The significance of morphological changes in cerebral arteries after subarachnoid hemorrhage

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A porcine model was developed to allow quantitative assessment of morphological changes in cerebral arteries after subarachnoid hemorrhage and to determine the significance of structural changes in producing arterial narrowing. Whole blood was selectively applied to the middle cerebral artery (MCA) of seven pigs. After 10 days, vessels were perfusion-fixed and examined by light and transmission electron microscopy and immunohistochemistry. The MCA's exposed to whole blood for 10 days showed prominent luminal narrowing associated with profound ultrastructural changes affecting all layers of the vessel wall. Morphometric analysis, however, demonstrated that significant reductions in the luminal cross-sectional area (-55.8% ± 12.5%, p < 0.005) and increases in radial wall thickness (75.1% ± 10.5%, p < 0.005) were associated with only minimal increase in the cross-sectional area of the vessel wall (12.5% ± 15%, p < 0.025). By stereological analysis, the volume density of individual components of the arterial wall was unchanged in MCA's exposed to blood. Vessels exposed to blood showed a 44% reduction in smooth-muscle cell immunoreactive actin and increased collagen in the extracellular matrix of the vessel wall. These data suggest that structural changes in cerebral arteries after subarachnoid hemorrhage do not directly contribute to vessel narrowing through increases in wall mass. Nevertheless, such changes may reflect pathological mechanisms which act to augment prolonged vasoconstriction or inhibit the maintenance of normal vascular tone.

KEY WORDS • vasospasm • subarachnoid hemorrhage • cerebral artery • smooth muscle • actin • morphometry

DESPITE extensive clinical and experimental investigation over 30 years, the pathophysiology of cerebral vasospasm after subarachnoid hemorrhage (SAH) is unknown. A fundamental question remains unanswered: namely, whether arterial narrowing results from vasoconstriction or from structural increases in the vessel wall mass. Although cerebral arteries in vitro constrict in response to several vasoactive substances in blood or serum,36-38 trials with agents directed at reversing smooth-muscle contraction have not proved consistently effective in reversing arterial narrowing with SAH.13,34,37

Numerous studies have described morphological changes in human cerebral arteries at autopsy11,12,16,30 or in animal models after experimental SAH.1,9,15,19,22,32-34 The evolution of such structural changes over days parallels the development of angiographic narrowing and the temporal course of clinically significant vasospasm in humans.15,21 To date, however, qualitative descriptions of morphological changes have failed to characterize the identity of the arteriopathic stimulus, the mechanism of the artery's response to injury, or the significance of the histological response compared to vasoconstriction in clinical vasospasm. Several features of the arteriopathic response to subarachnoid blood are similar to histological changes observed in extracranial arteries, especially with regard to endothelial injury,40 atherogenesis,24 and hypertension.3

A small-animal model has been developed in pigs to assess the structural responses of cerebral arteries exposed to blood for prolonged periods of time. In this model, whole blood applied directly to the middle cerebral artery (MCA) for 10 days elicited a reproducible narrowing of the vessel and a number of profound ultrastructural changes throughout the vessel wall. Morphometric analysis provides a method by which to quantitate changes in histological appearance and to identify potential loci of pathological changes. By this
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Means, we were able to assess the relative contribution of structural increases in vessel wall mass compared to vasoconstriction in arterial narrowing after SAH.

Materials and Methods

Application of Whole Blood to Cerebral Arteries

Seven immature male and female pigs (each weighing 8 to 12 kg) were pretreated with atropine sulfate (0.5 mg/kg), anesthetized with intramuscular ketamine hydrochloride (35 mg/kg) and xylazine (5 mg/kg), endotracheally intubated and ventilated with a small-animal respirator, and maintained on isoflurane (1% to 3%) during the surgical procedure. All procedures for animal surgery and care were approved by the Seattle Veterans Administration Medical Center Animal Rights Committee in accordance with National Institutes of Health guidelines. With sterile microsurgical technique, a left frontotemporal craniectomy was performed, the frontal lobe was elevated under magnified vision, and the arachnoid was widely incised at the base to expose the left internal carotid artery and proximal segment of the MCA. Fresh autologous arterial blood (2 ml) was directly applied to the MCA, then covered with a Silastic cup and sealed with petrolatum to prevent dissolution (Fig. 1 left).

Perfusion-Fixation of Vessels

At 10 days after application of blood, the animals were anesthetized as above and the descending aorta was cannulated retrogradely through a thoracotomy. To assess permeability, intravenous Evans blue dye (60 mg/kg) was injected at 30 minutes prior to fixation via a femoral catheter. The craniocervical circulation was perfused with 1 liter of heparinized iso-osmotic sodium phosphate buffer (0.1 M, pH 7.4), followed by 2.5 liters of 4% paraformaldehyde in 0.1 M phosphate buffer at a physiological mean arterial pressure. The brain was extracted and photographed, and matching 1-cm segments of both MCA's were removed. The vessels were placed in 1% glutaraldehyde for an additional 2 hours, then stored overnight in cold (4°C) 0.1 M phosphate buffer.

Tissue Processing

Prior to embedding, standardized 10-mm segments of both proximal MCA's were weighed and the lengths accurately measured; they were then subdivided into three 3.3-mm segments for processing. For light microscopy and morphometry, one vessel segment was embedded in hydroxyethyl methacrylate,* sectioned at 3-μ thickness, mounted on glass slides, and stained with hematoxylin and eosin. For transmission electron microscopy (TEM), the second vessel segment was postfixed in 2% osmium tetroxide in 0.1 M cacodylate buffer for 2 hours at 4°C, dehydrated in acetone, stained en bloc with 3% uranyl acetate, and embedded in Epon 812.† Ten sections 0.2 μ thick were poststained with uranyl acetate and Reynold's lead and were examined at 60 kV with a calibrated JEOL 100-S electron microscope. For immunohistochemical study, the third vessel segment was embedded in L-R White.‡ Mouse monoclonal antibodies (MAb's) directed at smooth-muscle actin (provided by Dr. Alan Gown, University of Washington, Seattle, Washington) were applied to sectioned arteries and stained using a colloidal gold/silver en-
hancement technique. Briefly, after blocking for non-specific binding, sections were incubated with the primary mouse anti-actin MAb at a dilution of 1:500 for 90 minutes, with secondary rabbit anti-mouse immunoglobulin (Ig)G (dilution 1:500) for 30 minutes, then with tertiary goat anti-rabbit IgG conjugated to colloidal gold (dilution 1:25) for 15 minutes. The sections were then reacted with silver nitrate solution to enhance particulate size, so that each antibody-conjugated gold particle was visible by both light and electron microscopy.

**Morphometric Techniques**

Morphometric analysis was performed on 20 microscopic fields for each vessel comprising the entire vessel in cross-section (magnification x 260) and high magnification (x 1040) views at 0°, 90°, 180°, and 270° of the circumference. Light microscopy and immunohistochemistry sections were projected as digitized video images using an automated image analysis system. By computer-aided tracing of the circumference of the digitized image of the vessel, planimetric measurements were made for: 1) the cross-sectional area of the lumen; 2) the cross-sectional area of the vessel wall (intima plus media); and 3) the radial thickness of the vessel wall (Fig. 2). The wall thickness was separately measured on the dorsal (brain surface) compared to the ventral (subarachnoid space) aspect of the vessel. To account for distortion imposed during embedding, the luminal cross-sectional area was standardized to a circular shape from the measured circumference. Assessment of permeability changes was made by gross and microscopic examination of vessels for the presence of the chromagen Evans blue dye in the vessel wall.

For stereological analysis, light microscopic sections at high magnification were projected at x 1040 as video images onto a test grid of 144 sampling points (Fig. 3). The volume density (Vv) of any component within a structure is represented as its proportional volume compared to the total volume. By the point-counting method of Weibel and Bolender, inherent variations in planimetric analysis such as orientation of the section or irregular borders are mathematically eliminated (Fig. 3). With this technique, volume densities were determined for endothelium, internal elastic lamina, media, and perivascular axons by counting the sampling points overlaying these components. The density of immunogold-labeled actin in smooth-muscle cells was expressed as the percentage ratio (volume density) of labeled particles per high-power light microscopic field from 20 representative areas of media for each vessel, using the automated image analysis system. Assessment of immunoreactive actin and extracellular collagen in TEM specimens was qualitative.

**Sampling and Statistical Analysis**

Twenty sections at 50-μ intervals were taken from each specimen for planimetric, stereological, and densitometric analysis. Data for each animal were expressed as percent change for the experimental versus control...
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By light microscopy, pig MCA not exposed to blood was consistent in appearance with normal porcine cerebral arteries, as described elsewhere. Endothelium formed a continuous monolayer overlying a thin non-convoluted internal elastic lamina. Six to eight layers of concentrically oriented smooth-muscle cells surrounded the intima, and no smooth-muscle proliferation or necrosis was apparent in control specimens. Typical perivascular axons were present in all specimens, and no adventitial inflammatory infiltrate was noted. Electron microscopy similarly demonstrated the normal appearance of these features at the ultrastructural level.

Experimental Vessels

At sacrifice, a well-circumscribed thrombus was present underlying the Silastic cup at the site of application to MCA (Fig. 1 right). The soft cup did not distort or compress the MCA, and no evidence of blood or hemosiderin was observed beyond the margins of the cup. After 10 days of exposure to periadventitial whole blood, pig MCA demonstrated marked morphological changes. Endothelial cells appeared to be contiguous, but were distorted by the marked convolutions of the adjacent elastica. Nevertheless, permeability (assessed by Evans blue dye exclusion) was not altered, and no regions of desquamation or adherent blood elements occurred. Electron microscopy substantiated endothelial distortion and heterogeneous loss of cytoplasmic density.

The internal elastic lamina of the vessels exposed to blood was markedly convoluted and appeared to be thicker compared to comparable controls. On TEM, frequent disruption of the elastica was associated with increased deposition of collagen in the intercellular space (Fig. 4 right). Occasional eosinophilic cells, probably representing migrating “myointimal” cells, were occasionally seen on the luminal aspect of the elastica. Immunohistochemical examination identified the pres-
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FIG. 6. Percent change in cross-sectional area of lumen and vessel wall, and radial wall thickness on dorsal (brain) and ventral (subarachnoid) aspects of a pig middle cerebral artery exposed to whole blood for 10 days.

FIG. 5. Immunogold histochemical studies with anti-actin antibody in pig middle cerebral artery exposed to whole blood (left) compared to a control vessel (right). There is a prominent reduction in actin density within smooth-muscle cells of the vessel exposed to blood. × 267.

ence of actin in these subendothelial cells, substantiating their probable myogenic origin. Electron microscopy revealed that the “myointimal” cells were not associated with loss of endothelium, and demonstrated nuclei and rough endoplasmic reticulum characteristic of transformed smooth-muscle cells. Smooth-muscle cell pallor and necrosis with prominent vacuole formation were occasionally present in vessels exposed to blood, and were associated with increased collagen in the extracellular matrix. Compared to matched controls, there was a marked reduction in smooth-muscle immunoreactive actin in vessels exposed to whole blood (Figs. 4 and 5).

The adventitia of vessels exposed to blood demonstrated a mild to moderate inflammatory reaction adjacent to the subarachnoid thrombus; the degree of inflammation, however, was not related to the presence or severity of arterial narrowing or morphological changes in the vessel wall. Perivascular axons were degenerated and markedly reduced in number compared to control vessels.

Morphometric Analysis

Figure 6 shows the planimetric analysis of changes in measured radial wall thickness and cross-sectional areas of the lumen and vessel wall for MCA’s after application of whole blood for 10 days compared to contralateral control vessels. There was a 55.8% ± 12.5% reduction in luminal cross-sectional area in vessels exposed to subarachnoid blood (p < 0.005), which corresponded to an increase in wall thickness of 56.2% ± 31.3% (p < 0.01) on the dorsal (brain) aspect and 75.1% ± 10.6% (p < 0.005) on the ventral (subarachnoid) aspect of the artery. Despite observed increases in MCA wall thickness, the cross-sectional area of the vessel wall increased only slightly (12.5% ± 15%, p < 0.025) for treated compared to control vessels.

Table 1 shows the volume densities for individual components of the vessel wall. The volume density between treated and control vessels was similar for endothelium (6.1% vs. 5.5%), internal elastic lamina (12.7% ± 11.2%), and media (81.2% vs. 83.3%). The volume density of perivascular axons, however, was markedly diminished in vessels exposed to blood (3.1% vs. 8.5%).

The volume density of immunogold-labeled actin in the media of MCA’s exposed to whole blood was compared to that of control MCA’s. There was marked reduction in smooth-muscle cell immunoreactive actin after chronic application of whole blood (35.6% ± 9.0%) compared to controls (64.8% ± 6.4%, p < 0.001).

Discussion

It has been assumed that vasoactive agents in subarachnoid blood permeate into the vessel wall and promote a prolonged receptor-mediated contraction of cerebral arterial smooth muscle, as reflected by changes in vessel contractility in vitro. Several substances in whole blood and cerebrospinal fluid from humans after SAH elicit contraction in isolated cerebral artery. Although the response of cerebral arteries in vitro reflects certain mechanisms determining vascular caliber after SAH, other components of this response cannot be fully determined ex vivo. An isolated vessel is denervated and removed from normal local intravascular and extravascular influences, and structural changes occurring over several days in arteries exposed to blood may influence the abnormal contractile response in vitro. The porcine model of vasospasm described in this report was used to examine the response of cerebral arteries to whole blood in vivo after...
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<table>
<thead>
<tr>
<th>Component</th>
<th>Volume Density of Vessel Wall Component</th>
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<tbody>
<tr>
<td></td>
<td>Whole Blood</td>
</tr>
<tr>
<td>endothelium*</td>
<td>6.1 ± 3.5</td>
</tr>
<tr>
<td>elastica*</td>
<td>12.7 ± 5.3</td>
</tr>
<tr>
<td>media*</td>
<td>81.2 ± 6.6</td>
</tr>
<tr>
<td>perivascular axons†</td>
<td>3.1 ± 5.8‡</td>
</tr>
</tbody>
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* Expressed as mean percentage of media plus intima ± standard deviation (SD).
† Expressed as mean percentage of total vessel wall ± SD.
‡ Difference from control value: p < 0.05.

10 days of exposure, and to assess the relative contribution of structural changes to luminal narrowing.

Morphometric analysis provides a quantitative measure of both the nature and degree of changes occurring in cerebral arteries after SAH. For small arteries, direct measurement of vessel cross-sectional area after perfusion-fixation may be more sensitive than angiographic determination of lumen diameter for measuring relative changes in vessel caliber, and the correlation between such measurements has been previously demonstrated. Although angiographic comparison to morphometric measurement was not employed in this experiment, the contralateral MCA in each animal served as a matched control vessel, suggesting that observed changes were not artifacts of the fixation procedure. In subsequent experiments using the same technique, arteries treated with blood components not containing hemoglobin were indistinguishable from control vessels, suggesting that the Silastic cup preparation was not responsible for any of the observed changes in vascular morphology. Stereological analysis provides a quantitative means to express changes in histological morphology and accounts for variations in sectioning or orientation which might otherwise distort planimetric analysis. Volume density determinations reflect proportional changes within individual components of a structure (such as the media volume in proportion to that of the total wall), and may identify specific morphological processes otherwise not discernible by planimetric measurement. In this experiment, comparison of volume densities demonstrated no significant change for any single component of the vessel wall after exposure to whole blood, suggesting that the small observed increase in vessel wall mass was not restricted to one portion of the artery.

Light and electron microscopic alterations in cerebral artery structure after SAH have been consistently described in human postmortem and intraoperative specimens, which correlated with angiographic vasospasm and cerebral infarction in the territory of the affected vessel. These changes included apparent swelling of the intima and media within days after SAH, a progressive myonecrosis with intimal proliferation and luminal narrowing after 1 to 6 weeks, and subsequent medial fibrosis and increases in luminal diameter at 3 to 15 months. Smith, et al., reported similar postmortem angiopathic changes in 24 of 28 patients autopsied after SAH from ruptured cerebral aneurysm. Although postmortem fixation introduces artifact in vascular morphology, and preexisting atherosclerotic changes may have been present in the patients studied, there is nevertheless considerable evidence that arteriopathic changes do occur in human arteries after SAH.

A number of animal models of SAH have shown that continuous exposure of large cerebral arteries to clotted blood over several days was associated with consistent ultrastructural changes in the vessel wall. In several species, SAH produced by arterial puncture, intracisternal injection of blood, or direct application of blood caused angiographic narrowing of cerebral arteries which corresponded to qualitative morphological changes on TEM and light microscopy. From as early as 3 hours after SAH, there was marked vasoconstriction of the vessel with corrugation of the elastica, distortion of the endothelial cells, and decrease in the luminal cross-sectional area. Permeability studies demonstrated impaired endothelial integrity at 3 days after SAH. From 3 to 14 days after SAH, cerebral artery morphology was characterized by alterations in endothelial cell morphology, thickening and discontinuities of the elastica, smooth-muscle vacuoles with occasional frank myonecrosis, proliferating “myointimal cells” migrating into the intima, and periadventitial inflammation. At 2 weeks to 6 months post-SAH, regression of the subintimal proliferation, increases in luminal diameter, and deposition of collagen in all three vessel layers were observed. The similarities between these findings and those seen in human postmortem specimens suggest that blood-induced arteriopathic responses in animal models are analogous to the structural component of vasospasm in humans.

In the current experiment, the majority of structural changes described above were present at both the light and electron microscopic levels in pig MCA’s 10 days after application of whole blood. The observed increase in thickness of the media, however, was not reflected in increases in the volume fraction of this component, and probably represented configurational changes related to smooth-muscle contraction rather than structural increase in vessel wall mass. Although a prominent inflammatory response was noted in the adventitia adjacent to the subarachnoid thrombus, subsequent experiments have shown that vessel narrowing and structural changes are not related to the presence of perivascular white blood cells.

The response of vascular smooth muscle to injury has been characterized in a number of conditions. Nonconfluent vascular smooth-muscle cells in culture undergo a phenotypic change over several days characterized by loss of myofilaments, a 26- to 45-fold...
increase in collagen production, and the ability to proliferate in response to mitogens such as platelet-derived growth factor. Similar changes in smooth-muscle structure and function have been observed in vivo in association with atherosclerosis and hypertension and after endothelial damage; in these settings the arteriopathic response is maximal 5 to 7 days after acute injury. It has been proposed that disruption of the smooth-muscle cell basal lamina by various stimuli initiates a response to injury in which the smooth-muscle cell assumes certain features of a fibroblast. In the current experiment, similar alterations in MCA smooth-muscle cells after exposure to blood (with loss of contractile proteins and increased collagen deposition) may have reflected such a response.

Although arterial narrowing in the current experiment was not associated with increases in the volume of vessel wall components, structural changes in cerebral arteries after SAH may nevertheless reflect critical pathophysiological processes. Although we did not observe altered permeability after exposure to blood for 10 days, a transient loss of endothelial integrity in the first 48 hours after SAH may expose the vessel to arteriopathic serum constituents during that period. Profound structural alterations in cerebral arteries have been observed within the first 4 hours after exposure to blood, and further experiments are required to delineate the time course of endothelial permeability changes after SAH. Similarly, prolonged vasoconstriction after SAH may be related to an inhibition of endogenous vasodilators such as endothelial-derived relaxing factor. Alternatively, loss of contractile protein and increases in vessel wall collagen after SAH may influence physiological responses of the artery. Primate cerebral arteries exposed to subarachnoid blood for several days were less distensible than controls, although other investigators have noted increased vascular compliance after SAH. Although the concept of cerebral artery hypersensitivity to vasoconstrictors after SAH has been proposed, recent data have suggested that arteries are less sensitive in vitro to both vasoconstrictors and vasodilators after chronic exposure to blood. In addition, recent reports have documented the effectiveness of angioplasty in dilating narrowed cerebral arteries after SAH, indicating that "fibrosis" of the vessel in its contracted state may represent one component of prolonged vasospasm. Alterations in cerebral artery ultrastructure after SAH are analogous to changes observed in extracranial arteries after hypertension, endothelial damage, or atherogenesis; these changes may represent variants of the same reparative response of arteries to injury.

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