB, ×2.5).* C: *The lumen of this microvessel is filled with HRP. Some of the HRP particles can also be seen in the cytoplasm of the endothelial cell (arrowhead) 3 hours after the arterial occlusion (HRP/DAB, ×13,300).*

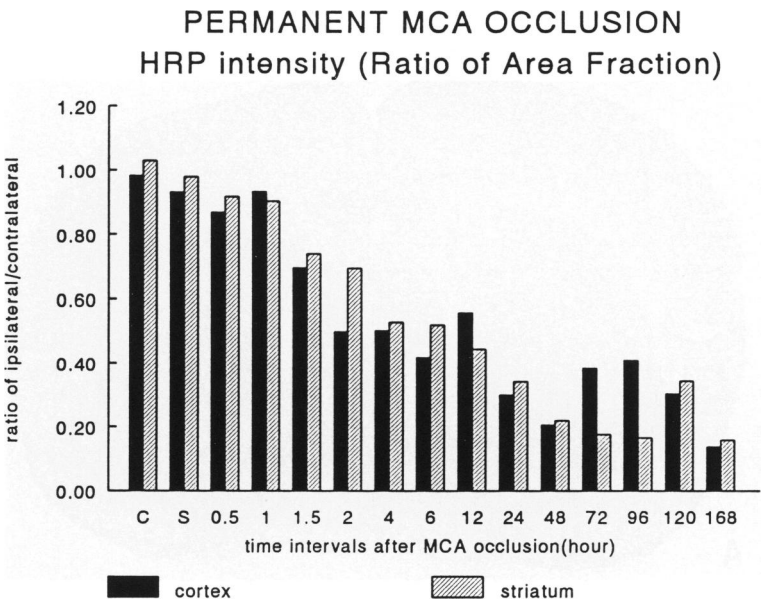

Figure 9. *Extent of HRP penetration into the brain microvessels, as measured by area fraction. Significant differences in the area fraction did not appear until 90 to 120 minutes after the arterial occlusion. C, control group A (normal); S, control group B (sham-operated).*

surface.[2] Moreover, non-human primates that had a MCA transiently occluded and were pretreated with an inhibitor of the leukocyte endothelial cell receptor showed significant improvement in microvascular patency compared with animals that had received a placebo.[11]

The number of microvessels containing polymorphonuclear leukocytes in rats with permanent MCA occlusion peaks at 12 hours.[2] However, at selected sites of the arterial territory, where the blood flow is known to be very low,[12] close to 50% of the microvessels show occlusion of the lumen (mainly by erythrocytes) as early as 4 hours after the arterial occlusion.[2] This suggests that other biological phenomena, in addition to leukocytes adhesion to endothelial cells, may contribute to the impairment of the circulation of erythrocytes. Leukocytes that adhere to endothelial cells are an effective impediment to the circulation of erythrocytes because white blood cells are comparatively large (average diameter 10 μ) and have a relatively stiff cytoskeleton.[10, 13] However, the influx of large numbers of leukocytes into the vessels of the ischemic brain territory does not peak until approximately 12 hours after the arterial occlusion,[2, 14] and other factors acting at earlier times may contribute to a progressive deterioration of the erythrocytes' circulation.

The results of these experiments suggest that progressive swelling of both endothelial cells and astrocytes may contribute to reduce the microvascular lumen and in this manner this type of cellular swelling may interfere with the erythrocytes circulation through the territory of the occluded cerebral artery. Swelling of both cell types (endothelial cells and astrocytes) was documented within 30 minutes of the arterial occlusion. Through the interference that this swelling may present to the flow of blood, cell swelling could contribute to the widespread neuronal necrosis that occurs in this model 12 to 24 hours after the arterial occlusion.[3]

The patency of the microvessels was tested in these experiments by estimating the number of microvessels that filled with a marker intravenously injected 20 minutes before death. The cerebral distribution of this marker was entirely dependent on cardiovascular functions, including pressure and vascular patency. The number of vessels that filled with HRP (as measured in terms of area fraction) dropped for the first time approximately 120 minutes after the arterial occlusion and thereafter this number continued to decrease. Two factors may be responsible for this continued decrease in circulating HRP: progressive increase in the number of vessels occluded by either swollen cells or leukocytes and a progressive increase in the number of necrotic, nonperfusable microvessels. Crowell et al[15] and Little et al[16, 17] have described impaired microvascular filling in experiments of MCA occlusion in non-human primates. However, in all of those experiments the marker (carbon black) was injected postmortem with the fixative, the number of samples examined under the microscope was limited to brain areas showing pallor to the naked eye inspection, and sequential chronological observations at short intervals were not

conducted. For these reasons, quantitation of the phenomenon was limited to a few, random observations.

Swelling of brain endothelial cells has been documented (by electron microscopy) under conditions of global brain ischemia and reperfusion.[18] In those experiments, adult Wistar rats had both carotid arteries clamped and systemic blood pressure was decreased for a period of 30 minutes and this was followed by reperfusion of either 5 or 90 minutes duration.[18] Two factors negatively influenced the internal diameter of cerebral microvessels: duration of reperfusion (90 minutes was worse than 5 minutes) and levels of serum glucose. Brain capillaries had a narrower lumenal diameter in hyperglycemic animals than in those receiving normal saline instead of an intravenous glucose injection.[18] The authors attributed this endothelial cell swelling to the effects of lactic acid; concentration of this metabolite in the brain tissues of hyperglycemic animals was 20 *versus* 14 $\mu mol/g^{-1}$ in the control subjects.[18] Capillary lumenal narrowing was demonstrated in the rabbit tenuissmus muscle by intravital microscopy. In this experiment 1 hour after a single rapid withdrawal of 40% of the blood volume, erythrocyte flux decreased by 60%.[19] This capillary endothelial swelling was completely prevented when a selective inhibitor of Na^+-H^+ activity was given before inducing shock.[20]

Lactic acid is an effective cause of astrocyte swelling, as demonstrated *in vitro* under a variety of experimental conditions,[21] and morphological evidence of astrocyte swelling has been noted under several circumstances in which the brain circulation was altered by various means. Some of these experiments included occlusion of an MCA in either cats[22] or non-human primates[23, 24] and bilateral carotid artery clamping in rats.[25–27]

Changes in brain concentration of ATP, which occur promptly after occluding an MCA in non-human primates,[28, 29] are likely to be promptly accompanied by excessive lactic acid formation. However, lactacidosis is but one of the potential causes of astrocyte swelling; Noble et al[30] have demonstrated *in vitro* swelling of astrocytes exposed to glutamate. The interstitial concentration of this neurotransmitter is known to increase in the hippocampus after carotid ligation in gerbils.[31]

The results of the experiments reported here suggest that in addition to lumenal obstruction by circulating cells, swelling of astrocytes and endothelial cells may impair the patency of the microvessels during the initial 30 to 60 minutes after an arterial occlusion. There was no exact chronological correlation between time of astroglial/endothelial swelling and time when deficits in circulating HRP occurred. Interference with the circulation of a plasma component may require additional factors such as influx of abundant leukocytes. Based on these and previous observations, we suggest the following chronology of events: directional changes in blood flow that develop after a large cerebral artery is occluded elicit endothelial cell swelling in the microvessels of the corresponding vascular bed. This effect is accompanied by swelling of astrocytes and narrowing of the capillary lumen at the same time that there is a progressive increase in the number of microvessels that become occluded by the adhesion that develops between circulating polymorphonuclear leukocytes and endothelial cells. These biological phenomena, which become apparent during the initial 60 minutes, may influence the extent of the subsequent neuronal necrosis. Therefore, therapeutic interventions aimed at improving cerebral perfusion in the acute stage (<6 hours) of an ischemic stroke might be directed to altering the biological responses of these three cell types: endothelial cells, astrocytes, and leukocytes.

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