Death of Smooth Muscle Cells and Expression of Mediators of Apoptosis by T Lymphocytes in Human Abdominal Aortic Aneurysms

E. Lynne Henderson, MD; Yong-Jian Geng, PhD; Galina K. Sukhova, PhD; Anthony D. Whittemore, MD; James Knox, MD; Peter Libby, MD

Background—Thinning of the tunica media and rarefaction of smooth muscle cells (SMCs) characterize aneurysmal aortas. Apoptosis determines the cellularity and morphogenesis of tissue. Macrophages and T lymphocytes infiltrate the wall of abdominal aortic aneurysms (AAAs) and produce death-promoting proteins (perforin, Fas, and FasL). This study investigated whether apoptosis occurs in association with the expression of these proteins.

Methods and Results—We examined signs of apoptosis and expression of death-promoting mediators in segments of AAAs from patients undergoing elective repair (n=20). Anti–α-actin immunostaining showed a reduced number of SMCs in AAAs. In situ terminal transferase-mediated dUTP nick end-labeling (TUNEL) showed higher levels of DNA fragmentation in AAAs than in controls (n=5). The AAA walls contained more cells bearing markers of apoptosis than normal aorta (P<0.05, Student’s t test). Double immunostaining identified SMCs and macrophages as the principal cell types displaying fragmented DNA. Immunohistochemistry revealed that AAAs but not normal aorta contained CD4+ and CD8+ T cells that expressed well-characterized cytotoxic mediators: perforin, which produces membrane damage, and Fas, which acts by ligand-receptor interaction. Double immunostaining also identified SMCs that expressed Fas. Immunoblotting confirmed the presence and, in the case of Fas, activation of these proteins in aneurysmal tissue.

Conclusions—Many medial SMCs in AAAs bear markers of apoptosis and signals capable of initiating cell death. Apoptotic death may contribute to the reduction of cellularity and to the impaired repair and maintenance of the arterial extracellular matrix in AAAs. Macrophages and T lymphocytes infiltrate the wall of AAAs, where they can produce cytotoxic mediators such as cytokines, perforin, and Fas/FasL. These death-promoting products of activated immune cells may contribute to elimination of SMCs, a source of elastin and collagen, during the pathogenesis of AAAs.

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Key Words: aneurysm ■ muscle, smooth ■ cells ■ lymphocytes ■ apoptosis

Aneurysms have been recognized causes of morbidity and mortality for centuries. Descriptions of “pulsatile masses” and the consequences of their injury appeared in the Ebers Papyrus (ca 2000 BC) and the works of Galen (131 to 200 AD).1 Epidemiological investigations have revealed a 3% prevalence of aneurysms in persons ≥60 years old.1–3 The past half century has provided a wealth of data concerning the natural history of aneurysm formation. Investigations of the pathogenesis of this disease, aimed at identification of a specific genetic defect, have so far yielded no clear pathogenic mechanism of formation. Although aneurysms can develop in any artery, the most striking morphological alterations occur in the abdominal aorta and other large arteries. Aortic aneurysms weaken and distort arterial architecture and, in certain patients, progressively enlarge and rupture. In late-stage aneurysms, aortic structure changes, displaying breakdown of elastic laminae and disappearance of well-organized smooth muscle layers.

Smooth muscle cells (SMCs), the most abundant cell type in the aortic media, can synthesize proteins such as collagen, elastin, laminin, gelatin, and proteoglycan, which compose the extracellular matrix of the arterial tunica media and confer strength and elasticity to the aortic wall. A loss or distortion of these structural proteins characterizes aneurysms. Researchers have sought enzymes capable of causing such changes. The primary candidates have been matrix metalloproteinases (MMPs) and certain serine proteases. Although many investigators have noted an increase in the levels of MMPs in aneurysmal and nonaneurysmal atherosclerotic tissue, recent evidence of elevation of both MMPs and their...
inhibitors (TIMPs) in aneurysmal tissue suggests that the overall balance of enzyme activity may be preserved. Thus, although proteolytic degradation of extracellular matrix may contribute to the structural changes found in the aneurysmal arteries, other mechanisms may pertain. For example, a decrease in number of SMCs could impair synthesis of the matrix proteins needed for repair. However, little information has been available regarding mechanisms that might deplete SMCs in these lesions.

We tested the hypothesis that local expression of death-promoting mediators by infiltrating immune cells may promote the formation of abdominal aortic aneurysms (AAAs), which are notable not only for the decrease in SMCs but also for an inflammatory infiltrate. The abundance of lymphocytes

<table>
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<tr>
<th>Antibody or Target</th>
<th>Cell Type</th>
<th>Source</th>
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mm indicates mouse monoclonal antibody; mp, mouse polyclonal antibody; IHC, immunohistochemistry; and IB, immunoblotting.

Figure 1. Paucity of smooth muscle cells in AAAs. Representative sections of human AAA (n=29) and normal (n=5) aortic tissue were incubated with murine anti-α-actin antibody, washed in PBS, and incubated with rabbit anti-mouse IgG conjugated with biotin. Color was developed with DAB. Nuclei were counterstained with hematoxylin. a, Normal aorta; b, Higher-power view of region indicated in a; c, AAA; and d, Higher-power view of region indicated in c. Low-power magnification ×100, high-power ×400.
and macrophages in these lesions suggests an ongoing, dynamic process of vascular remodeling. T lymphocytes initiate cell death by 2 pathways that involve distinct proteins, Fas and perforin. Fas is a member of the tumor necrosis factor (TNF) receptor family of membrane proteins. On binding Fas ligand (FasL), Fas initiates a cascade of signaling events that can kill the target cell. Activation of a series of proteases (caspases) figures prominently in this pathway. We have previously documented coexpression of the prototypic caspase, interleukin (IL)-1β–converting enzyme, with SMCs bearing markers of apoptosis in human atheromata. The second death-promoting pathway of T lymphocytes uses perforin. When released from storage granules in cytotoxic T cells, perforin creates holes in the membranes of target cells that alter permeability and lead to cell death.

Apoptosis contributes to normal morphogenesis. Recent studies have furnished evidence for apoptosis of SMCs in carotid and coronary atheromata as well as AAAs. In addition, cytokines produced by activated T cells and macrophages can cause the death of cultured human SMCs. The present study provides a novel potential mechanistic link between SMC death triggered by activation of the immune cells and weakening of the aortic wall. These changes may contribute to the pathogenesis of aneurysms and other aspects of arterial remodeling.

### Methods

#### Aneurysmal and Normal Aortic Tissue

Human AAA segments were obtained from patients undergoing elective repair (n=20). The average age was 70.6 years (range, 53 to 81 years). The average size of the aneurysmal lesions estimated by CT scan and/or angiography was 5.8 cm (range, 4.5 to 8.0 cm). Eighty percent of patients were men and 20% women. Nonaneurysmal aortic specimens were obtained at autopsy from 5 subjects who had no evidence or medical history of aneurysmal or occlusive disease (average age, 61.6 years; range, 46 to 84 years; 60% women, 40% men).
40% men). Normal aortic tissue obtained from organ donors or from the collection of aortic tissue in the Pathological Determinants of Atherosclerosis in Youth archive was used as control. Fresh aneurysmal tissue was divided into 2 parts: 1 for frozen or paraffin sections for histochemistry or immunohistochemistry and detection of apoptotic cells and 1 for protein isolation. The study of normally discarded human tissue was approved by the Human Investigation Review Committee at Brigham and Women’s Hospital.

In Situ End-Labeling of DNA Fragments (TUNEL)
Limited fragmentation of genomic DNA characterizes apoptosis. To evaluate numbers of cells with such damage to genomic DNA, we performed in situ labeling of DNA fragments by use of TUNEL with an ApopTag in situ apoptosis detection kit (Oncor, Inc). Briefly, sections were deparaffinized, rehydrated, and incubated with 0.3% H2 O2 in PBS to quench endogenous peroxidase activity. The slides were then incubated with 20 mg/mL of proteinase K (EM Science) and 10 mmol/L EDTA in PBS. DNA fragments were labeled with digoxigenin-dUTP. The terminal transferase and the labeled DNA fragments were detected with peroxidase-conjugated antibody against digoxigenin. Diaminobenzidine (DAB) was used as the substrate for peroxidase, yielding a brown color in nuclei. The sections of AAAs (n = 20) and the controls (n = 5) were viewed via light microscopy. For each section, the cells of 10 contiguous fields were counted independently by 2 investigators, and their observations were averaged. Cells with visible nuclear condensations were counted as positive.12 Apoptotic index was determined by the formula $100 \times \frac{\text{number of TUNEL-positive cell nuclei}}{\text{total number of cell nuclei}}$. Using parallel slides, we stained for α-actin (HHF-35). The same investigators counted the number of SMCs present in the wall of normal and aneurysmal aortas. A second index was determined with the same numerator and a denominator of α-actin–positive SMCs. The number of nuclei per cross-sectional area was calculated from the formula $(\text{perimeter} \times \text{thickness} \times \text{number of nuclei})/\text{high-power field}$ divided by the area of the high-power field. The perimeter was the average aortic diameter (normal, 2.3 cm; AAA, 5.8 cm), average aortic wall thickness (normal, 0.25 cm; AAA, 0.75 cm).

Immunohistochemistry
Cellular markers and products of immune cells in AAAs and control tissues were analyzed by immunohistochemistry with monoclonal antibodies (Table 1). Serial cryostat sections (6 μm) were prepared and air-dried on poly-l-lysine–coated slides. After fixation in acetone at −20°C for 5 minutes and pretreatment with 0.3% H2 O2 and proteinase K (EM Science), sections were incubated for 20 minutes in a blocking solution of 5% normal horse serum and then stained for 1 hour with a panel of primary antibodies (Table 1). Sections were washed and incubated with biotin-conjugated horse antibody (Vector Laboratories, Inc), which recognizes mouse IgG (1:200), for 30 minutes at room temperature. An avidin–alkaline phosphatase–fast red reagent (Vectastain ABC kit, Vector) visual-
Lymphocytes, and to a lesser degree, but still above baseline with the normal controls (n = 5) (Figure 1c and 1d). Cell counting revealed a 44% (25% to 1%) in the media of normal control aortas (P < 0.05). In contrast, the aneurysmal aorta exhibited fewer actin-positive cells (Figure 1c and 1d). Certain areas of AAAs appeared hypocellular, with little actin immunostaining. In the AAA regions with a relatively higher cell density, SMCs were often fragmented and adjacent to areas of acellular matrix (Figure 1c and 1d). These cells displayed weak immunoreactivity toward HHF-35, indicating a lower content of α-actin. AAAs lacked the orderly structure of elastic laminae observed in normal aortic media and showed a haphazard array of remaining SMCs interspersed with mononuclear leukocytes (Figure 1c and 1d). Cell counting revealed a 44% (25% to 66%) reduction in SMC density in AAAs (n = 29) compared with the normal controls (n = 5) (Table 2). To evaluate whether there was an actual decrease in the absolute number of SMCs in the aneurysmal wall as opposed to a similar number of cells dispersed over the enlarged aneurysmal wall, we calculated the number of nuclei per cross-sectional area. This calculation showed a 36% decrease in the nuclei per unit area in AAAs compared with that of normal aorta.

**Immunoblot Analysis**

A portion of the tissue samples was snap-frozen, crushed in liquid nitrogen, and mixed with 0.5 mL of SDS protein extraction buffer (20 mmol/L NaCl, 100 mmol/L Tris-HCl, pH 7.6, 10% SDS). After centrifugation at 4°C for 20 minutes at 13 000 g, the supernatant was collected and protein concentration determined with BSA as a standard. Proteins (30 μg/lane) were loaded into a 12.5% SDS-PAGE minigel and separated at 100 V for 1.25 hours. For determination of activated Fas aggregate, the samples were separated on a 7.5% SDS-PAGE gel. After electrophoresis, proteins were transferred to a membrane by use of a Trans-Blot SD semidry electrophoretic transfer cell (Bio-Rad). The membranes were blocked with 5% fat-free milk solution and incubated in PBS with primary antibodies (Table 1) against different T-cell antigens overnight. After staining, the membranes were washed in PBS with 0.2% Tween 20 and incubated with horseradish peroxidase–linked secondary antibody (goat anti-mouse, 1:10 000) for 1 hour. The membranes were washed again and developed with the Renaissance Western Blot Chemiluminescence Reagent (DuPont) or enhanced chemiluminescence system (Amersham).

**Statistics**

Significant differences between means were determined by Student’s t test. A value of P < 0.05 was considered significant.

**Results**

**Paucity of SMCs in AAAs**

In normal aortic media, almost all SMCs showed immunoreactive α-actin and formed homogeneous layers separated by elastic laminae in an orderly pattern (Figure 1a and 1b). In contrast, the aneurysmal aorta exhibited fewer actin-positive cells (Figure 1c and 1d). Certain areas of AAAs appeared hypocellular, with little actin immunostaining. In the AAA regions with a relatively higher cell density, SMCs were often fragmented and adjacent to areas of acellular matrix (Figure 1c and 1d). These cells displayed weak immunoreactivity toward HHF-35, indicating a lower content of α-actin. AAAs lacked the orderly structure of elastic laminae observed in normal aortic media and showed a haphazard array of remaining SMCs interspersed with mononuclear leukocytes (Figure 1c and 1d). Cell counting revealed a 44% (25% to 66%) reduction in SMC density in AAAs (n = 29) compared with the normal controls (n = 5) (Table 2). To evaluate whether there was an actual decrease in the absolute number of SMCs in the aneurysmal wall as opposed to a similar number of cells dispersed over the enlarged aneurysmal wall, we calculated the number of nuclei per cross-sectional area. This calculation showed a 36% decrease in the nuclei per unit area in AAAs compared with that of normal aorta.

**Infiltration of Inflammatory Leukocytes**

We examined the aneurysmal tissue for monocyte/macrophages or lymphocytes by immunohistochemistry (Table 1). The media of normal control aortas contained few CD68+ macrophages or CD3+ T cells. However, aneurysmal tissue showed focal accumulation of monocyte/macrophages, T lymphocytes, and to a lesser degree, but still above baseline control levels, B cells and natural cells (histology not shown). To clarify which subsets of T cells existed in the arterial tissues, we stained the sections with antibodies that recognize the CD4 and CD8 antigens characteristic of T helper and T cytotoxic cells, respectively. We found both CD4+ and CD8+ T-cell subsets in the aneurysms (Figure 2). We found more CD4+ cells than CD8+ cells. Negative controls using nonimmune IgG showed no positive staining (Figure 2d). These immunohistochemical results indicate coexistence of aortic degeneration and infiltration by inflammatory cells in AAAs.

**AAAs Exhibit Increased Markers of Apoptosis**

The lower number of SMCs in aneurysmal aortic walls suggested that cell death might mediate elimination of SMCs. To test this hypothesis, we analyzed DNA fragmentation using the TUNEL technique. Nonaneurysmal aortic tissue contained few TUNEL-positive cells (<1%) in the media (Figure 3a and 3b), whereas AAAs contained numerous TUNEL-positive cells. Cells bearing this marker of apoptosis localized within the lesions with inflammatory infiltrate, particularly in the region of intima-media junction (Figure 3c and 3d). Double staining with a combination of TUNEL and immunohistochemistry using antibodies against SMCs and macrophages determined which cell types bore this apoptotic marker (Table 1). Both α-actin–positive SMCs and CD68+ positive macrophages were TUNEL-positive (Figure 3). Cell counting revealed an increase in apoptotic index in AAAs (6.78 ± 3.1 SD) compared with normal tissue (0.57 ± 0.3 SD) (Table 2). To ensure that the index was not skewed by the absence of cells in the aneurysms, a second index was calculated using only SMCs as the denominator (Figure 4),
which demonstrated consistently increased numbers of SMCs with TUNEL-positive nuclei in AAAs (13.5 ± 6.2 SD) versus control (no appreciable change from noncorrected index, SD).

Candidate Mediators of Cell Death in AAAs

Further studies explored the nature of sources of signals that might trigger cell death in AAAs. The normal aorta contains little Fas antigen, limited to regions of the intima, with occasional Fas- cells in the media (Figure 5). In contrast, AAA tissue showed elevated levels of Fas throughout, prominently in both SMCs and leukocytes within the media. Double immunostaining of AAA specimens with α-actin and Fas demonstrates that SMCs express this receptor (Figure 5). Many TUNEL-positive cells also stain for Fas. AAA lesions but not control aortas contained FasL-positive T cells (histology not shown). Colocalization of both Fas and its ligand in AAA tissue suggested operation of the Fas death-signaling pathway. We sought evidence that the T cell–derived effector of cell death, perforin, might also participate in cell death within AAAs. Normal aortas contained little or no perforin, but in AAAs, immunoreactive perforin colocalized with activated T cells (Figure 6).

Immunoblotting evaluated levels of perforin, Fas, or FasL proteins in extracts of AAA or control tissue (Figures 7a through 7c and 8). To evaluate the specificity of the immunobLOTS, a negative control was incubated in blocking media and secondary antibody alone (Figure 7d). Consistent with the data obtained by immunohistochemistry, AAAs but not normal aortas contained appreciable amounts of perforin, Fas, and FasL (Figure 7a through 7c). Recent work by Kamitani et al13 has shown that certain anti-Fas antibodies recognize activated aggregates of human Fas. Indeed, immunoblots using these antibodies (3D5 and G254-274) disclosed higher-molecular-weight Fas aggregates, particularly a 97-kDa fragment in extracts of

Figure 5. Expression of death-promoting molecule Fas in AAAs. Cross sections of human AAAs were incubated with murine anti-Fas antibody. After a washing in PBS, sections were incubated with rabbit anti-mouse IgG conjugated with biotin. Color was developed with DAB. Nuclei were counterstained with hematoxylin. a and b, Normal aorta stained for Fas: (a) low magnification and (b) higher magnification of region indicated in a. AAA stained for Fas (c), and (d) higher-power magnification of region indicated in c. Double immunostaining of α-actin and Fas (e) and TUNEL and Fas (f) without counterstaining.
AAAs but not normal aortas, indicating Fas activation in the diseased tissue (Figure 8).

Discussion

Exploration of the pathogenesis of aneurysmal disease has evolved considerably over the past 30 years. Early studies focused on prevalence and natural history, attempting to define risk profiles for developing aneurysms and to determine whether additional factors might precipitate rupture.1,3,14,15 The association of some aneurysms with connective tissue disorders led to genetic evaluations of families with multiple members affected and to the search for culprit genes.1,2,16,17 Experiments have sought to identify enzymes in the aorta that might break down collagen and elastin, which compose major structural elements of the vessel wall. Recently, Knox et al4 compared MMP expression in aneurysmal and occlusive aortic diseases with normal controls and found no difference in MMP or TIMP expression that would explain aneurysm formation versus occlusive disease. It remains obscure why patients with seemingly identical risk profiles develop opposite manifestations of atherosclerosis.

Lack of SMCs, the source of the arterial extracellular matrix, might favor aneurysm formation. Cell death occurs in nonaneurysmal atherosclerosis.5,18,19 Recent studies have shown evidence of apoptosis of SMCs in AAAs. Because loss of SMCs might contribute etiologically to aneurysmal disease, we sought the potential mechanisms of this process.

A paucity of SMCs in AAAs compared with the accumulation of SMCs in occlusive disease is one of the most striking histological distinctions between these 2 conditions. Histological examination of AAAs consistently shows distinct loss of cellular structures and nuclei, arguing against a substantial population of \(\alpha\)-actin-negative SMCs.

The present results with perforin and Fas/FasL point to a role for immune cells in induction of apoptosis of SMCs in aneurysmal tissue.20 In situ observations cannot provide direct evidence for this possibility. However, our recent in vitro work has demonstrated that SMCs can undergo apoptosis triggered by activation of Fas after priming with proinflammatory cytokines such as interferon-\(\gamma\), TNF, and IL-1.11 Ligation of Fas caused aggregation, reflected by the appearance of higher-molecular-weight bands in immunoblots.13 Our observation that such 97-kDa Fas aggregate fragments exist in AAA but not normal aortic tissue provides further biochemical evidence for the role of the Fas/FasL system in cell death in AAAs. Macrophages and T cells within evolving aneurysms most likely produce these cytokines locally; these mediators may act in concert with Fas/FasL to induce SMC apoptosis. The low levels of inflammatory cells in normal aorta probably explain the lower expression of Fas/FasL than in AAAs, because T cells are considered the primary source for these molecules. In addition to SMCs, the inflammatory cells themselves may be targets for apoptosis, a potential mechanism of self-limitation of the immune response.

The present data support the view that the pathogenesis of aneurysm formation as well as their distinction from aortoocclusive disease may depend on the inflammatory infiltrate, including T cells expressing FasL and perforin. Not every T cell produces every cytokine, and activated T cells can be subtyped according to their cytokine profile. Th1 cells secrete IL-2, TNF-\(\beta\), and IFN-\(\gamma\), whereas Th2 cells typically produce IL-4, -5, -6, and -10. Because Th1 cytoto-

Figure 6. Expression of death-promoting molecule perforin in AAAs. Cross sections of human AAAs were incubated with murine anti-perforin antibody. After a washing in PBS, sections were incubated with rabbit anti-mouse IgG conjugated with biotin. Color was developed with DAB. Nuclei were counterstained with hematoxylin. Normal aorta stained for perforin (a) at low magnification and (b) higher magnification of region indicated in a. AAA stained for perforin (c), and (d) higher-power magnification of region indicated in c.
kines can promote apoptosis in SMCs, Th1 cells may mediate loss of SMCs in AAAs. Conversely, the cytokines elaborated by Th2 cells may promote a less cytolytic response that could culminate in the clinical picture of aorto-occlusive disease. Continuing studies should further characterize the T cell subtypes and the cytokines present in the 2 diseases.

It is important to acknowledge the limitations of a study that uses surgically removed human aneurysmal vessels. AAAs are largely asymptomatic until they have reached a size that requires intervention. This usually precludes obtaining fresh specimens from other than end-stage disease. Therefore, we cannot extrapolate from observations on late-stage specimens to earlier phases of aneurysm formation. In particular, the high apoptotic index for SMCs (Figure 4) may not reflect an actual instantaneous rate of apoptosis. As previously stated, care was taken during the cell counting to ensure that condensed nuclei were identified, avoiding the false-positives generated by staining of calcium-phospholipid vesicles.12 However, as recently reported, cells undergoing RNA splicing may stain positive with TUNEL, rendering this technique selective but not specific, because apoptotic nuclei contain an increased degree of fragmentation compared with nonapoptotic cells.21 Existing animal models use exposure of arteries to exogenous enzymes or xenografting of arterial tissue, 2 procedures that may not mimic the usual pathogenesis of human aortic aneurysms16, (E. Allaire, MD, unpublished data, 1997). These limitations make it difficult to draw conclusions regarding signaling and initiation of events in relation to the human disease. The present identification of 2 signals that may be involved in altering SMC function in human aortic aneurysms may aid in the development of an improved animal model for this important arterial disease.

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References


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