Blood Flow Decrease Induces Apoptosis of Endothelial Cells in Previously Dilated Arteries Resulting From Chronic High Blood Flow

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Abstract—We investigated apoptosis of endothelial cells during the arterial narrowing process in response to reduction in flow. The decrease in flow was created in the carotid artery by closure of an arteriovenous fistula (AVF), which had been established for 28 days in rabbits. The endothelial cell apoptosis in the carotid artery was studied at 1, 3, 7, and 21 days of flow reduction after closure of the AVF by use of terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) with laser scanning confocal microscopy and transmission and scanning electron microscopy. After AVF closure, arterial lumen diameter was reduced by 36%, and compared with endothelial cells before the closure, the number of endothelial cells was decreased by 45% at 21 days. Endothelial cell apoptosis was observed at 1 day, peaked at 3 days (381.3 ± 87.1 cells per square millimeter), and decreased at 7 days. These cells had irregular protrusions under scanning electron microscopy and were characterized by fragmented nuclei under transmission electron microscopy. Apoptotic cells were mainly beneath the endothelium and were occasionally within smooth muscle cells and endothelial cells. The results suggest that apoptosis of endothelial cells may play a role in the arterial remodeling in response to a reduction in flow. (Arterioscler Thromb Vasc Biol. 2001;21:1139-1145.)

Key Words: blood flow ■ wall shear stress ■ endothelial cells ■ apoptosis ■ arterial remodeling

Arteries enlarge in response to an increase in blood flow.1-5 They become narrowed when the high flow is normalized6,7 or reduced toward the normal baseline level. Flow-induced arterial remodeling is endothelial cell dependent.8 Endothelial integrity is important in flow-induced arterial enlargement and intimal thickening.5-8 It has been shown that endothelial cells proliferate to cover the increased lumen surface when the arteries dilate.5,9 Endothelial cell lining is also required for reduction in arterial diameter resulting from decreased flow during animal development.10 It is assumed that the number of endothelial cells decreases during the narrowing of the arterial lumen through physiologically time-scheduled cell death.11

Physiological cell death, or programmed cell death, is termed apoptosis. It is governed by cell-survival and cell-death signals that contribute to many fundamental biological processes. Apoptosis has been studied in many different organs and tissues.12-14 In the arterial wall, smooth muscle cell apoptosis has been demonstrated during arterial development and remodeling after changes in blood flow after birth,15,16 in arterial intimal thickening after balloon catheter injury, and in atherosclerotic plaque induced in cholesterol-fed animals,17,18 as well as in human atherosclerotic plaque.19-21 In addition, a large number of apoptotic endothelial cells have been observed in transplanted coronary arteries.22 However, little is known about the role of endothelial cells in the arterial narrowing process during the normalization of previously established high-flow–induced arterial enlargement. The present study investigated the role of endothelial cell apoptosis in the arterial remodeling during the normalization of high flow, which had no apparent injury to the vessel wall. The results revealed that endothelial apoptosis contributed to the reduction in the number of endothelial cells, which was associated with the narrowing of previously dilated lumen induced by high flow.

Methods

Animals and Experimental Design
Fifty-eight male Japanese White rabbits were used (weighing 3 to 4 kg). In 50 animals, dilatory remodeling by chronic high blood flow was induced in the left common carotid artery (CCA) by creation of an arteriovenous fistula (AVF) between the left CCA and the left external jugular vein in the same manner as stated previously.5 Briefly, animals were anesthetized with inhalation anesthesia of sevoflurane (1% to 1.5% in O₂/N₂O, 2:1 [vol/vol]) after premedication of xylazine (4 mg/kg IM) and ketamine (25 mg/kg IM). Under sterile conditions, a side-to-side anastomosis was created between the artery and the vein at the segment 10 to 15 mm distal to the thyroid artery branching point. The animals were kept for 28 days.
At 28 days of chronic high flow, 10 animals were euthanized (chronic high flow [28d-HF] group), and 40 animals were subjected to closure of AVF (flow reversal [Rev] groups) to induce flow normalization by applying a surgical clip as described previously. Animals were kept for 1 day (1d-Rev, n=10), 3 days (3d-Rev, n=10), 7 days (7d-Rev, n=10), and 21 days (21d-Rev, n=10) after the closure of the AVF. Blood flow of the left CCA was measured before AVF, after AVF, before AVF closure, after AVF closure, and at euthanasia by use of an electromagnetic flowmeter (Nikon Kohden Co). Eight animals served as nonoperated controls.

Animal care followed the Japanese Community Standard on the Care and Use of Laboratory Animals. The Animal Research Committee, Akita University School of Medicine, approved the protocols for animal experimentation. All subsequent animal experiments adhered to the Guidelines for Animal Experimentation of the University.

Artery Fixation and Sampling
Before the animals were killed, they were anesthetized as described above. After laparotomy, a catheter was introduced into the abdominal aorta at the segment 2 to 3 cm distal to the renal arteries. Animals were then killed by injection of an overdose of pentobarbital solution (100 mg/kg) through the aortic catheter.

Five animals in each group were pressure perfusion–fixed with 3% glutaraldehyde solution in 0.1 mol/L PBS (pH 7.4) at 20°C via the catheter at a pressure of 100 mm Hg for 30 minutes. After fixation, the aortic arch and carotid arteries were carefully excised. The left CCA was divided equally into 6 segments (Nos. 1 through 6) from the proximal to the distal location as described before. The most distal segment (No. 6) was used for morphological study. The other 5 animals in each group were pressure perfusion–fixed with 8% paraformaldehyde solution in 0.1 mol/L PBS (pH 7.4) in the same manner as described. After fixation and excision, the left CCA was divided equally into 6 segments. The most distal segment (No. 6) was postfixed with 8% paraformaldehyde overnight at 4°C and used for detecting DNA fragmentation during apoptosis with the use of terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL). The segment was divided into 2 smaller segments without opening the lumen. One was used for en face preparation, and the other was processed as frozen cross sections.

Morphology
Arteries fixed with 3% glutaraldehyde were processed for histology, scanning electron microscopy (SEM), and transmission electron microscopy (TEM). Specimens for histology were stained with hematoxylin and eosin as well as elastica Masson’s trichrome. Specimens for SEM were dehydrated through alcohol and dried by the critical-point technique. After trimming, mounting, and coating with gold-platinum, the specimens were observed with SEM (JSM-5200, JEOL Co). Specimens for TEM were dehydrated through alcohol and embedded in epoxy resin (Epon). Semithin sections were examined to confirm proper cross-sectional orientation before ultrathin sectioning for TEM. The sections were stained with lead citrate and uranyl acetate and observed with TEM (LEMM2000, Topcon Co).

Histometry
Histometry was performed on routine paraffin sections stained with elastica Masson’s trichrome (1 section for each case). The sections were projected at ×50 magnification by using a Profile projector (Nikon V-16, Nikon Co). Contours of the lumen were traced and digitized with a Cosmomezco-1 digitizer (Nikon Co) to obtain lumen diameters. The lumen diameters were corrected for shrinkage during fixation and staining procedures with a shrinkage factor of ×1.25.1–7

Wall Shear Stress
Wall shear stress (WSS) in newtons per square meter (Pa) was calculated as follows assuming Poiseuille flow: WSS = 0.1×4×μ×BF/60m², where μ is the blood viscosity (0.03 poise),1–7 BFR is blood flow rate (milliliters per minute), and r is arterial lumen radius (centimeters).

Endothelial Cell Density
Endothelial cell density (cells per square millimeter) was calculated from 5 SEM photographs (∗×2000) taken from the No. 6 segment of each case.

Endothelial Cell Number
The total number of endothelial cells in the No. 6 segment was calculated by the following formula: total number of endothelial cells = endothelial cell density ∗ LSA, where LSA is the arterial luminal surface area of the No. 6 segment. LSA was 2πr²/6, where r is lumen radius (millimeters) and L is the length of left common carotid artery from the aorta to the thyroid artery branch.8

Detection of Apoptotic Cells
The TUNEL method was used to detect DNA fragmentation of apoptosis by the following 2 methods.

En Face Observation of Immunofluorescence Labeling Under LSCM
The prepared segments were washed in PBS 3 times for 5 minutes each. DNA fragmentation was labeled with FITC and was detected by using an in situ apoptosis detection kit (ApopTag Plus S7111-KIT, Oncor, Inc) according to the supplier’s instructions. Nuclei were counterstained with propidium iodide. The specimens were then opened and mounted on glass slides with the lumen side facing up. The specimens were dipped with 1,4-diazabicyclo[2.2.2.]octane glycercin solution (50%) and covered with a coverslip. To keep the coverslip from directly contacting the endothelial surface, bilateral adhesive tape (NW-20, Nichiban) was used as a spacer. To protect the specimens from drying, the edges of the coverslip were sealed with nail polish. The whole wall–mounted specimens were observed en face with a laser scanning confocal microscope (LSCM, Carl Zeiss LSM 410) with a HeNe laser (488 nm) and argon laser (530 nm). The former detects FITC on TUNEL-positive nuclei, and the latter detects counterstain of propidium iodide on nuclei. To reduce deviation caused by artifacts, the segment surface within 1 mm from the cutting edge was excluded from the observation. Because the arterial lumen surface was usually slightly waving, serial images at different foci for the endothelium were evaluated to obtain the whole lumen surface for each specimen. The endothelial cell apoptosis rate was calculated as a mean of TUNEL-positive cells per square millimeter from 5 pictures for each case. Each picture was 0.1024 mm² in size.

Immunofluorescence Analysis of Apoptosis on Cross-Sectional Specimens
Frozen cross sections (6 μm thick) were mounted on glass slides. DNA fragmentation of apoptotic endothelial cells was labeled and observed by using the same method as described above.

Immunohistochemical Stain of CD31
Paraffin-embedded sections were deparaffinized and rehydrated. The sections were incubated with 1% H2O2 in methanol for 30 minutes, followed by washing in distilled water. The sections were blocked with goat serum in PBS for 20 minutes at room temperature. After a wash in PBS, the sections were incubated with rabbit anti-human platelet endothelial cell adhesion molecule-1 (CD31) polyclonal antibody (Santa Cruz Biotechnology, Inc) at a concentration of 1:20 in 1% BSA in PBS at room temperature in a humid chamber for 30 minutes. The sections were washed 3 times with PBS and incubated with biotinylated universal antibody (Vector Laboratories, Inc) for color development. Sections were finally counterstained for nuclei with hematoxylin, dehydrated in ethanol and xylenes, and mounted with coverslips. For negative controls, the sections were processed in the same way, but the primary antibody was replaced with PBS.
versus 2.1
increased significantly at 21 days after AVF closure (21d-Rev), gradually decreased. The number of endothelial cells decreased significantly at 21 days after AVF closure (21d-Rev, Table).

Table). Compared with control density, endothelial cell density was increased by 1.8-fold at 28d-HF. After the closure of AVF, endothelial cell density gradually decreased. The number of endothelial cells decreased significantly at 21 days after AVF closure (21d-Rev, Table).

SEM Findings of Endothelial Surface
Margins of the gaps of the internal elastic lamina were remarkable in 28d-HF, as shown previously. As early as 1d-Rev, the margins of the internal elastic lamina were no longer recognizable. At 3d-Rev, the endothelial luminal surface was almost flat. At 7d-Rev and 21d-Rev, the surface was entirely flat (online Figure I; please see http://atvb.ahajournals.org).

At 1d-Rev, the surface of an individual endothelial cell was mostly flat without protrusion, and contours of the endothelial cells did not appear to be elongated, as seen at 28d-HF. There were a few endothelial cells whose contours were polygonal, and they were much smaller than others with small protrusions. Some of the small endothelial cells formed clusters (Figure 1A and 1B). They were usually surrounded by larger

Statistical Analysis
Results were expressed as mean ± SD. ANOVA and the Fisher protected least significant difference test were used for analysis. Differences between values were considered significant at P < 0.05.

Results

Blood Flow
Compared with blood flow before AVF, blood flow of the left CCA was elevated ≈3-fold immediately after AVF and 16-fold at 4 weeks. It decreased significantly immediately after AVF closure and was reduced almost to the level before AVF, as reported previously (Table).

Luminal Diameter
The luminal diameter at the level of the No. 6 segment of the left CCA increased significantly 28 days after AVF (3.7 ± 0.3 mm versus 2.1 ± 0.2 mm before operation) as described previously. It was progressively reduced after the closure of AVF (2.7 ± 0.2 mm at 21d-Rev) (Table).

Wall Shear Stress
WSS was elevated significantly at 28 days after AVF, as described (3.3 ± 0.7 Pa at 28d-HF). At 1 day after AVF closure, WSS decreased to a subnormal level (0.35 ± 0.09 Pa) at 1d-Rev. It was still at a low level (0.36 ± 0.09 Pa) at 21d-Rev (Table).

Length
The length of left CCA was elongated significantly at 28d-HF. After the closure of AVF, it shortened (Table).

Changes in Density and Number of Endothelial Cells in No. 6 Segment
Compared with control density, endothelial cell density was increased by 1.8-fold at 28d-HF. The total number of endothelial cells in the No. 6 segment increased by 3.3-fold versus control. After the closure of AVF, endothelial cell density gradually decreased. The number of endothelial cells decreased significantly at 21 days after AVF closure (21d-Rev, Table).

Figure 1. SEM photographs at high magnification showing very small endothelial cells. Very small endothelial cells are usually clustered (A through D). Their luminal surfaces have many small protrusions (A and B, arrowheads) and sometimes appear bleb-like (C and D, arrowheads). These cells are suggested to be apoptotic endothelial cells. Larger endothelial cells surround their clusters with irregular or fine protrusions forming foci of 2 distinct cell populations. Mostly preserved endothelial cells (#) with smooth luminal surface surround these foci. Original magnification ×3500. Bar=5 μm.
polygonal endothelial cells, which showed a well-preserved larger smooth surface.

At 3d-Rev, endothelial cell clusters consisting of 3 to 5 very small endothelial cells were frequently observed (online Figure I, panel D). They were almost polygonal to round with irregular protrusions. These cells usually had irregular bleb-like protrusions. Larger endothelial cells with many fine protrusions surrounded these clusters, and well-preserved endothelial cells with larger smooth surfaces surrounded these foci (Figure 1C and 1D).

At 7d-Rev, most of the endothelial cells were regular in size and shape (hexagonal), whereas clusters of very small endothelial cells were very rare. There were no distinct areas consisting of endothelial cells with fine protrusions (online Figure I, panel E).

At 21d-Rev, the luminal surface consisted of regular hexagonal endothelial cells with smooth surfaces (online Figure I, panel F).

**TEM Observation of Endothelial Layer and Subendothelial Layer**

At 3d-Rev, some endothelial cells showed fragmental nuclei without distinct nuclear membranes (Figure 2A, 2B, 2C, and 2D, and online Figure II, panels A, B, C, and D; please see http://atvb.ahajournals.org). Their chromatin appeared to be condensed along the inner surface of the nuclear envelope, losing normal monotonous electron density. Their cytoplasm was condensed with vacuolization. These features are consistent with cell apoptosis. The LSA of the apoptotic cells was small compared with that of regular endothelial cells. Occasionally, some of them had no luminal surface, because adjacent endothelial cells covered them (Figure 2C, 2D, 2E, and 2F). These apoptotic cells were rarely observed at 1d-Rev, and very few could be found at 7d-Rev and 21d-Rev.

Apoptotic cells were also observed in the subendothelial zone at 1d-Rev, 3d-Rev, and 7d-Rev (Figure 2G and 2H). These cells had no direct contact with the endothelial layer and mostly appeared oval or round with rather fine cytoplasmic protrusions. Their nuclei were sometimes fragmented with no distinct nuclear membrane. The cytoplasm became condensed or vacuolated, although many organelles remained in it. Occasionally, they were located deep among smooth muscle cells in the intima (online Figure II, panel D, and Figure 2I and 2J). And very occasionally, these apoptotic cells were within endothelial cells and smooth muscle cells in the intima (online Figure II, panels B, D, and E).

**Detection of Apoptosis**

Apoptotic cells were detected by TUNEL in flow-reversal groups (Table and Figure 3), whereas there were no TUNEL-positive cells in the control and 28d-HF groups. Apoptotic cells were detected under en face observation at 1d-Rev, became frequent at 3d-Rev with a labeling rate of 38±87 cells per square millimeter, and were reduced at 7d-Rev with a labeling rate of 48.6±4.2 cells per square millimeter. On cross sections, TUNEL-positive cells were observed at 3 days and 7 days after flow reversal in the endothelial line and in the thickened intima (online Figure III; please see http://atvb.ahajournals.org).

**Immunohistochemical Stain of CD31**

All endothelial cells lined on the arterial lumen showed positive stain with CD31 in all experimental groups. In 1d-Rev and 3d-Rev groups, there were a few CD31-positive cells beneath the endothelium (online Figure IV; please see http://atvb.ahajournals.org).
Discussion

The present study revealed that narrowing of the arterial lumen was associated with a decrease in the number of endothelial cells after flow normalization. The LSA of the distal segment of the rabbit left CCA was reduced by nearly 36%, and the number of endothelial cells was dropped by almost 46% after 21 days of flow reversal. These results agreed well with our previous reports. Thus, reduced blood flow induces adaptive narrowing of the lumen by decreasing the number of endothelial cells. Yet, the mechanism for the decrease in the endothelial number is not known, and this decrease is assumed to be attained through physiologically time-scheduled cell death, or apoptosis.

Using the TUNEL method with en face observation, we found that some endothelial cells underwent apoptosis with TUNEL-positive nuclei soon after flow normalization. The number of apoptotic endothelial cells peaked at 3 days and was reduced at 7 days. At 21 days after flow normalization, no apoptotic endothelial cells were observed. These results suggest that the decrease in the number of endothelial cells during flow normalization may be mediated mainly by apoptosis.

It is well known that TEM findings are important in the detection and evaluation of apoptosis. In this experiment, from 3 days after flow normalization, some endothelial cells had compaction and segregation of chromatin at the periphery of the nuclei, and some had fragmented nuclei with condensation of the cytoplasm and budding off of cytoplasm. These features are consistent with typical morphological changes of apoptotic cells. The TEM findings confirmed that the apoptotic procedure occurred in response to flow normalization.

In accordance with the TEM findings, the SEM study revealed luminal irregular zones consisting of 10 to 20 irregular hexagonal endothelial cells with many fine protrusions after flow normalization. In the middle of the zone, there was a cluster of very small endothelial cells. They appeared oval or round with many short cytoplasmic protrusions compared with the fine protrusions of the other endothelial cells in the heterogeneous zones. Apoptotic endothelial cells maintain normal size and shape after flow normalization and were localized only in the subendothelial layer and among the smooth muscle cells in intima, because they had no contact with endothelial layer. However, they occurred together with apoptotic endothelial cells at 1, 3, and 7 days after flow normalization and were localized only in the subendothelial zone. Furthermore, immunohistochemistry revealed several CD31-positive endothelial cells in the subendothelial zone. We assume that these cells are apoptosis-bound endothelial cells. The destination of the apoptotic endothelial cells is yet to be defined. Our observations suggest that there are 5 stages in the apoptotic process of endothelial cells.

Stage 1: Appearance of Heterogeneous Zones

Apoptotic endothelial cells maintain normal size and shape but tend to have slightly larger luminal protrusions. They have slightly condensed nuclei with lightly stained nuclear membranes. Apoptotic cells remain in the endothelial layer with normal contact with other regular endothelial cells, forming heterogeneous zones. Morphological features of apoptosis are not yet distinct.

Stage 2: Apoptotic Endothelial Cells Cluster in Heterogeneous Zones

Apoptotic endothelial cells form clusters and have irregular cytoplasmic protrusions compared with the fine protrusions of the other endothelial cells in the heterogeneous zones. They show early apoptotic characteristics by TEM, such as chromatin condensation of the nucleus and slight condensation of the cytoplasm. Plasma membrane, cytoplasmic organelles, and intercellular junctions remain intact. They seem to be trapped in the endothelial layer.

Stage 3: Apoptotic Endothelial Cells “Sink” Beneath Endothelial Layer

Heterogeneous zones become distinct, and some apoptotic endothelial cells have “sunk” beneath the endothelial layer. Their cytoplasm and cytoplasmic organelles are further condensed. They contact with adjacent endothelial cells via intercellular junctions.

Stage 4: Apoptotic Endothelial Cells Are Embedded in Subendothelial Space

Apoptotic endothelial cells lose their contact with endothelial cells. They appear oval or round with many short cytoplasmic protrusions. Their nuclei are frequently fragmented, and their cytoplasm and organelles are markedly condensed. Some are in the superior layer of intima among smooth muscle cells.

Stage 5: Apoptotic Endothelial Cells Are Trapped by Endothelial Cells or/and Smooth Muscle Cells

Although apoptotic endothelial cells still keep their contact with intact endothelial cells, they are trapped among endothelial cells or their processes. Some apoptotic endothelial cells lose their relationship with endothelial layer and are...
trapped among smooth muscle cells. In the endothelial cells or smooth muscle cells, their nuclei are all fragmented, and the cytoplasm is very condensed.

The mechanism by which the apoptosis of endothelial cells is programmed during flow normalization is not very clear. Dimmeler et al.24 examined endothelial cell apoptosis related to shear stress in a study on human umbilical venous endothelial cells (HUVECs) in vitro. Exposure of HUVECs to laminar flow at 4.5 and 1.5 Pa significantly abrogated apoptosis induced by tumor necrosis factor-α, whereas 0.5 Pa was less effective in the reduction in endothelial cell apoptosis by tumor necrosis factor-α. These results demonstrate that shear stress significantly contributes to endothelial cell integrity by inhibition of apoptosis. Kaiser et al.25 also discussed that the lack of hemodynamic forces triggered the apoptosis of HUVECs in a culture chamber at a shear stress of 0.01 Pa. In the present study, wall shear stress was elevated significantly after AVF and dropped quickly to subnormal levels after AVF closure. It was under this low wall shear stress condition that the endothelial cell apoptosis rate increased significantly. Our in vivo study further supports the postulation that wall shear stress is one of the important local regulators of endothelial cell apoptosis in the arteries with flow alteration. Recently, a novel shear stress–stimulated signal transduction pathway, shear stress–induced Akt phosphorylation, in endothelial cells was defined,26 which may account for several functional and morphological alterations of endothelial cells after exposure to shear stress. Akt phosphorylation might contribute to maintaining endothelial cell viability in response to alterations in shear stress. On the contrary, it is well known that high flow or elevated WSS protects endothelial cells from apoptosis. It transcriptionally upregulates growth factors such as basic fibroblast growth factor and NO,28 which are considered to prevent endothelial cell viability in response to alterations in shear stress. On the physiological level of shear stress in maintain- ing the functional integrity of endothelial cells.38

Acknowledgments

This study was supported in part by a grant from the Morris and Alvinda Hyman Aneurysm Research Program and a grant from the Pacific Vascular Research Foundation, San Francisco, Calif.

References


