Appearance of Collagen Fibers in the Cerebral Vascular Wall Following Subarachnoid Hemorrhage

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Abstract

Ultrastructural examinations of the cerebral vascular wall following subarachnoid hemorrhage (SAH) demonstrated that collagen fibrils developed in the muscle layer near the adventitia in the early stage of SAH and increased in number and volume with time. These findings suggest that accelerating factors of collagen synthesis in the muscle layer may be released from the adventitial side, and collagen synthesis is induced by extravascular factors. The changes of collagen volume with time suggest collagen to be an important phenomenon of persistent vasospasm after SAH. Histoimmunological studies showed that increased collagen fibers in the tunica media were mainly type III collagen, implying that cerebral vasospasm may be related to chemical inflammation.

Key words: collagen, subarachnoid hemorrhage, ultrastructural study, vascular wall, vasospasm

Introduction

Vasospasm occurring after subarachnoid hemorrhage (SAH) has been extensively studied, but no conclusions about mode of occurrence, continuation, and recovery have been reached. Histological observations of the arterial walls in experimental animals have revealed vacuolar degeneration of the smooth muscle cells, as well as striking endothelial or other changes. Such histological modifications suggest an organic change in the arterial wall rather than a functional reaction to spasmogenic factors. Degenerative organic changes, however, cannot explain the phenomenon of reversible vasospasm several weeks after SAH. The degenerative changes of the smooth muscle cells of the artery must disturb the normal contractile activities. Therefore, another contractile mechanism may operate in the arterial wall other than the smooth muscle cells. Collagen fibers are a passive elastic component of the blood vessels, and have an essential supportive or mechanical role. An increase or decrease of collagen fibers in the vascular wall under various pathological conditions could lead to modification of passive elastic properties and so alter the contractile activity.

This study examined the mode of appearance, amount and type of collagen in vascular wall subjected to SAH, to elucidate the mechanism and mode of participation of collagen in vasospasm following SAH.

Materials and Methods

Thirty adult cats weighing 2.5–3.0 kg were anesthetized by intramuscular injection of 30 mg ketamine hydrochloride. An arterial catheter was inserted into the abdominal aorta through the femoral artery to measure the systemic blood pressure. A venous catheter was inserted via the femoral vein for withdrawal of blood and infusion of fluid. Intratracheal intubation was used for mechanical ventilation. Experimental SAH was produced in 25 cats by percutaneous administration of 2 ml/kg body weight of fresh non-heparinized autogenous blood into the cisterna magna. The injection rate of the
blood was 0.5 ml/min. When the animals became apneic, they were immediately resuscitated with a ventilator.

The five control and 25 SAH animals were sacrificed by transcardiac perfusion of primary fixative containing 1% glutaraldehyde and 1% paraformaldehyde at various intervals after SAH induction. The basilar arteries were dissected from cats sacrificed on the 3rd (n = 3) and 7th (n = 3) days after SAH induction and post-fixed in 2% osmium tetroxide with phosphate buffer (pH 7.4). The samples were then washed in phosphate buffer, dehydrated in a graded methanol series, and embedded in Quetol 812 (Nissin EM Co. Ltd., Tokyo). Thin sections were cut, stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (1200 EX; JEM, Akishima, Tokyo).

To estimate the amount of collagen quantitatively, materials obtained from animals sacrificed at 6 hours (n = 5), 12 hours (n = 5), the 3rd day (n = 5), and the 7th day (n = 5) were treated by the azan-staining method and evaluated by a morphometric technique. The areas of the collagen fibers stained blue were measured, and the volume ratio to the area of the tunica media was calculated. The amount of collagen was then expressed as the volume percentage (vol%).

Histoimmunological methods were used to identify the types of collagen in the vascular wall. Samples of arteries dissected from control animals (n = 3) and animals sacrificed on the 7th day after SAH induction (n = 3) were embedded in paraffin. Sections (4–5 μm thick) were cut and mounted on slides. The sections were dewaxed with xylol, and treated with 1% trypsin in phosphate-buffered saline (PBS) at 37°C for 60 minutes. They were then washed three times for 5 minutes in PBS. The slides were immersed in 0.03% H2O2 for 30 minutes, and washed three times in PBS for 5 minutes. The sections were then treated with 1% rabbit anti-type I–V collagen sera (Advance Co., Tokyo; Chemical Credential Co., Costa Mesa, Cal., U.S.A.) at 4°C overnight. All slides were then washed three times for 5 minutes in PBS. Hors eradish peroxidase (HRP)-labelled sheep anti-rabbit immunoglobulin was added to the sections and left for 30 minutes at room temperature. The slides were again washed three times for 5 minutes in PBS. The sections were then immersed in diaminobenzidine-H2O2 solution to demonstrate HRP. Counter staining with Mayer's hematoxylin was also performed.

All data are expressed as the mean ± SEM. The data were evaluated by Student's t-test for uncorrelated pairs, and probability values of < 0.05 considered significant.

Results

The ultrastructural appearances of basilar arteries obtained from the three control animals were essentially identical. The endothelial cells were in a single layer and flat and spindle shaped (Fig. 1). Collagen fibrils were barely recognized in the subendothelial region. The internal elastic lamina consisted of several stratified elastic fibers. Collagen fibrils were barely recognized in the lamina. The tunica media consisted of fusiform smooth muscle cells containing an elongated nucleus, several mitochondria, and endoplasmic reticulum forming a central core. Each muscle cell was surrounded by a basal lamina, with few collagen fibrils scattered between the basal laminae. However, the amount of collagen fibrils was small, and they were scarcely recognizable in the tunica media. The adventitia consisted of roughly arranged fibroblastic cells, abundant collagen fibrils, and occasional nerve fiber bundles containing both myelinated and unmyelinated fibers. The collagen fibrils in the tunica media and adventitia displayed characteristic periodic transverse bands repeated at intervals of about 65 nm.

All materials obtained from animals sacrificed on the 3rd day after SAH induction showed essentially identical findings. Infiltration by blood cells injected into the cisterna magna and loosening of the tissue elements were recognized in the adventitia of the basilar arteries. Fibroblastic cells with vacuoles of various sizes and collagen fibrils were loosely distributed. However, the collagen fibrils had apparently increased in number. The majority were parallel with periodic transverse bands identical to those observed in the normal vessels. The muscle cells in the tunica media exhibited irregular contours, with small vacuoles including electron-dense materials around the nucleus in the central core. Collagen fibrils were slightly increased between the muscle cells (Fig. 2), more predominantly near the adventitia, but a few were found in the immediate subendothelial region of the basilar arteries. Some collagen fibrils between the muscle cells were distributed irregularly, with relatively low electron density and less clear periodic transverse bands. The elastic fibers were loosely distributed, and small granules of 20–50 nm in diameter appeared in the internal elastic lamina. Collagen fibrils were barely recognizable in the lamina. The endothelial cells showed irregular aspects on both luminal and abluminal surfaces, and the nuclei had slightly changed contours. The organelles of the endothelial cells revealed little change, but small vacuoles were noted in a few cells.

Typical spastic findings involving the whole vas-
cular wall were clearly observed in all three animals sacrificed on the 7th day after SAH induction. In the adventitia, infiltrated blood cells had almost disappeared, and collagen fibrils had increased in number resulting in an increased adventitial thickness. In the tunica media, the smooth muscle cells displayed more marked changes in configuration with various vacuoles in the central core. Collagen fibrils were markedly increased among the muscle cells and, moreover, appeared near the internal elastic lamina (Fig. 3). Most were of normal electron density with periodic transverse bands as in the normal vessels. The internal elastic lamina had a tortuous appearance with loosened and discontinuous elastic fibers. Collagen fibrils were present in the luminal side of the muscle layer and extended into the abluminal side of the internal elastic lamina. The endothelium was corrugated, with strongly deformed contours. Although some cells were detached from the internal elastic lamina, the intercellular junctions were not disrupted.

The control animals (n = 5) demonstrated a mean volume of collagen fibers in the muscle layer of 8.3 \pm 0.8 \text{ vol}\%$. At 6 hours after SAH induction, the value was still at 8.5 \pm 1.2 \text{ vol}\% (n = 5). At 12 hours after SAH, however, the value had increased to 20.1 \pm 1.1 \text{ vol}\% (n = 5), significantly higher than the control (p < 0.001). The values reached 21.6 \pm 1.1 \text{ vol}\% (n = 5) on the 3rd day after SAH, and $22.6 \pm 1.4 \text{ vol}\% (n = 5)$ on the 7th day (Fig. 4).

The histoimmunological study revealed positive reactions to only anti-type I and III collagen in the basilar artery of the control animals. Reactions to anti-type I collagen were recognized in all layers of the basilar artery (Fig. 5 left). The reaction in the

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**Fig. 1** Electron micrograph of a control cat basilar artery. E: endothelial cell, EL: elastic lamina, M: muscle cell. Bar = 1 \text{ \mu m}.

**Fig. 2** Electron micrograph of the basilar artery obtained from a cat sacrificed on the 3rd day after SAH induction. C: collagen fibrils, E: endothelial cell. Bar = 1 \text{ \mu m}.

**Fig. 3** Electron micrograph of the basilar artery obtained from a cat sacrificed on the 7th day after SAH induction. C: collagen fibrils, E: endothelial cell, arrow: vacuole. Bar = 1 \text{ \mu m}.

**Fig. 4** Change in the volume of collagen fibers in the muscle layer with time after SAH induction. Significant difference from control by unpaired Student's t-test: *p < 0.01, **p < 0.001.

**Fig. 5** Left: immunohistochemical reaction for anti-type I collagen in the control basilar artery. Right: immunohistochemical reaction for anti-type III collagen in the control basilar artery.
tunica media occurred on the smooth muscle cells or near these cells. The reaction to anti-type III collagen occurred in the adventitia, but was barely recognized in the intima and tunica media (Fig. 5 right). The type III collagen in the adventitia generally appeared as roughly arranged small bundles.

On the 7th day after SAH induction, reactions to anti-type I collagen were apparent in the intima (Fig. 6 left). However, reaction in the tunica media and adventitia did not increase in density. In contrast, the type III collagen in the adventitia appeared to increase in density and was thickly arranged surrounding the tunica media (Fig. 6 right). The reaction was also increased in the tunica media and intima. The reaction in the tunica media appeared to be extracellular as small bundles or near the muscle cells. The reaction in the internal elastic lamina occurred in thin layers. These findings demonstrate that the amounts of both type I and III collagen were increased in the intima and internal elastic lamina. However, only type III collagen appeared in the tunica media and adventitia.

**Discussion**

Histological findings of the intracranial arteries following SAH include necrosis of the cortical arteries and subendothelial granulation in the trunk cerebral arteries. Myonecrosis of the basilar arterial wall in monkeys has also been reported. Accumulations of membrane-bound vesicles above the internal elastic lamina in the subendothelial region, probably representing debris, and various endothelial changes, such as vacuolation, the appearance of dense bodies, detachment from the internal elastic lamina, and thickening, have also been noted. Biochemical changes in the arterial wall or environs after SAH involve eicosanoids and the metabolism of cations, such as Ca++. Whether these pathological findings are actually related to the etiology of the vasospasm or result from contraction of the vessels remains controversial. However, functional arterial changes following SAH, such as an increased vascular smooth muscle tone due to changes in responsiveness to spasmogenic factors, have been described.

The present experiments revealed an apparent increase in collagen fibrils between the muscle cells in the tunica media of the basilar arteries following SAH. Hughes and Schianchi and Tanabe et al. also observed an increase of collagen fibers in the arterial walls after SAH, and proposed a resultant modification of the passive elastic properties of the vessels and eventually an altered degree of constriction.

Synthesis of collagen occurs in tissues during inflammatory processes and the collagen phase of the repair process of various injuries. The present experiments showed that an increase in collagen occurred as early as 12 hours following SAH. The ultrastructural study indicated that this time period was not sufficient to produce myonecrosis. The increase in collagen is thus not thought to represent a repair process of myonecrosis. The present experiments also demonstrated that collagen fibrils occurred in the muscle layer near the adventitia in the

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early stage of SAH and increased in number and volume with time. These findings suggest that accelerating factors of collagen synthesis in the muscle layer are released from the adventitial side, and collagen synthesis is induced by extravascular factors but not by muscular mechanically-induced histological changes invoked by contracting agents. Increased collagen synthesis in the blood vessel wall occurs in hypertension, responses to immunological or mechanical injury, and atherosclerosis. Bevan et al. suggested that evidence of increased collagen synthesis reflects an inflammatory response. This is supported by the present finding that the proliferating collagen fibrils in the tunica media are type III collagen, which is generally recognized to exist in inflammatory tissue, and the ratio of type III to type I collagen tends to be significantly higher than in normal tissue. The collagen in the intima and internal elastic lamina was both types I and III. Similar findings have occurred in vessels with atherosclerosis and aging intima. Smith et al. examined the location of type V collagen within the blood vessel wall after SAH and reported interstitial deposition, believed to be associated with the migration and proliferation of myofibroblasts. These findings support the idea that changes in the passive elastic components after SAH, such as collagen and elastin in the arterial wall, may be a factor which influences the development of cerebral vasospasm.

Rudolph et al. found that the contraction of tissue in a variety of wounds is primarily related to the presence of myofibroblasts and contractile elements such as collagen fibers. These cells appear early, and after several weeks disappear from the healing wound leaving type V collagen. Bevan et al. observed dysfunction in the cerebral arteries of monkeys with chronic vasospasm induced by experimental SAH associated with infiltration of inflammatory cells, macrophages, and fibroblasts. Nagasawa et al. also reported increased elastic modulus of the arteries in dogs with SAH, possibly due to a change in the composition of the connective tissues.

The smooth muscles in the tunica media are an active component of the vessel wall. The collagen in the blood vessel wall is a major constituent of the extracellular matrix, which provides the necessary passive properties of the vessel wall. The contracting forces generated by the smooth muscle are transmitted to the passive components to result in an increased tension of the vessel. Barnes proposed that collagens in the vessel wall have functions associated with distribution: Type I collagen is related to the tensile strength of the tissue; while type III is involved in the extensibility of the vessel wall. These observations indicate that the appearance of collagen may cause the distinctive phenomenon of lasting vasospasm after SAH.

Successful clinical management of cerebral vasospasm following SAH by transluminal balloon angioplasty has been reported. This may support the idea that vessels affected by SAH undergo a change in elastic properties resulting in a greater contractility and lower plasticity. These findings suggest that preventive therapy for mural collagenization may help prevent the development of cerebral vasospasm following SAH.

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