Vascular smooth muscle cell apoptosis in aneurysmal, occlusive, and normal human aortas

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Purpose: Apoptosis is a physiologic mechanism of cell death that regulates mass and architecture in many tissues. Apoptosis has been described as a feature in human vascular atherosclerosis and large vessel structural integrity. We examined the extent of vascular smooth muscle cell (VSMC) apoptosis in aneurysmal, occlusive, and normal human aortic tissue.

Methods: Tissue samples of aneurysmal, occlusive, and normal human infrarenal aorta were evaluated. DNA fragmentation detection methods, immunohistochemistry, and DNA electrophoresis determined VSMC density, VSMC apoptosis, and apoptosis markers. Apoptotic cells and VSMC nuclei were counted with the use of computer-generated image analysis. Aortic subtypes were compared statistically by analysis of variance.

Results: Seventeen aneurysmal, ten occlusive, and five normal human aortas were evaluated. By α_1-actin immunostaining, VSMC density was least in aneurysmal aortas (271.8 ± 13.5 cells / high-power field [HPF]) compared with occlusive aorta (278.2 ± 39.4 cells / HPF) and normal aortas (291.0 ± 25.4 cells / HPF; *P* = not significant). Presence of apoptotic VSMCs was demonstrated by terminal deoxynucleotidyl transferase fragment end labeling and propidium iodide nuclear staining. VSMC apoptosis was greatest within aneurysmal aortas with 11.7 ± 1.5 cells / HPF compared with occlusive aortas with 3.3 ± 0.8 cells / HPF ($P < .05$) and normal aortas with 3.75 ± 4.6 cells / HPF ($P < .05$). Significant differences in apoptosis markers, p53 or bcl-2, could not be demonstrated by immunohistochemistry or DNA electrophoresis in aortic subtypes.

Conclusion: Apoptosis of VSMCs is increased and VSMC density is decreased within the medial layer of aneurysmal aortic tissue. Structural degeneration of aortic tissue at the cellular level contributes to aneurysmal formation. (J Vasc Surg 2000;31:567-576.)

Apoptosis is the enigmatic, physiologic mechanism in which specific cells are programmed for death to regulate tissue mass and architecture. Kerr et al,[1] in 1972, introduced the word *apoptosis* (Greek [origin] meaning "falling off") into the scientific literature as a novel concept where cell death occurred in a suicidal manner. Reported differences in apoptotic cells, as opposed to necrotic cells included cell membrane blebbing, clumping of nuclear chromatin, and nuclear fragmentation. Alterations in the rate of programmed cell death have been thought to contribute to pathologic processes in a variety of tissues. Disease processes that involve malignant transformation, neurologic disorders, heart disease, and immune deficiency syndrome have been attributed to both accelerated and decreased rates of apoptosis.[2-7] Recent reports have linked apoptosis in human vascular atherosclerosis and large vessel structural integrity.[8-13] To evaluate mechanisms in large vessel pathologic conditions, we examined the extent of vascular smooth muscle cell (VSMC) apoptosis in aneurysmal, occlusive, and normal human aortic tissue. Our objective was to determine whether differ-

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ences in VSMC apoptosis and apoptosis markers varied between aortic disease subtypes.

MATERIAL AND METHODS

Tissue preparation. Tissue samples of aorta were collected intraoperatively from patients who were undergoing repair for abdominal aortic aneurysms (AAAs) and aortoiliac occlusive disease. Grossly normal aorta was harvested from cadaveric donors who were undergoing organ harvest for transplantation. All specimens were collected from the anterolateral section of infrarenal aorta and sectioned accordingly. Aortic tissue sectioned for light microscopy was placed immediately in 10% neutral buffered formalin for overnight fixation, transferred to 70% ethanol, and paraffin embedded by standard techniques. Aortic tissue for subsequent protein and nucleic acid extraction was immediately flash frozen in liquid nitrogen and stored at –70°C. The remaining aortic sections were processed for electron microscopy by placement in cacodylate buffer with 2.5% glutaraldehyde for 24 hours at 4°C then transferred to cacodylate buffer. Approval from the University of Tennessee Investigational Review Board was obtained for all aortic tissue collection.

Immunohistochemistry. Paraffin-embedded aortic specimens were cut into 5-μm serial sections and mounted on positively charged microscope slides. Tissue samples were cleared and rehydrated by sequential immersions in three changes of xylene, followed by baths of 100% ethanol, 95% ethanol, 50% ethanol, and distilled water. Tissue was treated with Protease 24 (BioGenex, San Ramon, Calif) and blocked with protein block, containing goat serum (BioGenex), at room temperature (RT). Specimens were incubated with the specific antibody for 1 hour at RT. The mouse monoclonal α-actin antibody (1:50; Dako Corporation, Carpenteria, Calif) was used for VSMC immunostaining. Binding of the primary antibodies was amplified with a biotin-streptavidin system (BioGenex). Immunoreactivity was observed with an alkaline phosphatase substrate (Vector Red; Vector Laboratories, Burlingame, Calif). Staining development was stopped by immersion in tap water, and the slide was counterstained with Harris hematoxylin. Tissue samples were dehydrated and coverslipped with mounting medium.

For correlation with the quantity of apoptosis within the aortic tissue, identification of p53 antibody and bcl-2 was performed. Localization of p53 protein, wild type and mutant, was accomplished by a citra buffer retrieval staining protocol. Briefly, tissue samples were cleared and rehydrated as described earlier. Slides were rinsed in 0.5% hydrogen peroxide followed by incubation with 1X Antigen Retrieval Citra (BioGenex) at 98°C for 15 minutes. After cooling, the sections were blocked with normal goat serum and incubated with mouse monoclonal p53 antibody (1 μg/mL; Santa Cruz Biotechnology Inc, Santa Cruz, Calif) for 20 minutes at RT then overnight fixation at 4°C. Immune complexes were amplified with biotin-streptavidin/peroxidase (BioGenex), detected with 3,3′-diaminobenzidine substrate (Sigma Laboratories, St Louis, Mo). Formalin-fixed, paraffin-embedded infiltrating ductal breast carcinoma sections served as positive controls for p53. Identification of bcl-2 was performed with the standard immunohistochemical techniques described earlier and incubation with mouse monoclonal anti-human bcl-2 oncoprotein (1:40; Dako Corporation) for 30 minutes at RT. Formalin-fixed, paraffin-embedded sections of lymph node served as bcl-2 positive controls.

All specimens were also stained with hematoxylin and eosin for tissue authentication.

Apoptosis assay. Embedded aortic sections were deparaffinized, rehydrated through graded alcohol, and treated with proteinase K (20 μg/mL) at RT. Slides were rinsed in 1X Tris-buffered saline solution (TBS) and blocked for endogenous peroxidase activity with 3% hydrogen peroxide in methanol. Equilibration with terminal deoxynucleotidyl transferase buffer for 20 minutes was followed by DNA end-labeling with terminal deoxynucleotidyl transferase labeling reaction mixture (FragEL Assay; Oncogene Research Products, Cambridge, Mass) at 37°C in a humidifying chamber for 1.5 hours. Labeling reaction was terminated with 0.5 mmol EDTA and multiple washes in 1X TBS. For detection, slides were incubated with peroxidase streptavidin solution followed by freshly prepared diaminobenzidine substrate in tap water for 12.5 minutes at RT. Slides were rinsed in hydrogen peroxide, counterstained in methyl-green solution, and dehydrated. Apoptotic nuclei were identified by the presence of dark brown staining. Positive controls were generated by application of DNAse (1 μg/mL) in 1X TBS/1 mmol/L $MgSO_4$ for 20 minutes at RT after protease incubation.

Colocalization with DNA end-labeling and secondary immunohistochemical staining with α-actin antibody was performed to identify apoptotic cells as VSMCs. Briefly, DNA end labeling was completed as described earlier, excluding the counterstain with methyl green. Tissue sections were then incubated with mouse monoclonal α-actin antibody (1:25) overnight at 4°C. Binding of the smooth muscle cell

antibody was amplified with the use of the biotin-streptavidin system. Immunoreactivity was observed with the use of an alkaline phosphatase Vector Red substrate. Tissue samples were then dehydrated and coverslipped with mounting medium.

Propidium iodide assay. Paraffin-embedded tissue sections were rehydrated by xylene and sequential alcohol immersions. Tissue was incubated with propidium iodide (PI; 50 μg/mL) in phosphate-buffered saline solution and 0.1% bovine serum albumin for 30 minutes at 37°C. Sections were washed in phosphate-buffered saline solution, dehydrated, and coverslipped. Positive-staining cells for apoptosis were identified under fluorescent microscope as bright orange condensed or fragmented nuclei.

Protein isolation of p53. Frozen aortic tissue was sliced thinly and placed in lysis buffer (1% Monodet 40, 1% Triton X, 0.01% sodium dodecylsulfate, 1% sodium deoxycholate, 150 mmol/L Tris, 1 mmol/L EDTA, 50 mmol/L nafcillin, 1 mmol/L sodium pyrophosphate, 1 mL/gm of tissue). After the addition of proteases, tissue samples were further homogenized at 0°C with 30 mL phenylmethylsulfonyl fluoride stock per 3 mL of homogenate and centrifuged twice at 15,000*g* for 20 minutes. Protein quantification was determined with the use of a Sigma Diagnostic Protein Assay kit (Sigma Laboratories). Twenty-five micrograms of denatured protein was electrophoresed on a 4% to 20% Tris-glycine gel. Protein was transblotted to positive-charged nitrocellulose. The membrane was blocked with 5% milk solution and incubated with mouse monoclonal p53 antibody (0.5 μl/mL; Santa Cruz Biotechnology Inc). The membrane was washed, and the immune complexes were linked with goat anti-mouse antibody conjugated with horseradish peroxidase. The nitrocellulose was washed and detected with a Pierce Super Signal Chemiluminescence detection kit (Pierce, Rockford, Ill). After a brief exposure to Amersham Hyperfil ECL (Amersham Life Science Inc, Arlington Heights, Ill), p53 protein was visualized in positive control lane (MCF-7 cell lysate).

Analysis. Analysis of the immunohistochemical characteristics was performed with a computer-generated image analysis. Slides were examined by light microscopy under ×100 to ×400 magnification (Zeiss Axioskop; Carl Zeiss Inc, Thornwood, NY), transmitted with a charge-coupled discharge color television camera to an image monitor. Interpretation of images was processed with Zeiss Image software (version 3.0; Carl Zeiss Inc). All specimens were reviewed by an independent pathologist to confirm tissue-staining authenticity. Staining characteristics (such as localization, distribution, and quantity of apoptotic cells) were determined for each sample. Four random measurement sites were identified within the appropriate staining area and calculated per sample. The average density medial VSMC of each specimen was determined by a count of the number of α-actin positive cells over four random ×100 power fields. Mean apoptotic cells within the medial layer (identified as brown staining cells) were calculated in a similar manner under ×200 power fields. Staining specifications of the specimens were compared by aortic subtype with the one-way analysis of variance by the Student-Newman-Keuls method and Dunn's test (SigmaStat; Jandel Corporation, San Rafael, Calif).

RESULTS

Seventeen aneurysmal, ten occlusive, and six normal human aortas were evaluated. Patients with ruptured, thoracoabdominal, or inflammatory aneurysms were excluded from the study. The average size of the AAAs before operation measured 5.9 cm (range, 4.2-9.0 cm). The average age was 71.5 years (range, 55-87 years) for patients with AAA, 50.8 years (range, 28-63 years) for patients with occlusive aortas, and 45.5 years (range, 34-64 years) for organ donors. All specimens used in the study had a storage time of less than 12 months.

Staining characteristics. α_1-Actin antibody immunostaining confirmed the predominance of VSMC in the medial layer of all aortic tissue types (Fig 1). Consistent with previous reports by other investigators, the architecture of the medial layer of aneurysmal aortas exhibited a more disrupted and disorganized pattern of VSMCs than normal or occlusive aortas.[12,14-16] Cellular staining for α_1-actin antibody was concentrated in the perinuclear region of the VSMCs. Aneurysmal aortas consistently revealed fewer VSMCs within the medial layer compared with occlusive and normal aortas. The mean VSMC count was 271.8 ± 13.5 cells/high-power field (HPF) for aneurysmal aortas, compared with 278.2 ± 39.4 cells/HPF for occlusive aortas and 291.0 ± 25.4 cells/HPF for normal aortas (Figs 2-4) (Table I). No statistical significance difference was seen for multiple comparisons of aortic subtypes.

Apoptotic cells were visualized within the aortic medial layer with DNA end-labeling methods and PI nuclear staining. Obvious endothelial-cell apoptosis was not apparent. VSMC apoptosis was transmural in nature and not inherent to any particular anatomic portion of the medial layer (Fig 5). Apoptotic cells appeared homogeneously throughout tissue sections

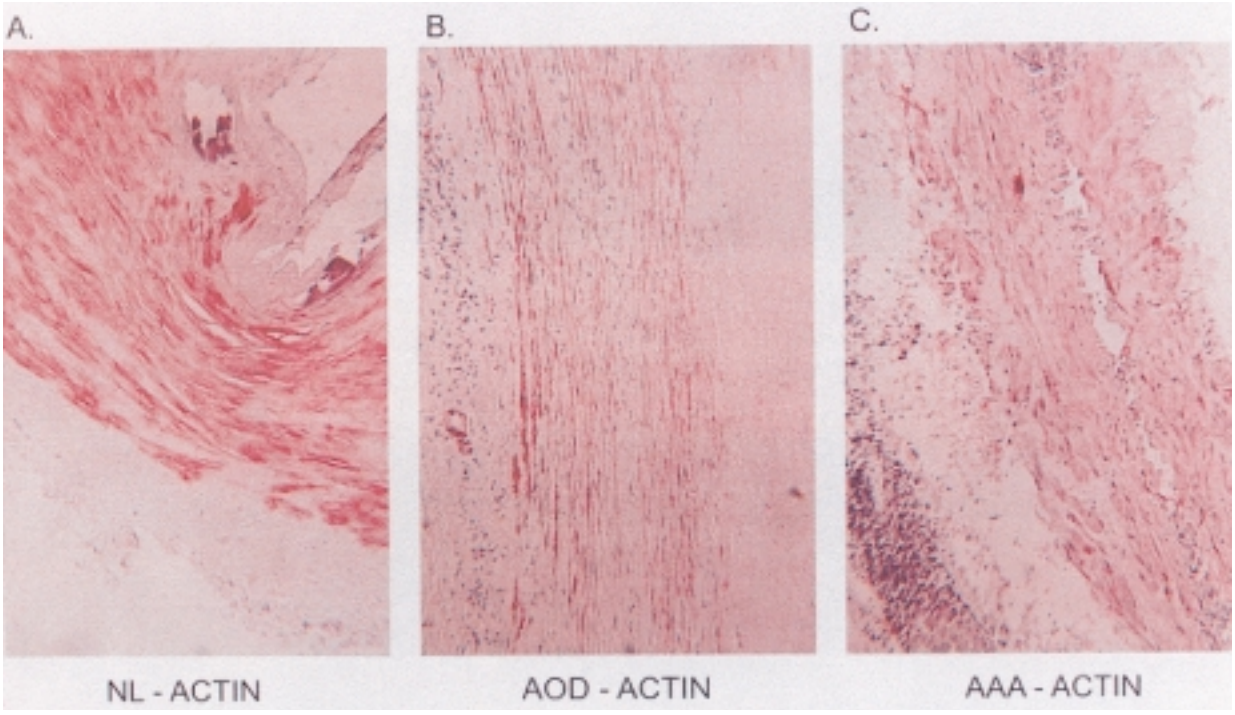

Fig 1. α_1-Actin antibody staining. A, Normal aorta (*NL*). Note the increased staining within the medial layer and the uniformity of the VSMC; the luminal surface is towards *upper right hand corner*. B, Occlusive aorta. Note the similar staining distribution; the luminal surface is towards the *right*. C, AAA. Note the disrupted architecture within the medial layer and the inflammatory cell and red blood cell infiltrate within the *lower left corner*; the luminal surface is towards the *upper right*. (Original magnification of all tissue, ×100). *AOD*, Aortic occlusive disease.

Table I. Aortic data: values* of average VSMC apoptosis and VSMC density for each aortic subtype

Aorta type	*N*	*Apoptotic VSMC*	*VSMC density*
Aneurysmal	17	11.7 ± 1.5	271.8 ± 13.4
Occlusive	10	3.3 ± 0.8	278.2 ± 39.4
Normal	6	3.8 ± 1.4	291.0 ± 25.4

*Values are expressed as mean and standard error of the mean.

and not pocketed to particular segments. Colocalization staining methods demonstrated α-actin staining in the perinuclear portion, and the dark-staining condensed nuclei of apoptotic cells were confirmed to be VSMCs (Fig 6). PI nuclear staining was added to verify the presence of apoptotic cells. Positive PI-stained cells showed intense concentration of bright orange color within fragmented or condensed nuclei of VSMCs (Fig 7).

Apoptosis of VSMCs was greatest within aneurysmal aortas with 11.7 ± 1.5 cells / HPF, compared with occlusive aortas with 3.3 ± 0.8 cells / HPF ($P < .05$) and normal aortas with 3.75 ± 4.6 cells / HPF ($P < .05$; Table I). No significant difference was noted between normal and occlusive aortic tissue for VSMC apoptosis.

Immunohistochemical staining with an antibody that recognizes both the wild type and mutant p53 protein was performed on representative sections of all aortic tissue, with ductal breast carcinoma serving as the positive control. Strong nuclear staining was observed in the positive control; however, no specific staining for the p53 antibody was noticed in the medial component of tissue sections reviewed. Positive nuclear staining of adventitial lymphocytes and rare mononuclear cells was observed in multiple sections. The protein was isolated, denatured, and electrophoresed on a 4% to 20% Tris-glycine gel and blotted for p53. An intense band at 53 kDa was detected in the control lane (MCF-7). Only faint banding was observed for p53 in aneurysmal and normal aorta samples. Because of such scant expression, no significant

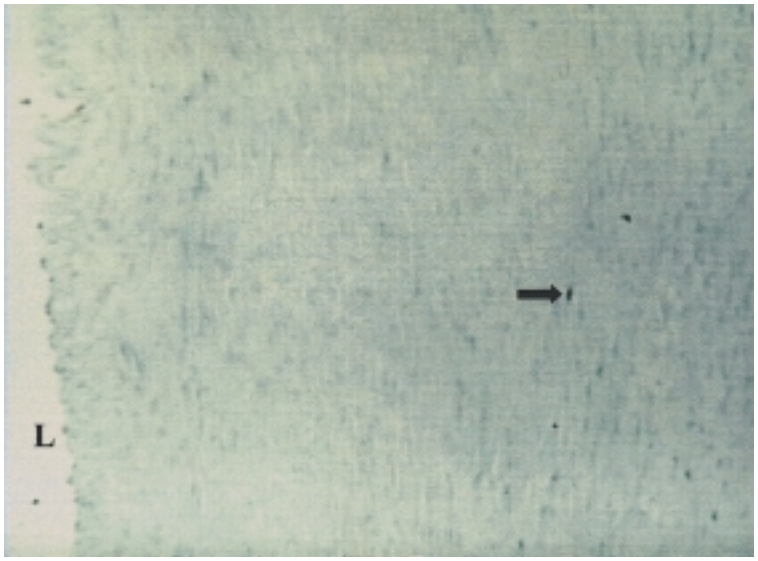

Fig 2. VSMC apoptosis staining in normal aorta. Cross-section of normal aortic tissue (original magnification, ×60). The *arrow* shows typical apoptotic VSMC within medial layer. *L*, Luminal surface.

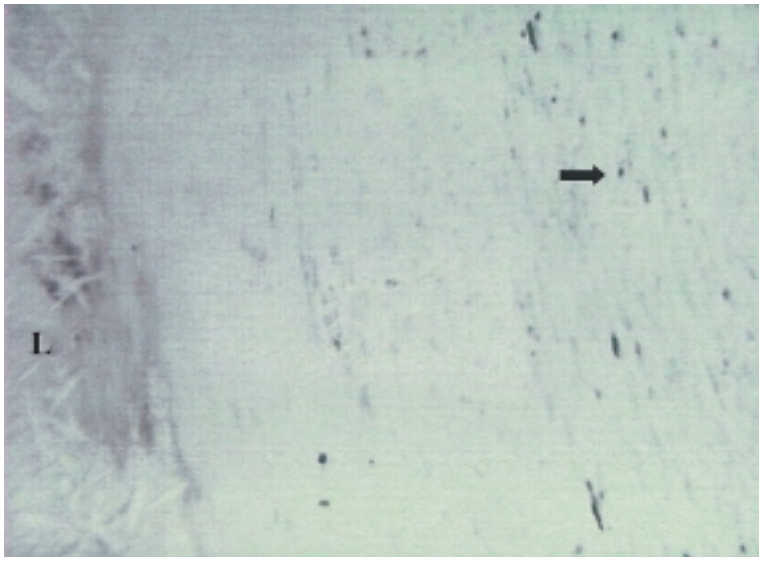

Fig 3. VSMC apoptosis staining in occlusive aorta. Cross-section of occlusive aortic tissue (original magnification, ×100). The *arrow* shows typical apoptotic VSMC within medial layer. Note the cholesterol plaque deposits on the lining of the endothelial layer. *L*, Luminal surface.

differences were observed between aortic subtypes. Similar staining techniques were used for identification of bcl-2. Mild to moderate positive staining of lymphoid follicle–associated lymphocytes was present in positive control tissue. No specific staining was detected for bcl-2 within the medial layer of aortic sections reviewed. Rare positive nuclear staining of adventitial lymphocytes was observed.

Specimens were matched to certain patient characteristics to assess the possible clinical significance of VSMC apoptosis. No linear correlation between the amount of VSMC apoptosis and the size of the aneurysm or the age of the patient existed.

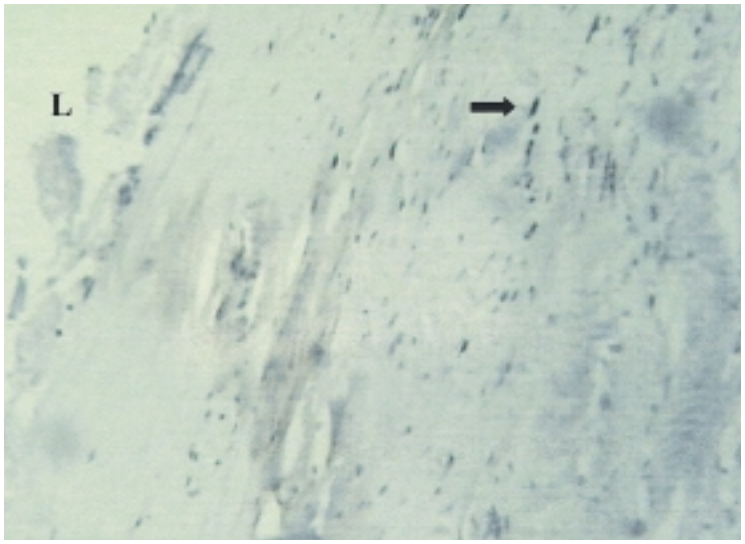

Fig 4. VSMC apoptosis staining in aneurysmal aorta. Cross-section of aneurysmal aortic tissue (original magnification, ×100). The *arrow* denotes typical apoptotic VSMC within the medial layer. Nonlinear distribution of medial VSMC is apparent. *L*, Luminal surface.

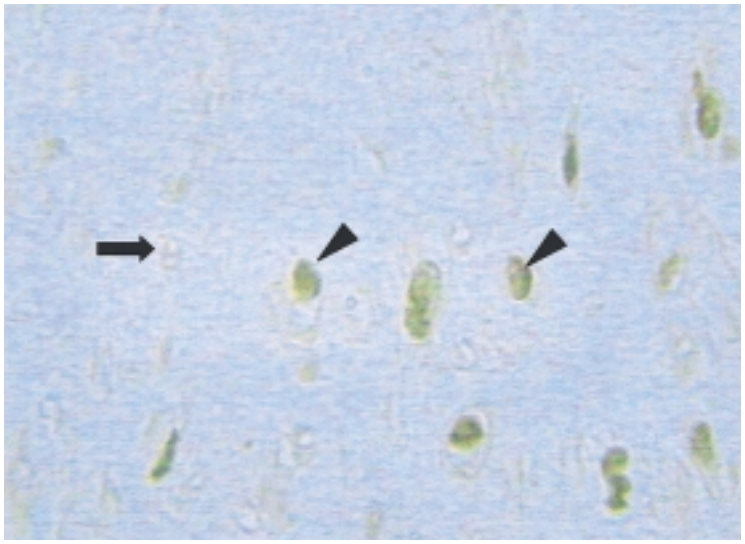

Fig 5. High-power VSMC apoptosis staining. Cross-section of medial layer AAA tissue sample (original magnification, ×400). The *arrowhead* is directed at positive nuclear staining for DNA end-labeling in apoptotic cells. Note the similar positive staining cells towards the periphery. The *arrow* points to the nonapoptotic cell. Note the lack of a dark brown–staining nucleus.

DISCUSSION

Human AAAs have distinct structural alterations compared with any other arteriopathy. Predominance of destructive proteases, diminished elastin content, and disruptive mechanical forces have been shown to contribute to the degenerative changes within the infrarenal aortic wall.[12,14-19] Our aim was to demonstrate that VSMC apoptosis was a plausible mechanism of medial layer degeneration. We observed an increase in apoptotic VSMCs and a corresponding decrease in VSMC density within the medial layer of AAA in contrast to occlusive and normal human aortas. Similar findings were reported by Lopez-Candales et al,[12] who documented a decreased VSMC density, increased VSMC apoptot-

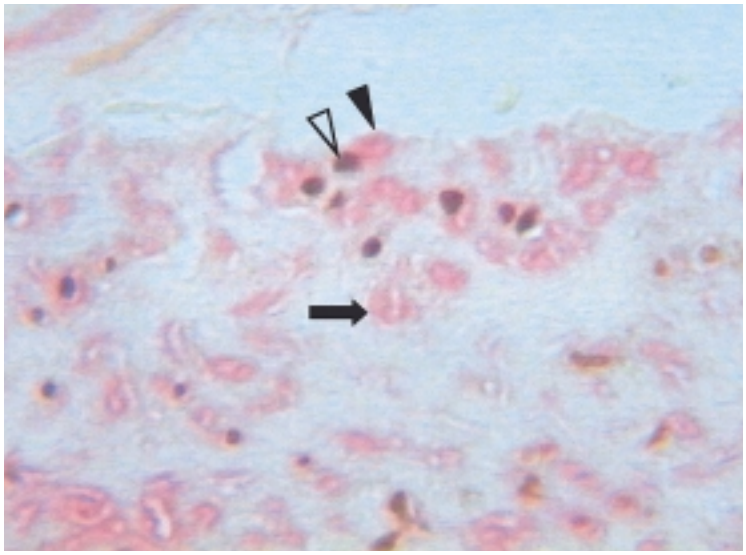

Fig 6. Colocalization of DNA end-labeling and α_1-actin antibody. Cross-section of medial layer AAA tissue sample (original magnification, ×400). The *hollow arrowhead* shows a typical apoptotic nucleus with dark-brown staining. The *solid arrowhead* designates perinuclear pink staining for VSMC. The *arrow* shows a cell with positive staining with α_1-actin antibody, but negative apoptosis staining. Note the disorganized array of VSMC orientation.

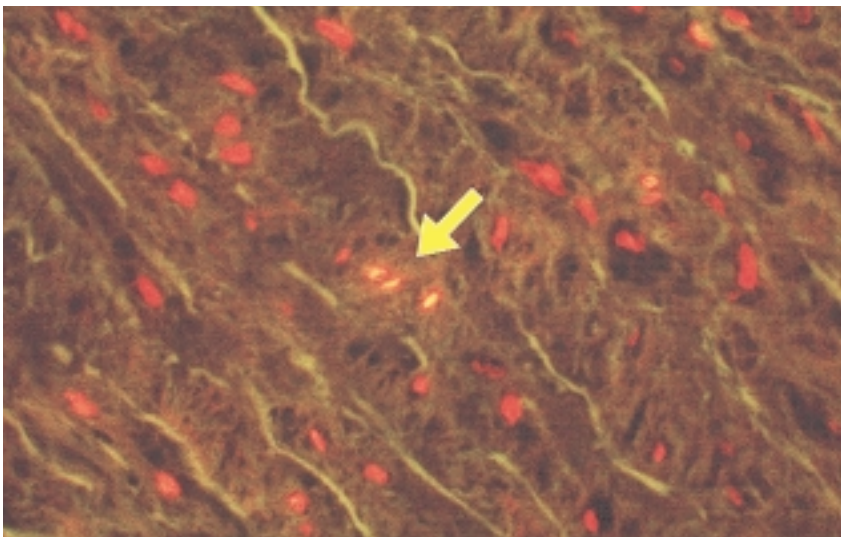

Fig 7. PI assay. Cross-section of medial layer normal aorta tissue sample under rhodamine fluorescence (original magnification, ×400). The *arrow* shows apoptotic VSMC with fragmented, condensed nuclei and bright orange staining.

ic cells, and an increase in the percent of apoptotic VSMCs within the aortic medial layer. Kondo et al[20] also supported these results. They prospectively followed induced aneurysm formation in cerebral arteries of rats and demonstrated the presence of VSMC apoptosis. In our study, VSMC apoptosis within the medial layer was similar for occlusive and normal aortas. Surprisingly, the structural integrity of the medial layer in the atherosclerotic aorta is relatively unaffected compared with the degenerative processes at the intimal surface associated with plaque formation. These findings suggest that a cellular signal mecha-

nism for VSMC apoptosis may influence aneurysmal formation and progression.

Controversy exists as to whether the observed apoptotic cells are truly representative of the amount of cells undergoing apoptosis. Geng and Libby[10] contend that the apoptotic cell population observed is falsely elevated by cells that underwent a programmed death long before the time of detection of apoptosis. These mummified cells are postulated to stain positively for fragmented DNA while awaiting clearance from the tissue by phagocytosis, thus causing an inaccurate quantification of apoptosis. The hypothesis of Geng and Libby may be correct, and the overall counts of apoptotic cells may be elevated; however, we contend that these mummified cells are present within all of the tissue studied, thereby negating the sampling error in a comparative study.

With apoptosis present, questions are raised about the initiating or regulatory factors involved in the manipulation of the rate of cell suicide. Two documented signal markers involved in apoptosis regulation are p53 protein and bcl-2.[12,21,22] The p53 marker was first described as an apoptosis initiator in cancer cells.[23] Since that time, numerous researchers have documented the universal role of p53 in the apoptosis cycle of diverse tissues.[12,22-25] In vascular biology, Bennett et al[22] were able to demonstrate that apoptosis in cultured VSMC occurred through p53-dependent and p53-independent pathways; Lopez-Candales et al[12] found higher levels of p53 protein within aneurysmal aortic tissue when compared with normal human aortas through reverse transcriptase. We were unable to conclusively demonstrate the differences in p53 protein within aortic tissue using immunohistochemistry or protein electrophoresis. In cells with positive staining for p53, we observed predominately nuclear staining as opposed to cytoplasmic staining reported by other investigators.[12] These findings are consistent with Bennett et al[22] who demonstrated that in vitro overexpression of p53 protein by rat aortic VSMCs was confined to nuclear staining. During the preparation for Western blotting, extensive tissue mincing is required. The inevitable inclusion of plasma proteins within blood-tinged fresh specimens may have diluted the sample to the extent that it precluded accurate isolation of scant p53 protein from the tissue. Similar findings occurred when isolation of bcl-2, a known inhibitor of apoptosis in various neoplastic processes, was attempted.[21] Perhaps, further investigation should focus on specific prevention factors of VSMC apoptosis rather than isolation of such elusive markers. Initial data from our laboratory indicates that vascular endothelial growth factor has a protective effect against ultraviolet radiation–induced apoptosis in cultured VSMCs.

No linear correlation was apparent between the amount of VSMC apoptosis and the size of the AAA. This could represent a distributional variation in the area of aneurysmal tissue sampled or variations in the rate of progression of medial degenerative changes. Kondo et al[20] prospectively followed induced aneurysm formation in the cerebral arteries of rats. When the aneurysmal arteries were closely examined, the greatest amount of VSMC apoptosis was observed within the medial layer at the neck (the transition zone) of the aneurysm as opposed to the area of maximum diameter. Our samples were taken from the anterolateral aneurysm wall where the diameter was the greatest because of the ease of harvest and minimal interference with the proximal anastomosis. Prospective comparison between these two anatomic areas and meticulous documentation of the progression rate may prove beneficial to further investigation. Laplace's law explains that for a given pressure, tangential wall stress increased exponentially as the radius of the tube enlarged. It is possible that the rate of AAA enlargement relates to detected VSMC apoptotic events. Unfortunately, our records and the referral pattern of patients with significant AAA restricted that aspect of the study. Also, most patients do not obtain the diagnosis of AAA until it is large in size, thus complicating construction of a longitudinal human study.

As the aging process matures, degenerative changes increase in multiple organ systems. This theory is partly supported by the higher incidence of AAA and atherosclerosis in the elderly population. Thompson et al[19] reasoned that an increase in age corresponded with more structural degeneration, leading to a cell population more susceptible to apoptosis. Our data could not demonstrate a correlation in VSMC apoptosis rates and age of the patients in the AAA subset.

CONCLUSION

Our study demonstrates that apoptosis of VSMCs does play a role in large vessel tissue architecture at the cellular level. When compared with normal aortic tissue, VSMC density within the medial layer shows a decreasing trend in diseased aortas (occlusive more than aneurysm). Correspondingly, VSMC apoptosis is significantly greater within the medial layer of aneurysmal aortas when matched against normal and occlusive aortas. Correlation of VSMC apoptosis in aneurysmal disease with patient characteristics still

warrants further investigation before any etiologic postulates can be confirmed. Such information may lead to novel therapeutic approaches in the prevention and treatment of aortic disease.

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DISCUSSION

Dr Thomas S. Huber (Gainesville, Fla). Apoptosis is a highly regulated mechanism of cell death controlled by interaction of the cellular receptors, cytoplasmic and nuclear gene products, and local cytokine and cellular environment. Apoptosis has been recently incriminated in a variety of processes, including aneurysms, atherosclerosis, and response of the vessel wall to injury.

Notably, Thompson et al[19] reported that the density of smooth muscle cells was decreased within the AAAs and that 30% of the smooth muscle cells stained positive for their marker of apoptosis. Furthermore, the protein concentrations of p53, an inducer of apoptosis, were increased four-fold in the aneurysmal tissue. Additionally, Sakaki et al[26] reported that smooth muscle cells within ruptured intracranial aneurysms were significantly more degenerated and had a higher incidence of apoptotic bodies when compared with intact aneurysms.

You harvested aortic tissues from patients with aneurysms who were undergoing aortoiliac bypass grafting for occlusive disease and organ harvest for transplanta-

tion. You reported a decrease in the density of smooth muscle cells within the aneurysmal tissue, although the differences did not reach statistical significance. You reported an increased incidence of apoptosis within the aneurysmal tissue relative to the other two types, although there was no difference among the aortic subtypes for the two regulator proteins for apoptosis analyzed. Based on the increased incidence of apoptosis and the decreased density of VSMCs, they concluded that the structural degeneration of the aortic tissue at the cellular level contributes to aneurysmal formation.

Although I am intrigued by the biologic phenomenon of apoptosis and its potential role in vascular pathology, I take exception to the authors' conclusions. As noted, there was no significant difference in the smooth muscle cell density among the aortic subtypes; to propose that the observed increased incidence of apoptosis contributes to aneurysm formation is possible, although at best, a reach. Clearly, the authors may be onto something very important, but the descriptive nature of the study and the single static image of the aortic tissue does not elucidate the underlying mechanisms of a very complex process that occurs over the course of a lifetime.

How was the aortic tissue harvested at the time of aneurysmal repair? I am somewhat bothered by studies in which aneurysmal aortic tissue is harvested, then subsequently analyzed, in light of the degenerative aortic tissue and the intraluminal debris frequently encountered.

How do you interpret your findings in view of the elegant study by Thompson et al,[19] who reported a decreased smooth muscle cell density and increased p53 levels within the aneurysmal tissue?

What do you hypothesize are the extracellular and intracellular signals for apoptosis within the aneurysmal tissue?

How do you explain your findings that no increased evidence of apoptosis was identified within the aortic tissue in patients with occlusive disease in light of the wealth of studies incriminating apoptosis in atherosclerosis?

Lastly, and perhaps most importantly, I would like you to comment on the clinical relevance of the increased number of apoptotic cells within the aneurysmal aortic tissue. Indeed, it is unclear to me whether apoptosis leads to aneurysms or vice-versa. In my fairly limited vascular surgery career, multiple causes have been incriminated for aneurysms, including copper metabolism, collagen, elastase, lysyl oxidase, inflammatory cytokines, and the metalloproteinases, although our overall understanding has advanced little.

Dr Rowe. First of all, in terms of the harvest, all of our tissues were harvested within the operating room, and the ones for molecular work were frozen in liquid nitrogen immediately within the operating room so that we did not have any delay in terms of decreasing our molecular content. For the paraffin-embedded specimens, we harvested them and put them immediately within neutral-buffered formalin and then transferred them to 70% alcohol. All of the specimens were processed that way.

In terms of the p53 marker, we did not identify a significant difference within the p53 marker within our tissue. I am well aware of the work by Dr Thompson and his group, and they did perform a very elegant study. They did note a small difference in p53 expression by protein work. The way that their largest difference was expressed was by doing a reverse transcriptase polymerase chain reaction, in which the signal of the p53 was amplified to 35 to 40 times before they found a difference. I think that shows that the p53 is in very scant amounts within the tissue and that to have to multiply the expression of that to such great levels shows that it may not have the exact significance that we really think.

In terms of the intracellular and extracellular signals, I cannot really comment on that at this time. Research is going to have to continue. Again, we have searched for the bcl-2, p53, TNF-α; and our search probably has not been as extensive as other laboratories, but so far no one is able to conclude exactly which signal is occurring within the aneurysmal tissue.

In terms of the increase that we did not note within our atherosclerotic tissue, I think the literature in terms of apoptosis and atherosclerotic tissue points to apoptosis occurring within the endothelial layer in plaque morphologic features and intimal hyperplasia. The focus of our research examined toward the medial layer of the aortic tissue, and we did not see much difference in medial layer apoptosis between vessels and normal vessels.

Regarding the clinical significance, at this point we believe that apoptosis does play a role. To say that it is the sole cause would be somewhat exaggerated, but we do feel that it plays a role. One study that seemed to stimulate us was a study in rats where they artificially induced aneurysm formation within the cerebral arteries and then followed these rats. Once the rats were killed, the researchers looked at the apoptotic rates within different samples of the aneurysmal tissue, and they found the apoptosis to be greatest within the transitional zone where the artery was just becoming aneurysmal, as opposed to the segment where the maximal diameter of the aneurysm was present. I thought that was very interesting because that is saying that it is occurring at the point where it is starting to change. Right now we are currently harvesting our samples from not only the dome of the aneurysm but also proximally and at the neck to see whether we can find a difference within human tissue.

The other key clinically will come from where we will be able to not only just identify markers but also inhibit the whole process or at least slow the aneurysmal progression. I think with aneurysmal disease it is going to be difficult to say that you can stop the formation, but once you find someone with a small aneurysm, the question will be whether you can inhibit the progression of disease. Right now we are looking at different markers that are able to slow down the rate of apoptosis within smooth muscle cells. We have been able to identify a couple of growth factors, which (when placed in cell culture) will inhibit apoptosis. Will that become clinically significant? We hope so, because if it is, then maybe we can take patients with a 3.5-cm aneurysm, give them a particular growth factor, and keep the progression rate at none.