American Journal of Pathology, Vol. 150, No. 3, March 1997

Decreased Vascular Smooth Muscle Cell Density in Medial Degeneration of Human Abdominal Aortic Aneurysms

Angel López-Candales,* Dennis R. Holmes,† Shixiong Liao,† Michael J. Scott,* Samuel A. Wickline,* and Robert W. Thompson†‡

From the Division of Cardiology, Department of Medicine, the Section of Vascular Surgery,† Department of Surgery, and the Department of Cell Biology and Physiology,‡ Washington University School of Medicine, St. Louis, Missouri*

Abstract

Abdominal aortic aneurysms (AAAs) are characterized by structural deterioration of the aortic wall leading to progressive aortic dilatation and eventual rupture. The histopathological changes in AAAs are particularly evident within the elastic media, which is normally dominated by vascular smooth muscle cells (SMCs). To determine whether a decrease in vascular SMCs contributes to medial degeneration, we measured SMC density in 21 normal and pathological human abdominal aortic tissue specimens using immunohistochemistry for α-SMC actin and direct cell counts (medial SMCs per high-power field (HPF)). Medial SMC density was not significantly different between normal aorta (n = 5; 199.5 ± 14.9 SMCs/HPF) and atherosclerotic occlusive disease (n = 6; 176.4 ± 13.9 SMCs/HPF), but it was reduced by 74% in AAA (n = 10; 50.9 ± 6.1 SMCs/HPF; P < 0.01 versus normal aorta). Light and electron microscopy revealed no evidence of overt cellular necrosis, but SMCs in AAAs exhibited ultrastructural changes consistent with apoptosis. Using in situ end-labeling (ISEL) of fragmented DNA to detect apoptotic cells, up to 30% of aortic wall cells were ISEL positive in AAAs. By double-labeling techniques, many of these cells were α-actin-positive SMCs distributed throughout the degenerative media. In contrast, ISEL-positive cells were observed only within the intimal plaque in atherosclerotic occlusive disease. The amount of p53 protein detected by immunoblotting was increased nearly fourfold in AAA compared with normal aorta and atherosclerotic occlusive disease (P < 0.01), and immunoreactive p53 was localized to lymphocytes and residual SMCs in the aneurysm wall. Using reverse transcription polymerase chain reaction assays a substantial amount of p53 mRNA expression was observed in AAAs. These results demonstrate that medial SMC density is significantly decreased in human AAA tissues associated with evidence of SMC apoptosis and increased production of p53, a potential mediator of cell cycle arrest and programmed cell death. Given the role that SMCs normally play in maintaining medial architecture and in arterial wall matrix remodeling, the induction of SMC apoptosis likely makes an important contribution to the evolution of aneurysm degeneration. (Am J Pathol 1997, 150:993–1007)

Human abdominal aortic aneurysms (AAAs) are characterized by profound histopathological changes in the aortic wall associated with progressive aortic dilatation and eventual rupture.[1–3] Although severe intimal atherosclerosis invariably accompanies AAA, structural degeneration of the

Presented in abstract form at a New York Academy of Sciences conference on Abdominal Aortic Aneurysms: Genetics, Pathophysiology, and Molecular Biology, March 7–9, 1996, New York, NY, and the 45th annual meeting of the American College of Cardiology, March 23–27, 1996, Orlando, FL.

Supported in part by a research grant-in-aid from the American Heart Association, Missouri Affiliate (R. W. Thompson) and grant HL-42950 from the National Institutes of Health (S. A. Wickline). A. López-Candales is the recipient of a Hewlett Packard Cardiology Fellowship Award, S. A. Wickline is an Established Investigator of the American Heart Association, and R. W. Thompson is a Faculty Fellow of the American College of Surgeons.

Accepted for publication November 14, 1996.

Address reprint requests to Dr. Robert W. Thompson, Section of Vascular Surgery, 5103 Queeny Tower, One Barnes Hospital Plaza, St. Louis, MO 63110.

elastic media plays a more unique role in the evolution of aneurysm disease.[4–6] Indeed, aortic wall dilatation is fundamentally caused by mechanical failure of collagen and elastin, the fiber-forming extracellular matrix proteins responsible for structural integrity of the arterial wall.[7–11] Functionally significant mutations in genes encoding type III collagen and elastic fiber components (ie, fibrillin-1) are responsible for several forms of inheritable aneurysm disease, such as Ehlers-Danlos type IV and Marfan syndromes.[12–14] The vast majority of AAAs, however, develop as acquired degenerative lesions in the absence of known connective tissue defects.[15] Because elastin is a particularly stable protein that normally undergoes little metabolic turnover in the adult,[16,17] recent investigations on the pathogenesis of AAA have emphasized alterations in aortic wall elastic matrices, elastin-degrading proteinases, and their inhibitors.[18–24]

In addition to accelerated degradation of structural matrix proteins, AAAs are associated with marked alterations in the cellular composition of the aortic wall. The most prominent of these changes include infiltration of the outer aorta by mononuclear phagocytes,[24–26] lymphocytes,[27] and proliferative endothelial cells.[20,28] Whereas any of these cells might mediate matrix degradation through local release of elastin- and collagen-degrading proteinases, vascular smooth muscle cells (SMCs) are normally the predominant cell type of the elastic media. SMCs thereby make substantial contributions to the elastic lamellar architecture of the arterial wall, both directly and indirectly through their production of elastin, collagen, and other matrix proteins.[29–31] SMCs may also participate in matrix remodeling through localized expression of various proteinases and their inhibitors.[26,32–34] Because SMC-mediated repair and resynthesis of structural matrix proteins might balance proteolysis induced by other cell types, the fate and function of vascular SMCs likely has an important influence on the progression of aneurysm degeneration.[35] The specific role of this cell population in aneurysm disease, however, remains uncertain.

The present study was undertaken to begin examining the relationship between medial SMCs and aneurysm degeneration in human aortic tissues. We found that medial SMC density is significantly decreased in AAAs compared with normal aortas and those with atherosclerotic occlusive disease (AOD) and that the apparent loss of medial SMCs in aneurysms is associated with ultrastructural and histochemical evidence of SMC apoptosis. We also found that SMCs in aneurysms exhibit increased production and accumulation of p53, a potentially important mediator of cell cycle arrest and physiological cell death. These observations indicate that molecular mechanisms involving the induction of medial SMC apoptosis contribute to the medial degeneration underlying AAA and therefore potentially act to destabilize the aneurysm wall.

Materials and Methods

Human Aortic Tissues

Fresh aortic wall specimens were obtained in the operating room from normal organ transplant donors without visible evidence of aortic atherosclerosis (n = 5), and from 16 patients undergoing aortic reconstruction for either AOD (n = 6) or AAA (n = 10). All tissue specimens were taken from the anterolateral aspect of the infrarenal abdominal aorta following a protocol approved by the Washington University School of Medicine Human Research Subjects Committee. For each specimen, one portion of the aortic wall was processed for light microscopy and an adjacent portion was snap-frozen in liquid nitrogen, stored at −80°C, and subsequently used for protein and nucleic acid extractions.

Immunohistochemistry

Tissues were fixed in 10% neutral buffered formalin at 4°C for 24 hours and then processed for routine paraffin embedding. By the convention used throughout this study, aortic wall specimens were oriented in cross section transverse to the axis of blood flow *in vivo*. Five-micron sections affixed to glass slides were deparaffinized and rehydrated, and endogenous peroxidase activity was quenched by incubation with 0.3% hydrogen peroxide for 30 minutes at room temperature. Sections were blocked with normal rabbit serum and then incubated with either mouse anti-α-SMC actin IgG (Sigma Chemical Co., St. Louis, MO; 1:20), goat anti-human p53 (Oncogene Science, Cambridge, MA; 1:200), or normal horse serum for controls. Immune complexes were detected with alkaline phosphatase/anti-alkaline phosphatase for SMCs or immunoperoxidase for p53, using Vectastain Elite kit reagents (Vector Laboratories, Burlingame, CA). After immunohistochemical staining, sections were counterstained with hematoxylin. To co-localize α-SMC actin and p53 in the same tissue sections, slides were sequentially incubated with goat anti-human p53 (1:200), peroxidase-conjugated rabbit anti-goat IgG, and fluorescein isothiocyanate (FITC)-conjugated mouse anti-α-SMC

actin IgG (1:500). After visualization of peroxidase-labeled immune complexes with diaminobenzidine to localize p53, sections were examined under epifluorescence to detect α-SMC actin.

Morphometric Analysis

Aortic sections stained for α-SMC actin were used to measure medial SMC density. Representative microscopic fields of the aortic media were selected under low-power light microscopy (×40). To avoid underestimating medial SMC density in aneurysm specimens with considerable regional variation, areas chosen for morphometric analysis were restricted to microscopic fields with the greatest number of SMCs and the least amount of inflammatory cell infiltration. The number of α-SMC actin-positive cells was measured for each of five contiguous ×400 high-power fields (HPFs, each covering an area of 8.75×10^{-2} mm^2), with individual cell counts made by two independent observers (D. R. Holmes and S. Liao). The overall mean SMC density for each specimen was determined as SMCs per HPF. For each type of aortic tissue (normal aorta, AOD, and AAA), the mean, standard error (SEM), and range of SMC density measurements were recorded. Statistical comparisons between each tissue type were made using analysis of variance (ANOVA) and the Newman-Student-Keuls multiple comparisons test.

Electron Microscopy

Tissue samples (2 mm^2) were fixed overnight in cold (4°C) Tyrode's buffer containing 2% glutaraldehyde. After rinsing with cold buffer for at least 1 hour, tissues were postfixed for 1 to 2 hours in 1% OsO_4 in cold Tyrode's buffer and rinsed for 30 to 60 minutes. Specimens were dehydrated through graded ethanols (70%, 95%, 100%; 30 minutes each at room temperature), incubated twice in propylene oxide (30 minutes each), and infiltrated overnight in a 1:1 mixture of propylene oxide/Polybed 812 resin. Tissues were embedded in Polybed 812 resin blocks for 1 hour at room temperature, incubated overnight at 45°C, and sectioned after cooling to room temperature. Thin sections were cut with a diamond knife, placed on Formvar-coated copper grids, and examined on a Phillips 201 transmission electron microscope (Eindhoven, The Netherlands).

In Situ *End-Labeling (ISEL)*

Formalin-fixed, paraffin embedded tissue sections were deparaffinized and rehydrated through graded alcohols. At room temperature, sections were rinsed in phosphate-buffered saline (PBS), pH 7.4, treated with 20 μg/ml proteinase K (Sigma) in PBS for 15 minutes, and then washed in four changes of distilled water. Sections were incubated with 2% hydrogen peroxide in PBS for 5 minutes and then rinsed twice in PBS. After incubating sections for 30 seconds in equilibration buffer supplied in the ApopTag kit (Oncor, Gaithersburg, MD), a working dilution of terminal deoxynucleotidyl transferase (TdT) was applied for 45 minutes at 37°C in reaction buffer containing digoxigenin-labeled nucleotides (Oncor). Sections were then incubated in prewarmed working strength ApopTag stop/wash buffer (Oncor) for 30 minutes at 37°C, with agitation once every 10 minutes. After washing sections in three changes of PBS for 5 minutes at room temperature, sections were incubated in anti-digoxigenin-peroxidase solution (60 μl/section) for 30 minutes at room temperature and then washed in three changes of PBS for 5 minutes each. Freshly prepared diaminobenzidine substrate solution was used for detection of immune complexes, and sections were counterstained with 0.5% methyl green (w/v) in 0.1 mol/L sodium acetate, pH 4.0. For positive controls, sections were treated before ISEL with 1 μg/ml DNAse (Sigma) in 30 mmol/L Tris/HCl, pH 7.2, containing 140 mmol/L potassium cacodylate, 4 mmol/L $MgCl_2$ and 0.1 mmol/L dithiothreitol, for 10 minutes at room temperature.

Immunoblot Analysis for p53

Aortic tissue samples were extracted at 4°C in 50 mmol/L Tris/HCl buffer, pH 7.5, containing 1 mol/L NaCl, 2 mol/L urea, 0.1% EDTA and 0.1% (w/v) Brij-35 for 20 minutes. After centrifugation at 10,000 × *g* for 1 hour at 4°C, the supernatant was dialyzed overnight at 4°C against 50 mmol/L Tris/HCl, pH 8.0, 1 mol/L NaCl, and 20 mmol/L $CaCl_2$ using a 12,000 to 14,000 molecular weight cut-off membrane. The total protein concentration of each sample was determined using a Bio-Rad protein assay with bovine serum albumin as the standard (Bio-Rad, Hercules, CA). Heat-denatured samples were resolved under reducing conditions by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 10% polyacrylamide gels (8 μg of total protein per lane). Proteins were electrophoretically transferred to nitrocellulose and washed three times in 20 mmol/L Tris/HCl, pH 7.5, 0.5 mol/L NaCl, 0.05% Tween-20 (TTBS). At room temperature, membranes were blocked overnight in TTBS containing 5% powdered milk and then incubated for 1 hour in TTBS/1% milk containing 2 μg/ml mouse

monoclonal anti-human p53 (Oncogene Science) recognizing both wild-type and mutant forms of human p53. Membranes were washed in TTBS/3% milk and incubated for 1 hour with horseradish-peroxidase-conjugated rabbit anti-mouse IgG (Amersham Life Science, Arlington Heights, IL) diluted 1:3000 in TTBS/1% milk. Immunoreactive proteins were visualized by enhanced chemiluminescence using kit reagents and instructions recommended by the manufacturer (Amersham). The relative density of immunoreactive p53 protein in each sample was determined with a GS 300 scanning densitometer (Hoefer Scientific Instruments, San Francisco, CA). For six samples of each tissue type, the mean ± SEM of relative p53 content was determined and compared using ANOVA and the Newman-Student-Keuls multiple comparisons test.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Given the limited yield of total RNA recovered from diseased human aortic tissue samples and the low abundance of p53 mRNA transcripts, RT-PCR assays were used to determine p53 gene expression. Total RNA was isolated from aortic tissue samples (each approximately 0.5 g) by guanidinium thiocyanate/phenol/chloroform extraction in RNAzol, according to the manufacturer's instructions (Cinna/Biotecx, Houston, TX). After verifying the integrity of the RNA by agarose gel electrophoresis, all samples were normalized to the same amount of total RNA template for RT-PCR analysis. First-strand cDNA synthesis was performed using 1 μg of total RNA, oligo (dT) primers, RNAse inhibitor, and 2.5 U/L Molony murine leukemia virus reverse transcriptase in a total volume of 20 μl, according to the manufacturer's recommendations (Perkin-Elmer-Cetus, Norwalk, CT). Samples were reverse transcribed on a Hybaid Omnigene system for 15 minutes at 42°C, and the reaction was terminated by heating to 99°C for 5 minutes. The RT reaction products served as the template for PCR amplification of p53 transcripts using primers for human p53 exons 7 through 9 (Clontech, Palo Alto, CA): 5′-GTGTTGTCTCCT AGGTTGGCTCTG-3′ (forward primer) and 5′-CCCAAGACTTAGTACCTGAAGGGTG-3′ (reverse complement primer). Each PCR amplification was performed in a 40-μl reaction volume with 1 μmol/L (each) 5′ and 3′ primers in 10 mmol/L Tris/HCl buffer containing 50 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.001% (w/v) gelatin, and 5 U/μl Amplitaq DNA polymerase. Because amplification of p53 transcripts was found to be linear at 35 cycles of PCR, typical PCR reactions included 5 minutes at 94°C for denaturation, 5 minutes at 60°C for annealing, 35 cycles of 1.5 minutes at 72°C, 45 seconds at 94°C, and 45 seconds at 60°C, and 10 minutes at 72°C for final extension. In all experiments, the possible presence of PCR contaminants was excluded by control reactions without reverse transcriptase or with extraction buffer alone (absence of template). PCR products (35 μl) were size-fractionated on 1.5% agarose gels together with molecular weight markers. DNA was visualized by ethidium bromide staining under ultraviolet illumination, and gels were photographed with positive/negative instant film (Polaroid Corp., Cambridge, MA). Photographs were examined using an Imagestone 7500 video densitometer (Ultraviolet Products, Cambridge, UK), and peak areas in each lane were calculated using ScanDO and NIH Image processing programs (Wayne Rasband, National Institutes of Health, version 1.44). For each tissue sample, the abundance of p53 mRNA was expressed as the relative density compared with other samples run on the same gel, and comparisons between results for normal aorta and AAA tissues were made using the Student's *t*-test.

Results

Medial SMC Density Is Reduced in AAA

As in previous studies,[4,6,9,24,26,28] the most conspicuous light microscopic features of AAAs were severe intimal atherosclerosis and architectural degeneration of the elastic media, exemplified by a pronounced decrease in aortic elastin fibers. The cellular composition of AAAs typically included chronic inflammatory cell infiltrates and medial neovascularization as previously described.[25–28] Importantly, aneurysms were associated with a markedly diminished population of medial SMCs as determined by immunohistochemistry for α-SMC actin (Figure 1). As shown in Table 1, morphometric analysis demonstrated a 74.5% reduction in mean SMC density in AAA compared with normal aorta ($P < 0.001$). In contrast, mean SMC density was not significantly different between normal aorta and AOD tissues.

The possibility that measurement of medial SMC density might have been influenced by tissue orientation was examined in a separate series of tissues by embedding the same tissue specimens in both transverse and longitudinal orientations. This revealed that tissue orientation did not contribute substantially to the differences in medial SMC density measured between different aortic tissue types (Ta-

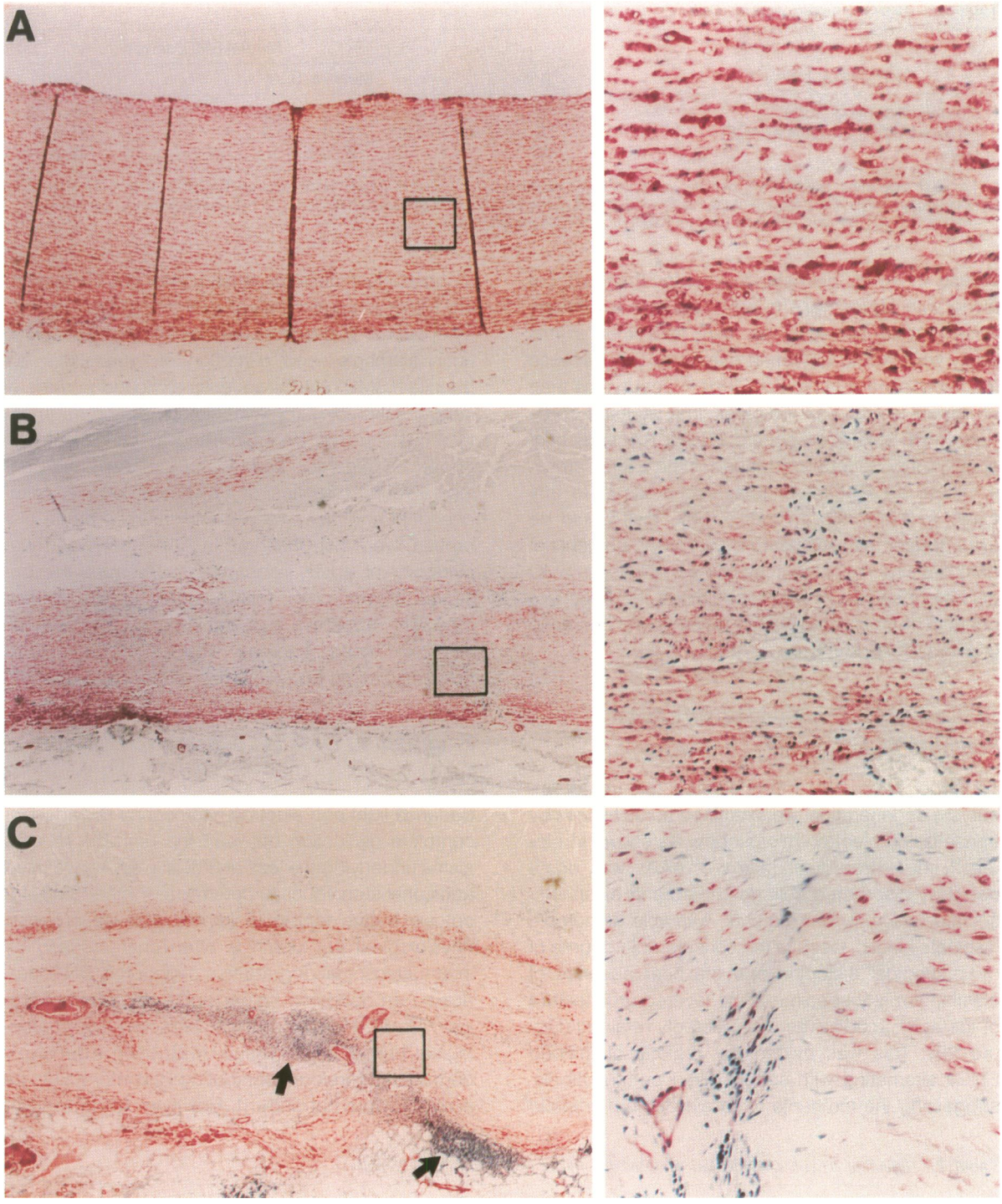

Figure 1. *Vascular SMCs in normal and diseased human abdominal aortic tissues. Formalin-fixed, paraffin-embedded sections were stained with anti-α-SMC actin monoclonal antibodies and alkaline phosphatase/anti-alkaline phosphatase (red reaction product). Transverse aortic sections are oriented with the lumen at the top.* A: *Normal aorta.* B: *AOD.* C: *AAA. The abundance of medial SMCs in normal aorta and AOD is contrasted with the decrease in medial SMCs density in AAA* (arrow). *Inflammatory infiltrates are present in AAA* (arrow). *Magnification, ×40* (left) *and ×100* (right).

ble 2). By estimating the extent to which SMC density might be expected to decrease in aneurysms of increasing diameter and varying wall thickness in the absence of actual cell loss, we also found that the reduction in medial SMC density in AAAs (from 200 SMCs/HPF to 50 SMCs/HPF) would require an in-

Table 1. *Medial Smooth Muscle Cell Density in Human Aortic Tissues*

Aortic tissue type	n	Medial SMC density	
		Mean ± SEM	Range
Normal abdominal aorta	5	199.5 ± 14.9	168–258
Atherosclerotic abdominal AOD	6	176.4 ± 13.9	140–235
AAA	10	50.9 ± 6.1*	16–80

For each specimen, medial SMC density was determined as the mean number of α-SMC actin-positive SMCs per ×400 HPF taken from five contiguous HPFs. Data represent the mean ± SEM and range of SMC density determinations for all tissue specimens in each group.
*$P < 0.001$; ANOVA and Newman-Student-Keuls multiple comparisons test.

crease in aortic diameter to greater than 5.5 cm along with an increase in wall thickness to at least twice normal. Because the AAAs examined in this study were 4.5 to 6.0 cm in diameter, and because medial thickness is typically less in aneurysms than in normal aorta, this indicates that at least some degree of SMC loss occurs during the course of aneurysm degeneration. Indeed, if the overall cross-sectional area of the media is decreased to any extent in AAAs, the amount of SMC loss would be even greater than estimated by our measurements of SMC density. The magnitude of the decrease in SMC density observed in AAA specimens therefore appears to be best explained by an authentic decrease in overall SMC number.

SMCs in AAA Display Ultrastructural Features of Apoptosis

To further assess the morphological features of aneurysm degeneration, we examined aortic tissues by transmission electron microscopy. As shown in Figure 2, the extracellular matrix of AAAs was dominated by disordered collagen fibers and elastin-associated microfibrillar material, with little detectable insoluble elastin. Despite the considerable degree of morphological distortion and the near absence of elastin, in many specimens the layered architecture of the aortic media was unexpectedly retained (Figure 2B). This was evident by regions of thinned SMCs alternating with spaces filled by microfibrils, suggesting the remnants of an elastic fiber scaffold stripped of its insoluble elastin. Intact SMCs remaining within aneurysms displayed loss of cell volume, cytoplasmic contracture, dissolution of cytoskeletal actin filaments, and distortion of intracellular organelles. SMC nuclei frequently exhibited peripheral condensation of chromatin, clumping, and fragmentation, which may be interpreted as consistent with apoptosis (Figure 2, C and D). In contrast, morphological characteristics of SMC necrosis, ie, mitochondrial and cell swelling and cell membrane disruption, were not observed in AAAs, nor was there evidence of acute inflammation. Because the observed ultrastructural changes are typically associated with apoptotic cell death rather than necrosis,[36–38] these findings suggested that SMC apoptosis might be a common occurrence in AAAs.

Fragmented DNA Is Localized to SMCs in AAAs

Because light and electron microscopy provided incomplete distinction between the possible mechanisms underlying the loss of SMCs in AAAs, we used additional techniques to determine whether SMC apoptosis might contribute to this disease process. Physiological cell death is often characterized by internucleosomal degradation of genomic DNA, producing an apoptotic ladder upon gel electrophoresis.[39,40] We found that DNA extracted from pathological human aortic tissues was often extensively degraded, therefore contributing little information for analysis (data not shown). However, using a histo-

Table 2. *Effect of Tissue Orientation on Measurements of Medial SMC Density*

	SMC density (SMCs/HPF)		
	Transverse	Longitudinal	
Normal aorta	245.6 ± 11.4	223.3 ± 9.8	9.1% reduction ($P = 0.04$)
Aortic aneurysm	43.5 ± 6.1	42.1 ± 2.9	3.2% reduction ($P = 0.42$)
	82.3% reduction ($P < 0.001$)	81.1% reduction ($P < 0.001$)	

Specimens of normal aorta and AAA (n = 3 each) were oriented transverse to the axis of blood flow, embedded, and sectioned. After morphometric measurement of medial SMC density in α-SMC actin-stained sections (SMCs/HPF), each specimen was re-embedded in an orientation longitudinal to the axis of blood flow and the morphometric analysis repeated. As compared by a one-tailed Student's *t*-test, orientation-dependent differences in medial SMC density were of minor significance compared with the magnitude of difference between the two tissue types.

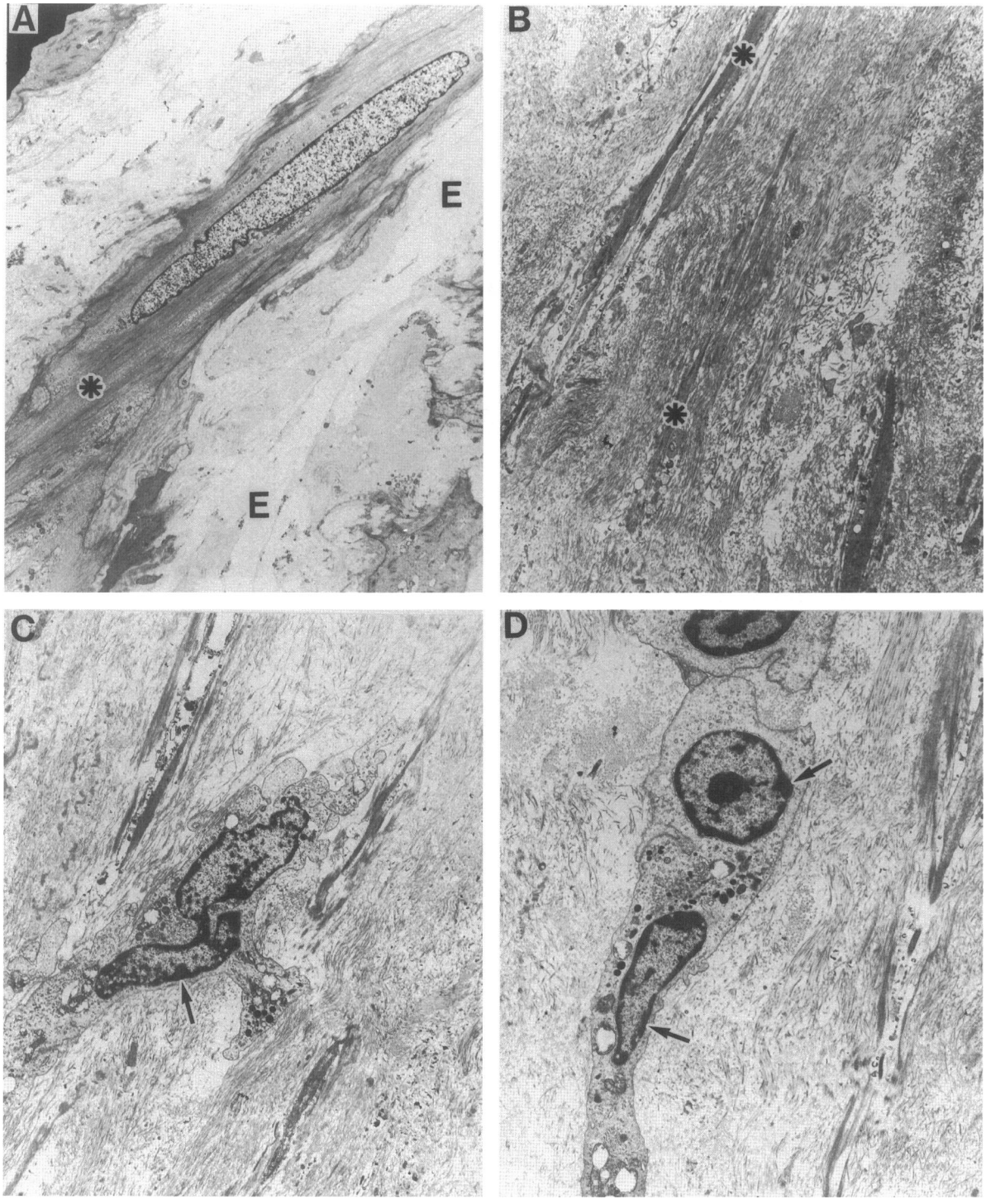

Figure 2. *Ultrastructural features of SMC apoptosis in AAA. Transmission electron micrographs demonstrate intact medial elastin fibers (E) in normal aorta* (A), *contrasted with their absence in AAA* (B *to* D). A: *SMC* (asterisk) *in normal aorta.* B *to* D: *SMCs in AAA display decreased cell volume, dissolution of the actin cytoskeleton, and membrane blebbing* (B *and* C) *as well as nuclear condensation, clumping and margination of chromatin, and fragmentation* (arrows, C *and* D). *Magnification, ×10,000.*

chemical method to detect apoptosis based on *in situ* 3′ end-labeling (ISEL) with terminal transferase,[41] we were able to effectively localize intact cells with fragmented DNA in aortic tissue sections. As illustrated in Figure 3, ISEL-positive cells were not detected in normal aorta and they were found only

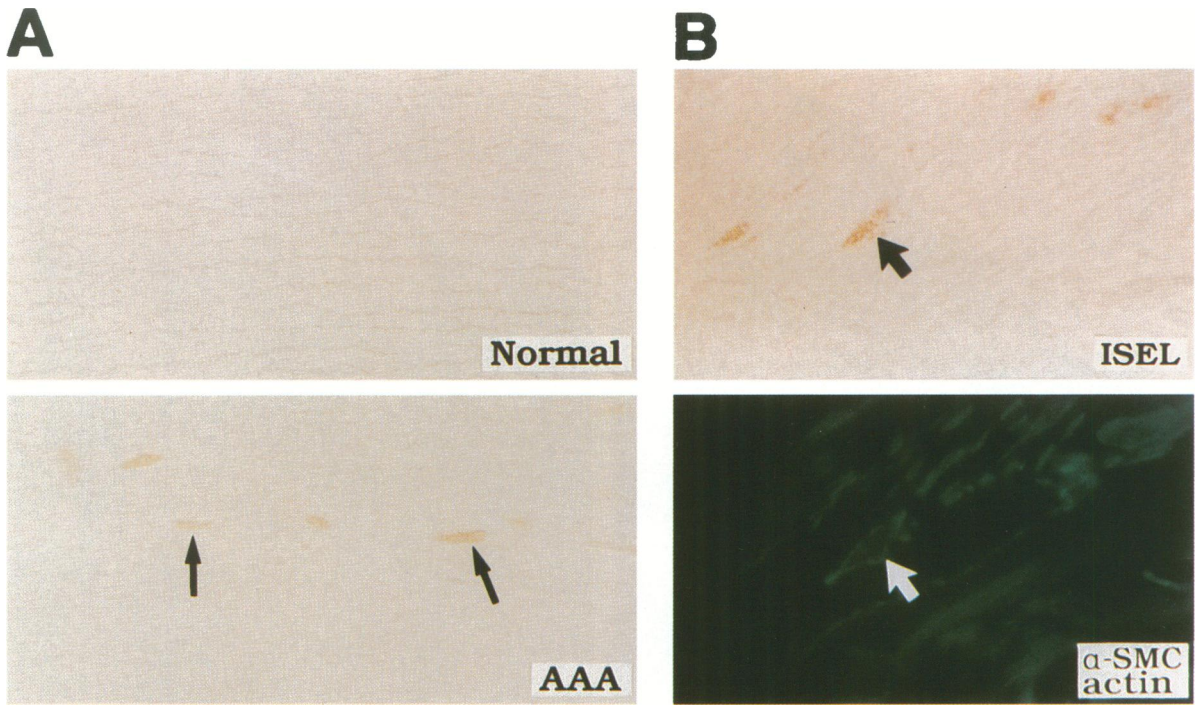

Figure 3. *ISEL of fragmented DNA localizes apoptotic SMCs in human aortic aneurysms.* **A:** *Formalin-fixed, paraffin-embedded sections of abdominal aorta were processed for ISEL and stained with immunoperoxidase (brown reaction product). ISEL-positive cells* (**arrows**) *are not observed in the media of normal aorta, but they are prevalent throughout the wall of AAAs. Magnification, ×200.* **B:** *Identification of apoptotic vascular SMCs in AAA. Formalin-fixed, paraffin-embedded sections of AAA were processed for ISEL and stained with immunoperoxidase (light field, brown reaction product). The same sections were incubated with FITC-conjugated antibodies against α-SMC actin (dark field, green reaction product under epifluorescence). Most ISEL-positive cells are identified as SMCs* (**arrows**). *Magnification, ×400.*

within the intimal atherosclerotic plaque in AOD. In contrast, a large number of aortic wall cells were ISEL positive in AAAs. These cells were located both within the intima and throughout the degenerative media, where a mean density of 8.6 ± 2.4 ISEL-positive cells per HPF was observed. By comparison with DNAse-treated positive control sections in which all cells present were stained for fragmented DNA, ISEL-staining nuclei represented up to 30% of the medial cells in AAAs. Using immunohistochemical co-localization of apoptotic cells with those expressing α-SMC actin, a large proportion of the ISEL-positive cells within the the media of AAAs were identified as vascular SMCs (Figure 3).

Production and Accumulation of p53 Protein

Because vascular SMCs exposed to serum-free conditions undergo apoptosis by p53-dependent and -independent mechanisms,[42] we used the production of p53 as a potential molecular marker of apoptosis in aortic tissues. As shown in Figure 4, p53 protein was detectable in extracts of all three types of human aortic tissue. The relative amount of immunoreactive p53 determined by densitometry was 3.9-fold greater in AAA than normal aorta ($P < 0.05$), whereas the amount of p53 in AOD was not significantly increased over that produced by normal aorta. By immunohistochemistry using paraffin-embedded tissue sections, p53 protein was undetectable within the media of normal aorta or AOD tissues (Figure 5). In nearly all AAA specimens examined, immunoreactive p53 protein was readily detectable in cells distributed throughout the degenerative aortic wall. p53 was localized to the cytoplasm and nuclei of SMCs, as verified by co-localization with antibodies to α-SMC actin, and to lymphocytes within the aneurysm wall (Figure 5). These studies revealed that approximately 20 to 30% of the α-actin-positive cells also accumulated p53 protein.

Expression of p53 mRNA

The steady-state expression of p53 mRNA was assessed in human aortic tissues using RT-PCR. As shown in Figure 6, the expected amplification product derived from p53 mRNA (470 bp) was detected in all aortic tissue specimens. Based on measurements of relative density in samples normalized to the same amount of total RNA template, the amount

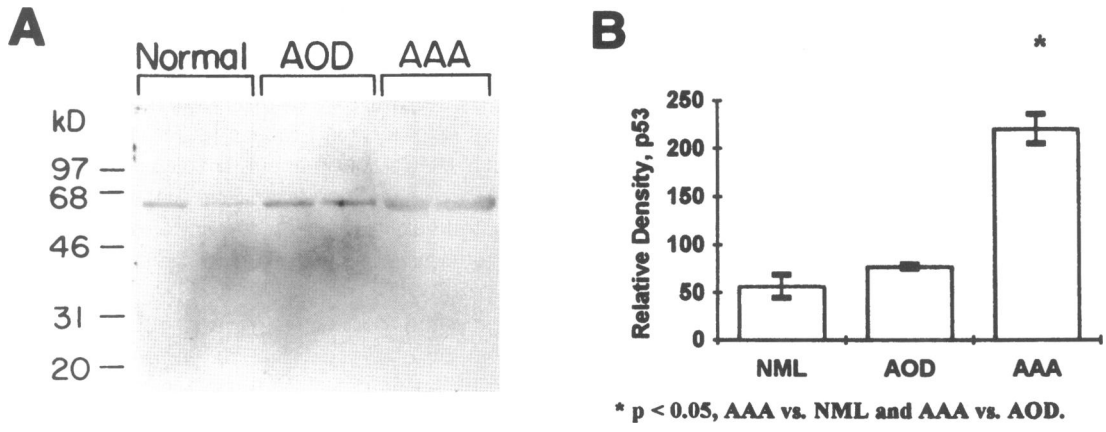

Figure 4. *Immunoblot analysis of p53 in human aortic tissues.* A: *Aortic tissue extracts were normalized to total protein, resolved by SDS-PAGE, and then incubated with monoclonal antibodies recognizing human p53. Immune complexes were detected by enhanced chemiluminescence. Representative samples of normal aorta, AOD, and AAAs.* B: *Mean (± SE) relative density of immunoreactive p53 protein produced in normal and diseased human aortic tissues (n = 6 each).*

of p53 expressed in AAA tissues was approximately two- to threefold higher than that in normal aorta.

Discussion

In contrast to occlusive atherosclerosis, the histopathological changes in human aortic aneurysms predominantly affect the elastic media.[3–5] Whereas this tissue layer is normally dominated by elastic fibers and vascular SMCs, previous studies have focused on the decrease in aortic elastin concentration in AAAs.[7,9] Thus, one of the contributions of the present study is the demonstration that medial degeneration in human AAA is also accompanied by a quantitative decrease in SMC density compared with normal and atherosclerotic aortic tissues. Because the magnitude of decreased SMC density was far greater than that expected if the total cell number per cross-sectional area of the vessel had remained constant, the decline in SMC density in AAA appears to reflect an authentic and significant decrease in total SMC number. The loss of this cell population, including its architectural contributions to the elastic lamellae and its matrix remodeling functions within the aortic wall, likely bears a significant influence on the structural and functional deterioration of the aneurysmal aorta. Additional knowledge of the cellular mechanisms underlying medial SMC death is therefore important to understanding the pathological events that occur during the genesis and evolution of AAA.

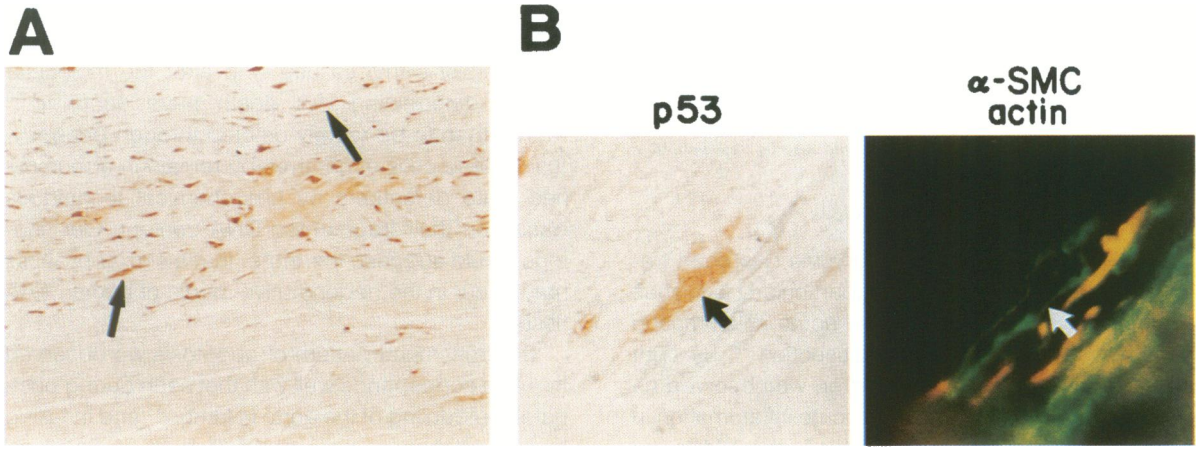

Figure 5. *Immunolocalization of p53 protein in AAA.* A: *Formalin-fixed sections of AAA were incubated with anti-p53 antibodies and stained with immunoperoxidase (brown reaction product). Positive-staining cells are indicated by* arrows. *Magnification, ×200.* B: *Formalin-fixed sections of AAA were simultaneously stained with antibodies against p53 and α-SMC actin. p53 was detected by immunoperoxidase and visualized under light-field microscopy as a brown reaction product* (arrow). *In the same sections, α-SMC actin was detected with FITC-conjugated antibodies visualized under dark-field microscopy and epifluorescence as a green signal. The yellow autofluorescence of residual elastin fibers is also apparent. Magnification, ×400.*

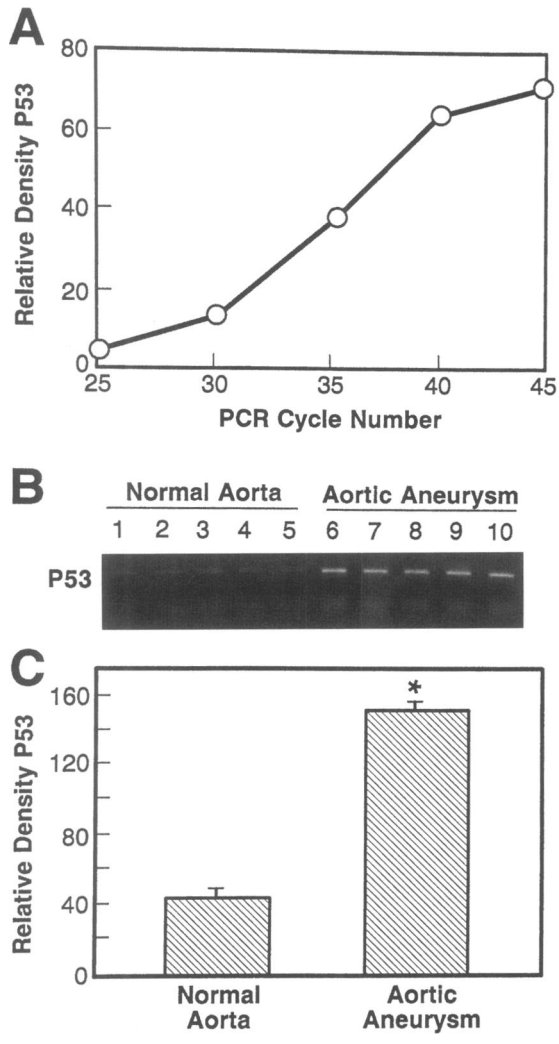

Figure 6. *RT-PCR analysis of p53 expression in human aortic tissues. Total RNA extracted from human aortic tissues was subjected to RT followed by PCR using primers specific for human p53 exons 7 to 9.* **A:** *Linear amplification of p53 transcripts (470-bp expected amplification product) was achieved at 35 cycles of PCR.* **B:** *Comparison of p53 amplification products detected in samples derived from normal aorta* (**lanes 1** *to* **5**) *and AAAs* (**lanes 6** *to* **10**). *Each sample was normalized to the same amount of total RNA as template for the RT-PCR assay.* **C:** *Mean (± SE) relative density units of p53 amplification products obtained from specimens of normal aorta and AAA (n = 5 each; *P < 0.001, Student's* t*-test).*

Medial degeneration in aneurysms has been traditionally attributed to necrosis, particularly in tissues exhibiting cystic changes or the acute inflammation often associated with aortic dissection.[43] By light and electron microscopy, however, we observed no evidence of cellular necrosis or acute inflammation in established infrarenal AAA. Indeed, the SMCs remaining within the degenerative media of aneurysms were typically intact, lying between layers of degraded elastic lamellae. Furthermore, at the ultrastructural level these SMCs frequently displayed cytoplasmic and nuclear features characteristic of apoptosis, or physiological cell death. Recent studies indicating that apoptosis plays a significant role in vascular wall biology prompted us to further investigate whether this process might contribute to the loss of SMCs during aneurysm degeneration.[42,44–55]

Although we found that DNA electrophoresis contributed little to the assessment of apoptosis in the degenerative tissues studied here, histochemical staining by ISEL demonstrated that intact cells with fragmented nuclear DNA are frequently present within the atherosclerotic intima of AOD specimens and in the media of AAAs. Although ISEL alone is insufficient proof that cells have undergone programmed cell death,[56,57] together with other data these findings provide evidence suggesting that apoptosis contributes to cell death in AAAs. Furthermore, our observations of apoptotic cells within atherosclerotic plaques are similar to those presented by Bennett and associates,[48] Geng and Libby,[49] Han et al,[50] and Isner and colleagues.[53] In contrast to previous studies, however, we found a broader distribution of apoptotic cells in AAAs. Thus, ISEL-positive cells in aneurysms were apparent both within the atherosclerotic intima and, more importantly, throughout the degenerative media. When compared with tissue sections treated with DNAse before ISEL staining, the ISEL-positive cells in the media of aneurysms represented up to 30% of the cells remaining in the tissue. This appears to be a surprisingly high rate for a process reflective of ongoing cell death, yet others have observed a similar proportion of apoptotic cells in human atherosclerotic plaques.[49,50,53] Indeed, Geng and Libby have suggested, that under certain circumstances, such as those existing within the degenerative local environment of atherosclerotic plaques, apoptotic cells may persist within tissues long after the molecular events determining cell death have occurred.[49] Rather than to be rapidly deleted through phagocytosis, these cells might thereby remain in a mummified state detectable by histochemical techniques based on their fragmented DNA content. Our findings would suggest, therefore, that a similar process may occur in the degenerative media of human AAA tissues.

The transmural pattern of apoptosis we observed in AAAs corresponds well with that seen during postnatal remodeling of the aorta in lambs[47] and in failing human saphenous vein grafts,[44] both of which involve degeneration of the elastic media. We identified the apoptotic cells in human AAAs by double immunohistochemical staining with ISEL and antibodies recognizing α-SMC actin. The immunofluo-

rescent staining pattern for α-actin was atypical for vascular SMCs, exhibiting localization of actin filaments in a peripheral pattern at the margins of the cell membrane. Because a similar pattern was observed in AAA tissues stained for α-SMC actin in the absence of ISEL, the lack of diffuse cytoplasmic staining was not a technical artifact of the double-staining methodology. Indeed, although vascular SMC expression of α-actin may vary depending on the growth state and differentiation of the cell population,[58,59] the altered histochemical appearance of medial SMCs in AAAs likely also reflects a degree of cellular injury resulting in disassembly and redistribution of cytoskeletal actin filaments. Interpretation of these findings is necessarily limited by the chronic degenerative nature of human AAA tissues available for study. Nonetheless, taken together with our observations of decreased SMC density and the ultrastructural appearance of SMCs, these results support the hypothesis that medial SMC apoptosis contributes to the loss of this cell population during the evolution of aneurysm disease.

Cellular production of p53 has been implicated in the induction of cell cycle arrest and apoptosis in many cell types, including vascular SMCs.[42,60–64] For example, vascular SMCs in culture undergo apoptosis during serum deprivation or in response to various cytokines, through both p53-dependent and -independent mechanisms.[42] To determine whether SMC death in human AAAs might be associated with similar processes, we therefore examined the production of p53 as a potential molecular marker of apoptosis. The amount of p53 protein extracted from AAAs was nearly fourfold greater than that from normal and atherosclerotic tissues. Using RT-PCR assays normalized to total RNA, steady-state expression of p53 mRNA also appeared higher in AAA than in normal aorta, although as used here this method cannot accurately demonstrate quantitative differences. Perhaps more importantly, immunoreactive p53 was readily detected in the degenerative media of most AAA tissues, but it was not observed in the media of normal or atherosclerotic aortic specimens, and cellular accumulation of p53 in AAAs was predominantly localized to lymphocytes and spindle-shaped cells co-expressing α-SMC actin. These findings provide additional evidence for the development of physiological SMC death in aneurysms and they suggest that this process may occur, at least in part, through mechanisms involving increased SMC expression of p53. Although the ability to detect p53 by immunohistochemistry is often taken as evidence of p53 mutations, such as those seen in various malignancies,[65,66] we predict that SMC overproduction of p53 in AAAs reflects normal physiological processes occurring in response to cellular injury and/or DNA damage[67] rather than neoplastic transformation.

Given the evidence that apoptosis contributes to the decreased vascular SMC density in human AAA, a number of different mechanisms may be proposed by which to explain the induction of physiological SMC death in the media of aneurysm tissues. Through close spatial relationship with the diseased intima, for example, medial SMCs might be subject to high local concentrations of oxidants produced in atherosclerotic tissues, such as nitric oxide, oxygen free radicals, and oxidized low-density lipoproteins. Indeed, recent studies confirm that both nitric oxide and highly oxygenated forms of low-density lipoprotein induce vascular SMC apoptosis *in vitro*.[54,68] Diffusion of these compounds into the aortic media might thereby result in cytotoxic effects on medial SMCs with the concomitant induction of physiological cell death.

Ischemic injury to medial SMCs might also induce a process of apoptosis rather than overt tissue necrosis. In contrast to the thoracic aorta where vasa vasorum are prevalent, the media of the infrarenal human aorta is normally devoid of an intramural blood supply.[69] SMCs in the infrarenal aorta are thereby entirely dependent on nutrient diffusion from the lumen. The inherent susceptibility of the media to ischemic injury also corresponds with clinical patterns in which the vast majority of AAAs arise in the infrarenal aorta.[1–3] Nutrient diffusion from the aortic lumen into the medial SMC layers may also be significantly limited by the development of intimal thickening and atherosclerotic plaques in this region, resulting in chronic medial ischemia. Like serum-deprivation *in vitro*,[42,45,46,48,54] this environment might be particularly conducive to the induction of SMC apoptosis.

Chronic inflammation is commonly observed in AAA, characterized by mononuclear cell infiltration of the outer aortic wall.[25–27] Inflammatory cytokines known to induce SMC apoptosis *in vitro*, such as interleukin-1β, tumor necrosis factor-α, and interferon-γ,[45,55] are also produced in atherosclerotic lesions and in aneurysm tissues.[70–74] High local concentrations of these mediators, such as that expected near the inflammatory cell infiltrates prevalent within the outer aortic wall of aneurysms, might have a deleterious influence on medial SMCs. In addition, it is possible that SMC apoptosis is induced by direct interaction with inflammatory cells, particularly T lymphocytes and macrophages. Although the factors eliciting an immune response in AAA tissues

remain poorly understood, the finding that SMCs in AAAs express class II histocompatibility antigens[75] supports the possibility that medial SMCs may also be the target of cell-mediated immune responses resulting in apoptotic cell death. Indeed, recent observations linking inflammatory AAAs with human cytomegalovirus infection,[76] and the association of cytomegalovirus with p53 expression in human coronary restenosis lesions,[77] may be particularly relevant in this respect. Additional investigation will clearly be needed to distinguish which of these potential mechanisms might be most important in human aneurysm disease.

Finally, it is important to revisit the fact that vascular SMCs normally exist within an extracellular matrix rich in elastin and that they typically lie adjacent to mature elastic fibers. During early atherogenesis and intimal thickening after arterial injury, SMCs migrate into the intima where they proliferate and deposit large amounts of extracellular matrix protein. The development of SMC apoptosis in atherosclerosis is largely associated with advanced plaques undergoing degeneration, where little if any elastin exists. Like the environment of the atherosclerotic intimal plaque, the development of SMC apoptosis in AAAs also occurs in a tissue environment depleted of elastin. We speculate, therefore, that the association of medial SMCs with elastin or other components of the intact elastic fiber may provide a measure of resistance to apoptotic stimuli that, in the absence of an elastin-rich extracellular matrix, might otherwise induce physiological SMC death.[78,79] Studies using cultured SMCs and elastase-induced animal models of AAA[80] will be particularly useful toward testing these possible mechanisms.

Acknowledgments

We gratefully acknowledge the assistance of Drs. Gregorio A. Sicard, Charles B. Anderson, Brent T. Allen, Todd K. Howard, Jeffrey A. Lowell, Surendra Shenoy, and Steven M. Strasberg in procuring human tissue specimens for this study. We thank Drs. William C. Parks, Robert P. Mecham, and Timothy J. Ley for their valuable suggestions.

References

1. Ernst CB: Abdominal aortic aneurysm. N Engl J Med 1993, 328:1167–1172
2. Mitchell MB, Rutherford RB, Krupski WC: Infrarenal aortic aneurysms. Vascular Surgery, ed 4. Edited by RB Rutherford. Philadelphia, WB Saunders, 1995, pp 1032–1060
3. Thompson RW: Basic science of abdominal aortic aneurysms: emerging therapeutic strategies for an unresolved clinical problem. Curr Opin Cardiol 1996, 11: 504–518
4. Tilson MD: Histochemistry of aortic elastin in nonspecific abdominal aortic aneurysm disease. Arch Surg 1988, 123:503–505
5. Zatina MA, Zarins CK, Gewertz BL, Glagov S: Role of medial lamellar architecture in the pathogenesis of aortic aneurysms. J Vasc Surg 1984, 1:442–448
6. White JV, Haas K, Phillips S, Comerota AJ: Adventitial elastolysis is a primary event in aneurysm formation. J Vasc Surg 1993, 17:371–381
7. Sumner DS, Hokanson DE, Strandness DE Jr: Stress-strain characteristics and collagen-elastin content of abdominal aortic aneurysms. Surg Gynecol Obstet 1970, 130:459–466
8. Dobrin PB, Baker WH, Gley WC: Elastolytic and collagenolytic studies of arteries: implications for the mechanical properties of aneurysms. Arch Surg 1984, 119:405–409
9. Campa JS, Greenhalgh RM, Powell JT: Elastin degradation in abdominal aortic aneurysms. Atherosclerosis 1987, 65:13–21
10. Rizzo RJ, McCarthy WJ, Dixit SN, Lilly MP, Shively VP, Flinn WR, Yao JST: Collagen types and matrix protein content in human abdominal aortic aneurysms. J Vasc Surg 1989, 10:365–373
11. Dobrin PB, Mrkvicka R: Failure of elastin or collagen as possible critical connective tissue alterations underlying aneurysmal dilatation. Cardiovasc Surg 1994, 2:484–488
12. Kontusaari S, Tromp G, Kuivaniemi H, Romanic AM, Prockop DJ: A mutation in the gene for type III procollagen (COL3A1) in a family with aortic aneurysms. J Clin Invest 1990, 86:1465–1473
13. Superti-Furga A, Steinmann B, Ramirez F, Byers PH: Molecular defects of type III procollagen in Ehlers-Danlos syndrome type IV. Hum Genet 1989, 82:104–108
14. McKusick VA: The defect in Marfan syndrome. Nature 1991, 352:279–281
15. Tromp G, Wu Y, Prockop DJ, Madhatheri SL, Kleinert C, Earley JJ, Zhuang J, Norrgard O, Darling RC, Abbott WM, Cole CW, Jaakola P, Ryynanen M, Pearce WH, Yao JST, Majamaa K, Smullens SN, Gatalica Z, Ferrell RE, Jimenez SA, Jackson CE, Michels VV, Kaye M, Kuivaniemi H: Sequencing of cDNA from 50 unrelated patients reveals that mutations in the triple-helical domain of type III procollagen are an infrequent cause of aortic aneurysms. J Clin Invest 1993, 91:2539–2545
16. Lefevre M, Rucker RB: Aorta elastin turnover in normal and hypercholesterolemic Japanese quail. Biochim Biophys Acta 1980, 630:519–529
17. Shapiro SD, Endocott SK, Province MA, Pearce JA, Campbell EJ: Marked longevity of human lung paren-

chymal elastic fibers deduced from prevalence of D-aspartate and nuclear weapons-related radiocarbon. J Clin Invest 1991, 87:1828–1834
18. Cohen JR, Mandell C, Chang JB, Wise L: Elastin metabolism of the infrarenal aorta. J Vasc Surg 1988, 7:210–214
19. Powell JT, Greenhalgh RM: Cellular, enzymatic, and genetic factors in the pathogenesis of abdominal aortic aneurysms. J Vasc Surg 1989, 9:297–304
20. Herron GS, Unemori E, Wong M, Rapp JH, Hibbs MH, Stoney RJ: Connective tissue proteinases and inhibitors in abdominal aortic aneurysms: involvement of the vasa vasorum in the pathogenesis of aortic aneurysms. Arterioscler Thromb 1991, 1667–1677
21. Baxter BT, McGee GS, Shively VP, Drummond IAS, Dixit SN, Yamauchi M, Pearce WH: Elastin content, cross-links, and mRNA in normal and aneurysmal human aorta. J Vasc Surg 1992, 16:192–200
22. Gandhi RH, Irizarry E, Cantor JO, Keller S, Nachman GB, Halpern VJ, Newman KM, Tilson MD: Analysis of elastin cross-linking and the connective tissue matrix of abdominal aortic aneurysms. Surgery 1994, 115:617–620
23. Newman KM, Malon AM, Shin RD, Scholes JV, Ramey WG, Tilson MD: Matrix metalloproteinases in abdominal aortic aneurysm: characterization, purification, and their possible sources. Conn Tiss Res 1994, 30:265–276
24. Thompson RW, Holmes DR, Mertens RA, Liao S, Botney MD, Mecham RP, Welgus HG, Parks WC: Production and localization of 92-kilodalton gelatinase in abdominal aortic aneurysms: an elastolytic metalloproteinase expressed by aneurysm-infiltrating macrophages. J Clin Invest 1995, 96:318–326
25. Brophy CM, Reilly JM, Walker Smith GJ, Tilson MD: The role of inflammation in nonspecific aneurysm disease. Ann Vasc Surg 1991, 5:229–233
26. Freestone T, Turner RJ, Coady A, Higman DJ, Greenhalgh RM, Powell JT: Inflammation and matrix metalloproteinases in the enlarging abdominal aortic aneurysm. Arterioscler Thromb Vasc Biol 1995, 15:1145–1151
27. Koch AE, Haines GK, Rizzo RJ, Radosevich JA, Pope RM, Robinson PG, Pearce WH: Human abdominal aortic aneurysms: immunophenotypic analysis suggesting an immune-mediated response. Am J Pathol 1990, 137:1199–1213
28. Holmes DR, Liao S, Parks WC, Thompson RW: Medial neovascularization in abdominal aortic aneurysms: a histopathologic marker of aneurysmal degeneration with pathophysiologic implications. J Vasc Surg 1995, 21:761–771
29. Rhodin JAG: Architecture of the vessel wall. Handbook of Physiology, Section 2. Edited by DF Bohr, AP Somylo, HV Sparks Jr. Bethesda, MD, American Physiological Society, 1980
30. Ross R, Klebanoff SJ: The smooth muscle cell. I. *In vivo* synthesis of connective tissue proteins. J Cell Biol 1971, 50:159–171
31. Rasmussen LM, Wolf YG, Ruoslahti E: Vascular smooth muscle cells from injured rat aortas display elevated matrix production associated with transforming growth factor-β activity. Am J Pathol 1995, 147:1041–1048
32. Cohen JR, Sarfati I, Danna D, Wise L: Smooth muscle cell elastase, atherosclerosis, and abdominal aortic aneurysms. Ann Surg 1992, 216:327–332
33. Okada Y, Katsuda S, Okada Y, Nakanishi I: An elastinolytic enzyme detected in the culture medium of human arterial smooth muscle cells. Cell Biol Int 1993, 17:863–869
34. Bendeck MP, Zempo N, Clowes AW, Galardy RE, Reidy MA: Smooth muscle cell migration and matrix metalloproteinase expression after arterial injury in the rat. Circ Res 1994, 75:539–545
35. He CM, Roach MR: The composition and mechanical properties of abdominal aortic aneurysms. J Vasc Surg 1994, 20:6–13
36. Kerr JFR, Wyllie AH, Currie AR: Apoptosis: a basic biologic phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 1972, 26:239–257
37. Majno G, Joris I: Apoptosis, oncosis, and necrosis: an overview of cell death. Am J Pathol 1995, 146:3–15
38. Hockenbery D: Defining apoptosis. Am J Pathol 1995, 146:16–19
39. Arrends MJ, Morris RG, Wyllie AH: Apoptosis: the role of the endonuclease. Am J Pathol 1990, 136:593–608
40. Kroemer G, Petit P, Zamzami N, Vayssière J-L, Mignotte B: The biochemistry of programmed cell death. FASEB J 1995, 9:1277–1287
41. Gavrieli Y, Sherman Y, Ben-Sasson SA: Identification of programmed cell death *in situ via* specific labeling of nuclear DNA fragmentation. J Cell Biol 1992, 119:493–501
42. Bennett MR, Evan GI, Schwartz SM: Apoptosis of rat vascular smooth muscle cells is regulated by p53-dependent and -independent pathways. Circ Res 1995, 77:266–273
43. Fox B: Cystic medial necrosis. The Cause and Management of Aneurysms. Edited by RM Greenhalgh, JA Mannick, JT Powell. London, WB Saunders, 1990, pp 79–87
44. Kockx MM, Cambier BA, Bortier HE, De Meyer GR, Declercq SC, Van Cauwelaert PA, Bultinck J: Foam cell replication and smooth muscle cell apoptosis in human saphenous vein grafts. Histopathology 1994, 25:365–371
45. Bennett MR, Evan GI, Newby AC: Deregulated expression of the c-*myc* oncogene abolishes inhibition of proliferation of rat vascular smooth muscle cells by serum reduction, interferon-γ, heparin, and cyclic nucleotide analogues and induces apoptosis. Circ Res 1994, 74: 525–536
46. Leszczynski D, Zhao Y, Luokkamäki M, Foegh ML: Apoptosis of vascular smooth muscle cells: protein kinase C and oncoprotein Bcl-2 are involved in regu-

lation of apoptosis in nontransformed rat vascular smooth muscle cells. Am J Pathol 1994, 145:1265–1270
47. Cho A, Courtman DW, Langille BL: Apoptosis (programmed cell death) in arteries of the neonatal lamb. Circ Res 1995, 76:168–175
48. Bennett MR, Evan GI, Schwartz SM: Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic plaques. J Clin Invest 1995, 95:2266–2274
49. Geng Y-J, Libby P: Evidence for apoptosis in advanced human atheroma: co-localization with interleukin-1β-converting enzyme. Am J Pathol 1995, 147:251–266
50. Han DKM, Haudenschild CC, Hong MK, Tinkle BT, Leon MB, Liau G: Evidence for apoptosis in human atherogenesis and in a rat vascular injury model. Am J Pathol 1995, 147:267–277
51. Schwartz SM, Bennett MR: Death by any other name. Am J Pathol 1995, 147:229–234
52. Bouchaton-Piallat ML, Gabbiani F, Redard M, Desmouliere A, Gabbiani G: Apoptosis participates in cellularity regulation during rat aortic intimal thickening. Am J Pathol 1995, 146:1059–1064
53. Isner JM, Kearney M, Bortman S, Passeri J: Apoptosis in human atherosclerosis and restenosis. Circulation 1995, 91:2703–2711
54. Pollman MJ, Yamada T, Horiuchi M, Gibbons GH: Vasoactive substances regulate vascular smooth muscle cell apoptosis: countervailing influences of nitric oxide and angiotensin II. Circ Res 1996, 79:748–756
55. Geng Y-J, Wu Q, Muszynski M, Hansson GK, Libby P: Apoptosis of vascular smooth muscle cells induced by *in vitro* stimulation with interferon-γ, tumor necrosis factor-α, and interleukin-1β. Arterioscler Thromb Vasc Biol 1996, 16:19–27
56. Grasl-Kraupp B, Ruttkay-Nedecky B, Koudelka H, Bukowska K, Bursch W, Schulte-Hermann R: *In situ* detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. Hepatology 1995, 21: 1465–1468
57. Mundle SD, Raza A: The two *in situ* techniques do not differentiate between apoptosis and necrosis but rather reveal distinct patterns of DNA fragmentation in apoptosis. Lab Invest 1995, 72:611–612
58. Owens GK, Loeb A, Gordon D, Thompson MM: Expression of smooth muscle-specific α-isoactin in cultured vascular smooth muscle cells: relationship between growth and cytodifferentiation. J Cell Biol 1986, 102: 343–352
59. Holifield B, Helgason T, Jemelka S, Taylor A, Navran S, Allen J, Seidel C: Differentiated vascular myocytes. Are they involved in neointimal formation? J Clin Invest 1996, 97:814–825
60. Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML, Wyllie AH: Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature 1993, 362:849–852
61. Wang Y, Szekely L, Okan I, Klein G, Wilman KG: Wild-type p53-triggered apoptosis is inhibited by bcl-2 in a v-myc-induced T-cell lymphoma line. Oncogene 1993, 8:3427–3431
62. Dulic V, Kaufmann WK, Wilson SJ, Tlsty TD, Lees E, Harper JW, Elledge SJ, Reed SI: p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. Cell 1994, 76:1013–1023
63. Symonds H, Krall L, Remington L, Saenz-Robles M, Lowe S, Jacks T, Van Dyke T: p53 dependent apoptosis suppresses tumor growth and progression *in vivo*. Cell 1994, 78:703–711
64. El-Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackman J, Pietenpol JA, Burrell M, Hill DE, Wang Y, Vogelstein B: WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. Cancer Res 1994, 54:1169–1174
65. Hollstein M, Sidransky D, Vogelstein B, Harris CC: p53 mutations in human cancers. Science 1991, 253:49–53
66. Vogelstein B, Kinzler KW: p53 function and dysfunction. Cell 1992, 70:523–526
67. Zhan Q, Carrier F, Fornance AJ: Induction of cellular p53 activity by DNA-damaging agents and growth arrest. Mol Cell Biol 1993, 13:4242–4250
68. Björkerud B, Björkerud S: Contrary effects of lightly and strongly oxidized LDL with potent promotion of growth *versus* apoptosis on arterial smooth muscle cells, macrophages, and fibroblasts. Arterioscler Thromb Vasc Biol 1996, 16:416–424
69. Wolinsky H, Glagov S: Nature of species differences in the medial distribution of aortic vasa vasorum in mammals. Circ Res 1967, 20:409–421
70. Pearce WH, Sweiss I, Yao JST, McCarthy WJ, Koch AE: Interleukin-1β and tumor necrosis factor-α release in normal and diseased human infrarenal aorta. J Vasc Surg 1992, 16:784–789
71. Koch AE, Kunkel SL, Pearce WH, Shah MR, Parikh D, Evanoff HL, Haines GK, Burdick MD, Strieter RM: Enhanced production of the chemotactic cytokines interleukin-8 and monocyte chemoattractant protein-1 in human abdominal aortic aneurysms. Am J Pathol 1993, 142:1423–1431
72. Newman KM, Jean-Claude J, Li H, Ramey WG, Tilson MD: Cytokines that activate proteolysis are increased in abdominal aortic aneurysms. Circulation 1994, 90: 224–227
73. Szekanecz Z, Shah MR, Pearce WH, Koch AE: Human atherosclerotic abdominal aortic aneurysms produce interleukin (IL)-6 and interferon-γ but not IL-2 and IL-4: the possible role for IL-6 and interferon-γ in vascular inflammation. Agents Actions 1994, 42:159–162
74. Barath P, Fishbein MC, Cao J, Berenson J, Helfant RH, Forrester JS: Detection and localization of tumor necrosis factor in human atheroma. Am J Cardiol 1990, 65: 297–302

75. Kosierkiewicz TA, Capella JF, Yin NX, Tilson MD: Expression of the major histocompatibility complex (MHC) class II (DR) antigen by smooth muscle cells of the media of abdominal aortic aneurysms. Surg Forum 1995, 46:365–367
76. Tanaka S, Komori K, Okadome K, Sugimachi K, Mori R: Detection of active cytomegalovirus infection in inflammatory aortic aneurysms with RNA polymerase chain reaction. J Vasc Surg 1994, 20:235–243
77. Speir E, Modali R, Huang ES, Leon MB, Shawl F, Finkel T, Epstein SE: Potential role of human cytomegalovirus and p53 interaction in coronary restenosis. Science 1994, 265:391–394
78. Meredith JE Jr, Fazeli B, Schwartz MA: The extracellular matrix as a cell survival factor. Mol Biol Cell 1993, 4:953–961
79. Juliano RL, Haskill S: Signal transduction from the extracellular matrix. J Cell Biol 1993, 120:577–585
80. Anidjar S, Salzmann JL, Gentric D, Lagneau P, Camilleri JP, Michel JB: Elastase induced experimental aneurysms in rats. Circulation 1990, 82:973–981