Ultrastructural evidence of arterial denervation following experimental subarachnoid hemorrhage

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Loss of catecholamine histofluorescence, increased sensitivity to norepinephrine, and changes in alpha_1 receptor binding have led to the proposal that denervation hypersensitivity may play a role in cerebrovascular spasm. Because the significance of these alterations has remained unclear, the present study was undertaken to determine whether there was direct ultrastructural evidence of arterial denervation following experimental subarachnoid hemorrhage. Under general anesthesia, adult cats were subjected to pre-pontine injection of blood or serum (5 to 7 ml) via a transcervical approach. The animals were sacrificed 4, 7, or 10 days later and basilar artery segments were prepared for electron microscopy. Control vessels appeared normal, whereas those bathed in blood revealed unequivocal changes in neural and supporting elements, including: 1) disintegration of both clear- and dense-core vesicles; 2) fragmentation of varicosities; 3) loss of Schwann cell cytoplasm; and 4) axonal degeneration. These changes were most pronounced 7 days after instillation of blood, and correlated in time with maximal injury of the media and endothelium. Although the development of smooth-muscle hypersensitivity remains unsettled, this study indicates that prolonged exposure to blood can cause extensive denervation of cerebral arteries.

KEY WORDS • subarachnoid hemorrhage • arterial denervation • basilar artery • ultrastructural study

Previous morphological studies have shown that subarachnoid hemorrhage (SAH) can produce dramatic pathological changes in both smooth muscle and endothelium of cerebral arteries. Anatomical evidence of injury to the nerve elements, however, has been more circumstantial, consisting of reduced catecholamine histofluorescence. This finding, coupled with physiological evidence of altered norepinephrine uptake and receptor binding, has led to the proposal that cerebrovascular spasm may be due, at least in part, to hemorrhage-induced denervation hypersensitivity of smooth-muscle cells. The present investigation was undertaken to ascertain whether there was direct ultrastructural evidence of arterial denervation following experimental SAH.

Materials and Methods

Fifteen adult mongrel cats, each weighing between 3 and 5 kg, were used in this study. For the induction of experimental SAH, animals were anesthetized by intramuscular injection of ketamine (35 mg/kg) and xylazine (1.0 mg/kg), then tracheal intubation and mechanical ventilation using 100% O_2 were instituted. Under sterile conditions, a midline vertical incision was made over the larynx. With the aid of an operating microscope, the lower 3 to 4 mm of the clivus was exposed via a transclival approach. After this portion of bone had been removed with microrongeurs, the basilar artery could be visualized through the dura. A No. 25 needle was inserted through the dura into the pre-pontine cistern so that its tip lay adjacent to the artery. Cerebrospinal fluid was allowed to fill the catheter tubing attached to the needle, and then 5.0 to 8.0 ml of fresh autologous blood, obtained from the exposed common carotid artery, was injected intermittently over a 3- to 5-minute period. Successful deposition of clotted blood could be determined by a persistent change in color and tension of the dura. Upon withdrawal of the needle, the puncture site quickly sealed with the first few drops of thickened blood. The region was observed for an additional 15 minutes in order to verify that a stable clot had formed. The wound was closed in layers, and after the endotracheal tube had been removed, the animal was returned to its cage and closely observed until the time of reexploration. In sham-operated animals, 5.0 to 8.0 ml of autologous serum, prepared 2 hours in advance, was injected in place of blood.
Arterial denervation after experimental SAH

**FIG. 1.** Electron micrograph of axons and varicosities (arrows) in the basilar artery of a normal cat. Varicosities tended to be arranged in clusters, with an intermingling of those containing dense-core and those containing clear-core vesicles. × 13,100.

**FIG. 2.** Electron micrograph showing red blood cells clustered in the adventitial layer of a basilar artery removed 4 days after instillation of blood into the pre-pontine cistern. × 1500.

Animals were reexplored at 4, 7, or 10 days following pre-pontine injections. Anesthesia was induced via mask with a N₂O:O₂ mixture, in the ratio of 3:1, and 5% halothane. The previous neck incision was reopened and the procedure described above was employed to expose the entire length of the clivus. After most of this bone had been excised, the dura was opened vertically to expose the basilar artery. The amount of clotted blood was quantified as large (> 75% of vessel length covered), medium (between 25% and 75%), or small (< 25%). The thorax was opened through the sternum, a stab wound was made in the right atrium of the heart, and a No. 18 needle was inserted into the left ventricle. Through this needle the animal was perfused with 500 ml of 0.10 M phosphate buffer, pH 7.4, followed by 1000 ml of 2% glutaraldehyde and 1% paraformaldehyde in 0.10 M buffer. Clotted material in the pre-pontine cistern was removed, and the basilar artery excised. The vessel was washed in buffer solution, post-fixed in 1% osmium tetroxide, dehydrated, and embedded in Durcupan. Ultrathin sections were mounted on copper one-hole grids and counterstained with 1% lead citrate and 1% uranyl acetate. Sections were then examined on either a JEOL 100-CX or Hitachi H-600 electron microscope. Specimens from three normal cats, not subjected to pre-pontine injection, were also examined.

**Results**

**Examination of the Pre-pontine Cistern**

In cats that had received pre-pontine injections of whole blood, gross inspection of the cistern at the time of sacrifice revealed large clot in the two animals sacrificed at 4 days, large or medium-sized clot in the four animals sacrificed at 7 days, and small or no clot in the three animals sacrificed at 10 days. However, in the last group of animals, diffuse xanthochromic staining of the arachnoidal and pial membranes was consistently seen.

**Ultrastructural Examination of Basilar Arteries**

Ultrastructural examination of arteries removed from normal animals revealed a rich neuronal network throughout the adventitia. The vast majority of axons were small and unmyelinated, and varicosities tended to occur in clusters, with those containing dense-core vesicles intermixed with those containing clear-core vesicles (Fig. 1). The mean number of such clusters per entire cross section of vessel was 21 (± 4.0, standard error), and the mean number of varicosities per cluster was four. Specimens from animals that had been injected with autologous serum showed no significant difference in these values, and, in addition, there was no evidence of injury to smooth-muscle cells, elastic lamina, or endothelium.

Red blood cells were seen in the adventitial layer in virtually every artery removed after instillation of blood. These cells occurred both as aggregates (Fig. 2) and sporadically (Fig. 6).
Basilar artery specimens removed from cats 4 days after blood injection generally revealed mild degrees of injury to neuronal elements. Approximately one-third of the varicosities showed alteration in ultrastructure, including outer membrane disruption, accumulation of electron-dense material, and swelling of vesicles (Fig. 3). Most axons appeared normal, but approximately 10% of fibers manifested clear signs of injury, with loss of clarity of microtubules and neurofilaments, and disintegration of mitochondria (Fig. 4). Other layers of the arterial wall demonstrated little evidence of pathological change.

The most dramatic morphological changes in neural structures occurred in vessels removed 7 days after instillation of blood. A large proportion of varicosities (70%) were markedly abnormal in these specimens, and in many instances it was not possible to identify a suspected varicosity with certainty (Fig. 5). Although 40% of axons appeared relatively normal, the majority had lost their usual detail and many had undergone extensive disruption (Fig. 6 upper). Myelinated fibers appeared to be more resilient to injury, but even these occasionally showed distortion and vacuolization of sheaths (Fig. 6 lower). Schwann cells also showed marked pathological changes, and frequently the nuclei were nearly devoid of surrounding cytoplasm (Fig. 7 center). Throughout the adventitia a variable amount of debris was intermingled with collagen fibers, and these too were occasionally found to be disintegrating. Although disruption of the adventitia was often suggestive of inflammatory changes, white blood cells were seen infrequently.

Other layers within these vessels likewise showed the most extensive damage at 7 days after blood injection. Smooth-muscle cells were characterized by scalloped outer membranes and vacuolization of the cytoplasm (Fig. 7 center), and the endothelium was corrugated and swollen (Fig. 7 right).

Electron micrographs of basilar arteries removed 10 days after pre-pontine injection of blood demonstrated less evidence of generalized disruption, suggesting that much of the damaged tissue and debris had been removed. This impression was reinforced by a striking decrease in the number of varicosities observed. Few fibers could be seen near the outer border of the media, and many of those located more peripherally showed later stages of injury, with globular collections in the axoplasm (Fig. 8). Schwann cells often appeared to be in the process of sending out new extensions (Fig. 9 left), and the cytoplasm of fibroblasts contained a rich abundance of endoplasmic reticulum (Fig. 9 right).

Discussion

Although several investigators have reported that cerebral vessels do not develop significant pathological alterations following either clinical or experimental SAH, the majority of studies, as noted above,
have demonstrated unequivocal changes in the endothelial and/or smooth-muscle cells and elastic lamina. With regard to the discrepancy in the results of experimental studies, the employment of a reliable model of chronic SAH is obviously of critical importance. It is worthwhile to note that relatively few published photographs of cerebral arteries subjected to experimental SAH show the presence of red blood cells within the adventitia, and this absence may well reflect failure to achieve adequate clot deposition. It seems unlikely that the commonly employed technique of injecting blood through a catheter inserted into the optic foramen or cisterna magna can assure adequate deposition of material in the desired location, even when coupled with “double injection” methods. Recognition of these problems may explain why certain investigators have resorted to craniotomy for modeling chronic SAH in the monkey.

The prolonged time course of cerebrovascular spasm prompted the initial proposal that this condition might reflect a state of smooth-muscle hypersensitivity, perhaps due to denervation. Over the past 15 years, there has been growing evidence of alterations in neurotransmitter levels, uptake, and binding constants, but morphological demonstration of injury to axons and varicosities has been lacking.

The present study indicates that the neural elements of cerebral arteries can be extensively damaged following experimental SAH. The significance of these results is twofold. First, the occurrence of SAH-induced denervation indicates that the possibility of consequent hypersensitivity of cerebral vessels must still be entertained. It should be recognized, however, that hypersensitive responses of cerebral vessels after sympathectomy have generally not been found to be as exaggerated as those occurring in other sympathetically innervated tissues or in denervated striated muscle. Moreover, the denervation observed in the present study involved both dense-core and clear-core varicosities, theoretically introducing the potential for hypersensitivity to both vasoconstricting and vasodilating agents. In short, although SAH is capable of causing extensive denervation of cerebral arteries, it is far from clear that hypersensitivity plays a role in cerebrovascular spasm. Second, the appearance of pathological changes in neural ele-
ments following SAH may provide important clues concerning the etiology of injury to smooth-muscle and endothelial cells. While some investigators have argued that the changes seen in these layers are a direct result of sustained vasoconstriction,\textsuperscript{14,22} it is very difficult to conceive how prolonged contraction could deleteriously affect the structural integrity of neural elements or adventitia. A more plausible explanation is that substances released from clotted blood either exert a direct toxic effect or initiate a chain of reactions that result in injury to the entire arterial wall.

Finally, it should be pointed out that the present study does not resolve the ongoing question as to whether cerebrovascular spasm is primarily a state of sustained vasoconstriction or one of vasculopathy. To view the issue in this manner may, however, be misleading, since it is quite conceivable that either condition eventually entails the other. The more central issue is whether SAH-induced arterial spasm is initiated principally by intensification of the contractile processes or by alteration of the structural components of cerebral vessels. At the present time, there seems little reason for excluding either possibility.

References

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Fig. 9. Left: Electron micrograph demonstrating numerous Schwann cell buds extending to the perimeter of an axonal cluster in a basilar artery removed from a cat 10 days after injection of blood. × 8730. Right: Increased endoplasmic reticulum and enlarged mitochondria are visible within the fibroblast cytoplasm. × 16,350.

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