Apoptosis of Medial Smooth Muscle Cells in the Development of Saccular Cerebral Aneurysms in Rats

Soichiro Kondo, MD; Nobuo Hashimoto, MD; Haruhiko Kikuchi, MD; Fumitada Hazama, MD; Izumi Nagata, MD; Hideo Kataoka, MT

Background and Purpose—Using an animal model, we examined the role of apoptosis in the disappearance of medial smooth muscle cells (SMCs) during the development and growth of cerebral aneurysms.

Methods—Various degrees of cerebral aneurysms were induced in the right anterior cerebral artery–olfactory artery bifurcations in 65 Sprague-Dawley rats with ligation of the left common carotid artery and renal hypertension. We performed in situ end labeling of fragmented DNA with the lesions in 45 rats and electron microscopic study in the other 20 rats.

Results—With in situ end labeling of fragmented DNA, 4±3 apoptotic medial SMCs were detected in 35 of the 45 bifurcations. Apoptotic SMCs appeared in the medial layer in the “preneurysm” group, the site speculated to show an aneurysmal change in the near future (6±3), and in the media in the “early aneurysm” group, which showed characteristics such as a small depression (5±3). In the “progressive aneurysm” group, they appeared more frequently at the aneurysmal neck (3±2) than the dome (1±1). By electron microscopic study, shrunken medial SMCs exhibiting morphological apoptotic changes such as chromatin condensation and fragmentation of the cytoplasm and nucleus were observed in the preaneurysm and early aneurysm groups and at the neck portion in the progressive aneurysm group. In the aneurysmal dome, SMCs showed late characteristics of apoptosis such as more advanced nuclear and cytoplasmic condensation and formation of apoptotic bodies.

Conclusions—The present findings indicate that there is an association between apoptosis of medial SMCs and the formation of saccular cerebral aneurysms. (Stroke. 1998;29:181-189.)

Key Words: apoptosis ■ cerebral aneurysm ■ pathology ■ muscle, smooth

In the pathogenesis of saccular cerebral aneurysms, thinning of the medial layer is important as well as degeneration of the internal elastic lamina. This medial change is accompanied with a decrease in cellularity of SMCs. The question arises as to what process is responsible for the disappearance of medial SMCs. It is difficult to elucidate the process of aneurysm formation or development with human specimens because their changes are already advanced or have been modified with other factors such as atherosclerosis. We have developed a method of inducing cerebral aneurysms in rats1–5 and monkeys,6,7 which have morphological and pathological similarity to human cerebral aneurysms, by ligating one common carotid artery and rendering the animals hypertensive, with or without feeding β-aminoproprionitrile. With the use of this animal model, it is possible to study the early and essential changes of aneurysm formation or development.

PCD, or apoptosis, is the process whereby cells are induced to activate their own death or cell suicide. PCD, which is an active, orderly process that often requires de novo gene expression and new protein synthesis,8 can be distinguished from necrosis, a passive form of nonprogrammed sudden cell death, both morphologically and biochemically. The term

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“apoptosis” has been used historically to refer to the unique morphology of cells undergoing PCD. The ultrastructural criteria include nuclear and cytoplasmic condensation, membrane budding, cell fragmentation, and phagocytosis of the apoptotic bodies. PCD occurs in a wide variety of cell types and is required for morphogenesis and homeostasis.9,10 Several studies have shown that PCD may have an important role in the pathogenesis and progression of various diseases, including cancer,9,11 neurological disorders,12 and cardiovascular diseases such as atherosclerosis and restenosis.13–15

In the present study we examined the association between apoptosis of medial SMCs and the formation of saccular cerebral aneurysms with in situ end labeling of fragmented DNA and electron microscopic study for these lesions in rats.

Materials and Methods

Procedures for Inducing Aneurysms

The left common carotid artery and posterior branches of both renal arteries of 65 male 5-week-old Sprague-Dawley strain rats each weighing 200 to 300 g were ligated to induce cerebral aneurysms. These procedures were performed with the rats under intraperitoneal

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Preparation for Light Microscopic Study

In 10 rats in the short-term group and 35 rats in the long-term group and all 15 control rats, a catheter was placed in the abdominal aorta, and the animals were perfused with heparinized saline, followed by Zamboni solution (2% paraformaldehyde containing picric acid in 0.15 mol/L phosphate buffer, pH 7.3). The circle of Willis was carefully removed from the brain and immersed in the same fixative at 4°C for 6 hours.

The right ACA-OA bifurcations, where aneurysmal changes of various degrees were speculated to be induced, were cut from the circles of all rats. The left ACA-OA bifurcations of experimental rats were also cut as a control. The specimens were then washed, dehydrated in a graded ethanol series, and embedded in paraffin. Semithin sections cut at 3-μm thickness were stained with hematoxylin and eosin or elastica van Gieson stain for the light microscopic study.

Preparation for Specific Labeling of Nuclear DNA Fragmentation and Immunohistochemical Analysis for SMCs

TUNEL16 was performed on the sections of these 45 experimental and 15 control rats for specific labeling of nuclear DNA fragmentation with Apop TagTM (Oncor Corp). Paraffin was removed from sections and nuclei of tissue sections were stripped from proteins by incubation with 20 μg/mL proteinase K for 15 minutes at room temperature. Endogenous peroxidase was inactivated by covering the sections with 0.5% H2O2 in PBS for 10 minutes at room temperature. After the excess liquid around the sections was removed, 10 μL of working TdT enzyme was applied. Then the sections were incubated in a humid atmosphere for 1 hour at 37°C. The reaction was terminated by transferring the slides to stop/wash buffer for 30 minutes at 37°C. The sections were rinsed with PBS and incubated in the presence of antidigoxigenin peroxidase in a humid atmosphere for 30 minutes at room temperature. After they were rinsed with PBS, the sections were exposed to filtered 0.05% diaminobenzidine with 0.02% H2O2 for 5 minutes. Rat large intestine and involuting prostates (2 days after castration), known to contain apoptotic cells, were analyzed as a positive control.16

Preparation for Electron Microscopy

In 5 rats in the short-term group and 15 rats in the long-term group, right ACA-OA bifurcations were excised from the circle after perfusion, then fixed with a solution of 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) at 4°C for 12 hours. Then the specimens were washed, postfixed in 1% osmium tetroxide in phosphate buffer (pH 7.4) for 1 hour, dehydrated in a graded ethanol series, and embedded in acrylic resin. Semithin sections (1 μm) were cut with glass knives and stained with toluidine blue for the light microscopic study. Thin sections were cut with diamond knives from areas selected in 1-μm sections. They were stained with uranyl acetate and lead citrate and viewed in a Philips 400 electron microscope.

Definitions and Judgments

We classified the bifurcations in the axial sections into four groups according to light microscopy findings: “no change” for no apparent changes, “preaneurysm” for fragmentation of internal elastic lamina without aneurysmal dilatation (Fig 1a), “early aneurysm” for the height of aneurysmal dilatations smaller than half the distance between the proximal and distal ends of the dilatation (magnification×400), “progressive aneurysm” (saccular aneurysm). In the wall of the aneurysm, most medial SMCs have disappeared. The wall consists mainly of connective tissue. Near the entrance of the aneurysm, the medial layer is tapered and the number of SMCs decreased. The internal elastic lamina at this portion is very thin and has nearly disappeared at the dome (magnification×400).

To identify SMCs, double staining was performed by combining TUNEL and immunohistochemistry with antibodies against α-smooth muscle actin (ZYMED Laboratories Inc), which specifically react with the α-smooth muscle isoform of actin.15 The cross-reaction to the antigen in rats was confirmed.16 Immunostains were visualized by using a streptavidin–alkaline phosphatase substrate system (HISTOSTAIN AP-Red kit, ZYMED Laboratories Inc). After the blocking of nonspecific binding with normal goat serum at a dilution of 1:10, the antibody without dilution was applied to the sections for 24 hours at 4°C. This was followed by incubation with a biotinylated second antibody. Streptavidin–alkaline phosphatase was then added, followed by addition of a mixture of substrate–chromogen solution. Counterstaining was done with hematoxylin or methyl green. Sections incubated with normal mouse serum served as negative controls.
The cells simultaneously stained with TUNEL and α-smooth muscle actin at or near the ACA-OA junctions were considered apoptotic SMCs and counted manually. These procedures were facilitated by projection of the microscopic image (Bausch and Lomb Inc). Three of us examined all sections (N.H., I.N., and S.K.). Each value was shown as mean±SD.

Statistical Analysis
The Mann-Whitney U test was used to compare the incidence of apoptotic SMCs between the short-term group and the long-term group and among the various stages of aneurysmal changes in ACA-OA bifurcations. The Wilcoxon signed rank test was used to compare the number of apoptotic SMCs at the distal side of the ACA branch adjacent to the apex and that at the distal side of the OA branch at each term and each stage of aneurysmal change. In the progressive aneurysm group, the difference between the number of apoptotic SMCs in the aneurysmal neck and that in the dome was also analyzed by this test.

Furthermore, the differences in systolic blood pressure just before operation and just before death in experimental and control groups were analyzed with the Wilcoxon signed rank test. A value of P<.05 on two-sided tests was considered to indicate significant difference.

Results
Systolic Blood Pressure
The systolic blood pressure of the 65 experimental rats just before operation was 104.2±5.4 mm Hg, and that just before death was 168.0±21.1 mm Hg, the difference being significant (P<.001). The systolic blood pressure of the 15 control rats (104.0±7.0 mm Hg and 102.8±5.6 mm Hg, respectively) was not significantly different (P=.460).

Light Microscopic Study
In all 15 control rats, the wall of the ACA-OA bifurcations on both sides consisted of normal arterial components, ie, endothelial cells, internal elastic lamina, medial SMCs, and thin adventitia. The medial layer consisted of three to five strata of SMCs that were similar in size and shape. No defect of the medial layer was found in any bifurcation.

In the short-term group, the bifurcations showed no change in 2 rats, preaneurysm in 5 rats, and early aneurysm in 3 rats. None of the bifurcations showed a progressive aneurysm. In the long-term group, the bifurcations showed no change in 6 rats, preaneurysm in 4 rats, and early aneurysm in 14 rats. The bifurcations in the other 11 rats were classified as progressive aneurysm.

The histological features of aneurysmal changes were thinning of the medial layer accompanied by fragmentation or disappearance of internal elastic lamina with wall dilatation. At the beginning, such changes tended to occur on the distal side of the ACA branch adjacent to the apex. In the preaneurysm group, discontinuity of the internal elastic lamina was observed. Although there was no apparent thinning of medial layer or wall dilatation, the disarrangement of medial SMCs had already begun (Fig 1a). The density of SMCs was preserved, but it was decreased in several rats in the preaneurysm group.

In the early aneurysm group, wall dilatation became apparent. In proportion to the dilatation size, the wall tended to become thinner. In the thinned parts of the wall, the decrease in SMC number and disarrangement advanced (Fig 1b). Volume reduction and distortion of SMC shape were found more frequently at the orifice of the dilatation. Nuclear shape was distorted like that of the whole cell, and the nucleus sometimes broke up into several fragments (Fig 2).

TUNEL of Fragmented DNA
In rat large intestine and involuting prostate 2 days after castration, apoptotic cells were detected at the tips of villi and in the acinar epithelium, respectively, as previously described (data not shown). The right ACA-OA bifurcations of the 15 control rats (5 in the short-term group and 10 in the long-term group) did not contain any apoptotic cells in the medial layer near the apex.

In the short-term group (Table 1), the mean number of apoptotic SMCs in the medial layer in the bifurcations was 6±4, 1±1 in 1 of the 2 bifurcations with no change, 8±2 in all 5 bifurcations in the preaneurysm group (Fig 3a), and 7±3 in all 3 bifurcations in the early aneurysm group (Fig 3b).

In the long-term group (Table 1), the mean number of apoptotic SMCs was 3±3. No apoptotic SMCs were detected in the 6 bifurcations with no change. In all 4 bifurcations in the preaneurysm group, 4±3 apoptotic SMCs were identified. In 12 of 14 bifurcations in the early aneurysm group and 9 of 11 in the progressive aneurysm group (Fig 3c), the mean number of apoptotic SMCs was 4±3 and 4±2, respectively.
**TABLE 1. Number of Apoptotic SMCs in Right ACA-OA Bifurcation in Short- and Long-term Groups**

<table>
<thead>
<tr>
<th>Grade*</th>
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<th>OA Side†</th>
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<td>Pre 9</td>
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<td>Mean ± SD</td>
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</table>

*Light microscopic grade of aneurysmal change: No indicates “no change” group; Pre, “preaneurysm” group; Early, “early aneurysm” group; and Progress, “progressive aneurysm” group.
†For the long-term group, at the neck portion of aneurysms of each side in progressive aneurysm group.
‡Value applies to the long-term group (P=.0096).
§Value applies to the OA side of the same term (P=.0074 in the short-term group and P=.00171 in the long-term group).
The mean number of TUNEL-positive SMCs in the bifurcations in the short-term group (6 ± 4) was significantly higher than that in the long-term group (3 ± 3) (P = .0096). In both groups, the number at the distal side of the ACA branch adjacent to the apex (5 ± 3 in the short-term group and 2 ± 2 in the long-term group) was significantly higher than that at the distal side of the OA branch (2 ± 1 in the short-term group and 1 ± 1 in the long-term group) (P = .0074 and .0017, respectively).

In total (Table 2), at least one TUNEL-positive SMC (mean number, 4 ± 3) was present in the medial layer in the bifurcations of 35 of the 45 experimental rats, 0 ± 0 in the 8 bifurcations with no change, 6 ± 3 in the 9 bifurcations in the preaneurysm group, 5 ± 3 in the 17 bifurcations in the early aneurysm group, and 4 ± 2 in the 11 bifurcations in the progressive aneurysm group.

The number of TUNEL-positive SMCs was highest in the bifurcation preaneurysm group, being significantly higher than that in the group with no change (P = .0005) and the progressive aneurysm group (P = .0043). However, there was no significant difference between the preaneurysm and early aneurysm groups (P = .148). The difference between the early aneurysm and the progressive aneurysm groups was not significant (P = .361).

TUNEL-positive SMCs appeared mainly in the medial layer of the preaneurysm bifurcation, the site speculated to show an aneurysmal change in the near future, or in the media of the early aneurysm group, which showed characteristics such as a small depression. The mean number of TUNEL-positive SMCs at the distal side of the ACA branch adjacent to the apex in the preaneurysm (5 ± 3) was significantly higher than that at the distal side of the OA branch (1 ± 1) (P = .0012), as well as in the early aneurysm group (3 ± 2 and 2 ± 1, respectively) (P = .0038). In the progressive aneurysm group, TUNEL-positive SMCs tended to be detected more frequently at the aneurysmal neck (3 ± 2) than the aneurysmal dome (1 ± 1) (P = .0074).

Among the 45 experimental rats, 3 had one TUNEL-positive SMC near the apex in the left ACA-OA bifurcation (the side of carotid ligation) (0 ± 0), which was significantly lower than the incidence in the right ACA-OA bifurcation (P = .0001).

Several apoptotic cells in the medial layer other than SMCs might be fibroblasts, although the number was very low compared with that of SMCs. Endothelial cells in the inner surface of the vessel wall were also occasionally labeled as apoptotic cells.

Electron Microscopic Study
The bifurcations for the electron microscopic study were classified as no change, preaneurysm, early aneurysm, and progressive aneurysm in 0, 2, 3, and 0 rats in the short-term group, respectively, and in 1, 1, 4, and 4 rats in the long-term group, respectively. In total, 1, 3, 7, and 4 rats with no change, preaneurysms, early aneurysms, and progressive aneurysms, respectively, were examined electron microscopically.

In the medial layer near the ACA-OA bifurcations in the preaneurysm or early aneurysm group, the number of SMCs decreased. Surface contact and normal arrangement was lost, and intracellular space was irregular. Under degenerated internal elastic lamina stained heterogeneously with uranyl acetate and lead citrate, shrunken SMCs exhibited early morphological changes associated with apoptosis such as margination or various degrees of condensation of chromatin (Fig 4a and 4b), and some SMCs showed dropping off and fragmentation of the cytoplasm and nucleus (Fig 4c).

At the neck portion of progressive aneurysms, SMCs also showed morphological changes of apoptosis: nuclear and cytoplasmic condensation and fragmentation (Fig 5a). In the thinned wall or aneurysmal dome, the structure of the vessel wall was markedly changed (Fig 5b). SMCs containing dense cytoplasmic actin fibers showed late characteristics of apoptosis such as more advanced nuclear and cytoplasmic condensation and formation of nuclear fragments with or without surrounding by a double membrane (Fig 5c). Some viable SMCs...
Discussion

Our group has successfully induced saccular cerebral aneurysms in rats, whose morphological and pathological features were very similar to those of human beings. Using these animal models, we investigated early changes in the cerebral arterial wall during the development of saccular cerebral aneurysms and found that thinning of the medial smooth muscle layer with fragmentation or disappearance of the internal elastic lamina may be the basis of aneurysm formation. In the thinned wall, the media consisted of a decreased number of medial SMCs, which varied in size and shape. The advanced aneurysmal wall, which consisted mainly of connective tissue and medial SMCs, had completely disappeared. These findings suggested that the reduction in SMC number in the aneurysmal wall was achieved to a great extent through apoptosis.

Identification of apoptosis in tissue sections has been greatly facilitated by specific immunolabeling of nuclear DNA fragmentation with terminal TdT. By this method, we proved that SMC death due to apoptosis occurred frequently in the medial layer near the right ACA-OA bifurcations in preaneurysm group, P = .0038 in the early aneurysm group, and P = .0116 in the "progressive aneurysm" group.

McKee et al. in rats1–5 or monkeys,6,7 whose morphological and pathological features were very similar to those of human beings. Using these animal models, we investigated early changes in the cerebral arterial wall during the development of saccular cerebral aneurysms. TUNEL (a-1, b-1, and c-1) and TUNEL and anti–smooth muscle actin (a-2, b-2, and c-2). The TUNEL staining yielded brown nuclei and the anti-actin immunostaining yielded blue nuclei. Counterstaining with hematoxylin yielded blue nuclei. Bar 5 μm. a, SMC with promoter is seen (magnification ×400). b, SMCs near the degenerated internal elastic lamina (asterisk) show volume reduction and distortion of cell shape. Their nuclear shape undergoes the same distortion as that of the whole cell, and some degree of chromatin condensation is visible. The medial layer is moderately thin. Bar = 2 μm. c, SMC with vacuole shows dropping off and fragmentation of the cytoplasm and nucleus. Bar = 2 μm.

Table 2. Mean No. of Apoptotic SMCs in the Right ACA-OA Bifurcation in Each Grade of Aneurysmal Changes in Short- and Long-term Groups

<table>
<thead>
<tr>
<th>Grade*</th>
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<th>OA Side‡</th>
<th>Aneurysmal Dome</th>
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<td>Progress (n=11)</td>
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<td>2.4±2.3§</td>
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*Light microscopic grade of aneurysmal change. No indicates "no change" group; Pre, "preaneurysm" group; Early, "early aneurysm" group; and Progress, "progressive aneurysm" group.
†At the neck portion of aneurysms of each side in progressive aneurysm group.
‡Value applies to the no change group (P=.0005) and the progressive aneurysm group (P=.0043).
§Value applies to the OA side of the same grade and "Total" (P=.0012 in the preaneurysm group, P=.0038 in the early aneurysm group, and P=.0116 in "Total").

were arranged in a disorderly fashion, and fibrous connective tissue. Some SMCs showed volume reduction and distorted cell shape. The advanced aneurysmal wall, which consisted mainly of connective tissue and medial SMCs, had completely disappeared. These findings suggested that the reduction in SMC number in the aneurysmal wall was achieved to a great extent through apoptosis.

Identification of apoptosis in tissue sections has been greatly facilitated by specific immunolabeling of nuclear DNA fragmentation with terminal TdT. By this method, we proved that SMC death due to apoptosis occurred frequently in the medial layer near the right ACA-OA bifurcations in preaneu-

Table 2: Mean No. of Apoptotic SMCs in the Right ACA-OA Bifurcation in Each Grade of Aneurysmal Changes in Short- and Long-term Groups

<table>
<thead>
<tr>
<th>Grade*</th>
<th>Total No.</th>
<th>ACA Side†</th>
<th>OA Side‡</th>
<th>Aneurysmal Dome</th>
</tr>
</thead>
<tbody>
<tr>
<td>No (n=8)</td>
<td>0.1±0.4</td>
<td>0.1±0.4</td>
<td>0</td>
<td>...</td>
</tr>
<tr>
<td>Pre (n=9)</td>
<td>6.1±3.0t</td>
<td>4.8±2.5g</td>
<td>1.3±1.0</td>
<td>...</td>
</tr>
<tr>
<td>Early (n=17)</td>
<td>4.6±2.9</td>
<td>2.9±2.0§</td>
<td>1.7±1.1</td>
<td>...</td>
</tr>
<tr>
<td>Progress (n=11)</td>
<td>3.7±2.2</td>
<td>1.5±1.3</td>
<td>1.6±1.0</td>
<td>0.6±0.5</td>
</tr>
<tr>
<td>Total</td>
<td>3.9±3.1</td>
<td>2.4±2.3§</td>
<td>1.3±1.1</td>
<td>0.6±0.5</td>
</tr>
</tbody>
</table>

*Light microscopic grade of aneurysmal change. No indicates "no change" group; Pre, "preaneurysm" group; Early, "early aneurysm" group; and Progress, "progressive aneurysm" group.
†At the neck portion of aneurysms of each side in progressive aneurysm group.
‡Value applies to the no change group (P=.0005) and the progressive aneurysm group (P=.0043).
§Value applies to the OA side of the same grade and "Total" (P=.0012 in the preaneurysm group, P=.0038 in the early aneurysm group, and P=.0116 in "Total").

contained small lysosomal bodies and showed phagocytosis of apoptotic bodies (Fig 6a). Macrophages containing many phagosomes were also identified (Fig 6b). At the innermost surface of the wall, endothelial cells were almost always present. Some of them had several microvilli and vacuoles (Figs 4a, 5b, and 6b).
Figure 5. Transmission electron photomicrographs show apoptotic SMCs in the progressive aneurysm group. a. The SMC at the orifice shows distortion and fragmentation of nucleus. Bar=2.0 μm. b. The structure of the wall is different from that of a normal vessel. The wall is very thin, and the internal elastic lamina has disappeared. Residual SMCs with vacuole are seen. Apoptotic bodies (arrows) of various sizes are seen. EC indicates endothelial cell; A, adventitia. Bar=4.0 μm. c. Late stage of apoptosis is seen in the wall. Margination of condensed nuclear chromatin (arrowheads) is seen. The cytoplasm (arrows) also appears electron-dense. Many fragmented electron-dense bodies (apoptotic bodies) are also seen in other portions. Bar=2.0 μm.

Figure 6. Transmission electron photomicrographs show phagocytosis in an aneurysmal wall. a. A viable SMC with many lysosomal bodies shows phagocytosis of apoptotic bodies. EC indicates endothelial cell. Bar=2.0 μm. b. A macrophage contains many phagosomes with membrane. Bar=2.0 μm.

rums or early aneurysms, especially at the distal side of the ACA branch adjacent to the apex, the site where aneurysmal change started. Furthermore, the frequency of the apoptosis was significantly higher at 3 months than at 6 months after the start of the experiment. Additionally, in progressive saccular aneurysms, medial SMCs had almost disappeared at the dome; apoptosis of SMCs took place in the neck portion. Thus, the aneurysmal wall expands and the aneurysm becomes larger.

Because apoptosis has been used historically to refer to the unique morphology of cells undergoing PCD, the presence of apoptosis is identified from ultrastructural characteristics including cell shrinkage, chromatin condensation, budding of cytosolic and nuclear components into membrane-bound apoptotic bodies, and phagocytosis by neighboring cells.19–22 We found several cells containing dense cytoplasmic actin fibers that showed different stages of typical apoptotic deterioration, including early chromatin condensation, membrane budding, and fragmentation of cells into apoptotic bodies. These findings demonstrate that medial SMCs are undergoing apoptosis during the formation of aneurysms. Apoptotic bodies constitute the most characteristic morphological feature of apoptosis.23,24

In the present study the frequency of apoptotic cells was not as high. This may have been caused by the fact that apoptotic cells can be seen light microscopically for only a few minutes; even ultrastructurally apoptotic bodies may be seen for only a few hours before they undergo phagocytosis.23 Thus, a small proportion of apoptotic cells visualized in a tissue section can represent a considerable magnitude of cell loss.25

Bennett et al26 noted that apoptosis could be observed in rat vascular SMCs in vitro. Bjorkerud et al27 also identified apoptosis in normal human vascular SMCs in culture. Apoptosis has been observed during the remodeling of the arterial wall after birth in animals.28

By the method of in situ labeling of DNA fragmentation and electron microscopic study, apoptosis in SMCs has been observed in proliferative diseases such as atherosclerosis,13,15 restenosis after percutaneous revascularization,13 saphenous vein grafts,14 and intimal thickening in a rat vascular injury model.15 These studies suggest that a balance between proliferation and apoptosis regulates the number of cells in the vessel wall, and apoptosis is potentially responsible for physiological arterial wall remodeling. With respect to genes that regulate apoptosis in SMCs, constitutive c-myc expression by SMCs induces continuous cell apoptosis as well as proliferation.26 Dominant proto-oncogenes, anti-apoptotic genes such as bcl-2, and tumor suppressor genes such as p53 have been reported to regulate apoptosis in SMCs.29

With regard to fragmentation or disappearance of the internal elastic lamina as another important factor in aneurysmal formations, we should consider an imbalance of elastic biosynthesis and elastinolysis. SMCs are known to synthesize and secrete connective tissue matrix, including elastic tissues.30 Therefore, the disappearance of viable SMCs through apoptosis may cause the decline of elastic synthesis. Elastin content as well as its synthesis in the vasculature has also been demonstrated to decrease with age.31 On the other hand, several proteinases such as elastase32 and matrix metalloproteinases33 are known to be elastinolytic enzymes. Elastase is produced and/or stored by polymorphonuclear neutrophils,34 monocyte/macrophages,35 and platelets.36 We previously demonstrated that many polymorphonuclear neutrophils and some platelets adhered to the interendothelial gap in this bifurcation.37,38 The lytic process of elastase from these cells might play a role in elastic degeneration. Furthermore, matrix metalloproteinase-2 has been reported to be localized within the cytoplasm of the SMCs.39 This enzyme from viable SMCs also may take part in elastic degradation.

Previously we reported that aneurysms tend to develop at sites of high wall shear stress.40 Kamiya and Togawa41 showed that autoregulation maintains constant wall shear stress in arterial walls. They mentioned that, with this regulation, increase of blood flow and wall shear stress may induce the adaptive enlargement of the vessel radius, which acts as negative feedback to reduce the stress itself. Additionally,
thinning of medial smooth muscle layer and degenerative changes of the internal elastic lamina are essential histological features of this change. We believe that aneurysm formation is the result of arterial remodeling under this regulation and that the death of medial SMCs through apoptosis plays an important role in this phenomenon.

The events that trigger apoptosis in the medial SMCs in the aneurysmal wall are unknown. However, the correlation between the endothelial cell, acting as a mechanosensor perceiving increased wall shear stress, and SMCs may be important. Several endothelium-derived factors such as nitric oxide, prostaglandin I₂, and other locally liberated yet important. Several endothelium-derived factors such as nitric oxide, prostaglandin I₂, and other locally liberated yet unknown factors have been speculated to induce apoptosis of SMCs. Release of SMCs from the extracellular matrix or deficiency of growth factors may also be a trigger.

Conclusions

The in situ end labeling of fragmented DNA and electron microscopy study showed an association between apoptosis of medial SMCs and cerebral aneurysmal formation; these findings require further investigation.

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References

The authors have demonstrated an association between the presence of apoptotic cells in the media of rat cerebral arteries where such arteries are made to give rise to aneurysms and within the wall of the aneurysm. The aneurysms are thought to be a model of berry aneurysms in humans. The apoptotic cells were identified with a double-labeling technique as SMCs. The disappearance of cells killed by apoptosis, or rather of the apoptotic bodies that such cell death leaves behind, is extremely rapid. Hence, the demonstration of relatively few apoptotic cells at any point in time, as in the accompanying article, is not evidence against the importance of apoptosis as a mode of cell death.

The article implies but cannot definitively prove that apoptosis is important in the production of the aneurysms, both in the rats and perhaps in humans. To test the hypothesis suggested by the data, it would be possible to attempt production of the aneurysms in rats pharmacologically treated with blockers of apoptosis.

Apoptosis is currently a topic of much interest in many disciplines including neuroscience and among students of vascular disease. Cell death in apoptosis is brought about by a mechanism that differs from that in necrosis. In apoptosis there is digestion of nuclear DNA, which leads to the death of the cell. It is thought that this digestion is the result of an action of intracellular endonucleases, enzymes whose synthesis depends on activation of a genetic program within the cells. Still unknown is which endonucleases are responsible and whether there are circumstances in which endonucleases already present within the cell may be activated to induce apoptotic death.

Apoptosis has been implicated as a cause of cell death in and around both myocardial and cerebral infarcts. The accompanying article extends the phenomenon to the vessel wall at the site of aneurysm formation. The precise cause of such aneurysms in humans remains an unsolved problem, although there is strong evidence that hemodynamic forces acting with greater strength at one point near the circle of Willis than at another are an important factor in producing aneurysms at sites that already lack an internal elastic lamella as a consequence of normal embryological development. The model used here increases the hemodynamic forces selectively at the sites of future aneurysm formation.

If the increased stress somehow induces synthesis of endonucleases through expression of the requisite genes and their message, this finding would represent an important step not only in our understanding of aneurysm development but also in the development of medical treatments to prevent formation of or further growth of aneurysms. At present only surgical treatment is available, and because of associated morbidity such therapy is not widely sought as a prophylactic measure before an actual bleed or rupture of the aneurysm. As medical science develops the means for local delivery of agents that block gene expression, one can envision the local delivery of such agents to the aneurysms without clipping or other disruptive surgeries. If such therapy could actually suppress a genetic mechanism responsible for local cell death and aneurysm production, the basis for such therapy presented in the accompanying article would be important indeed.

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Apopptosis of Medial Smooth Muscle Cells in the Development of Saccular Cerebral Aneurysms in Rats
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