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Short Communication

Apoptosis Participates in Cellularity Regulation during Rat Aortic Intimal Thickening

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Intimal thickening induced after endothelial denudation of rat aorta is thought to be due to migration and proliferation of smooth muscle cells (SMC). When the reendothelialization is achieved, intimal thickening shows an important decrease in cellularity. Using* in situ *end labeling of fragmented DNA and electron microscopy, we show that this remodeling is accompanied by apoptosis of SMC. The number of apoptotic SMC becomes important 15 days after endothelial injury and reaches a maximum at 20 days; at 45 days the intimal thickening is reendothelialized and no more apoptotic SMC are detected. Apoptotic SMC show nuclear and cytoplasmic condensation as well as cytoplasmic vacuolization. Our results indicate that apoptosis is an important mechanism in the regulation of intimal thickening evolution. (Am J Pathol 1995, 146:1059–1064)

Arterial intimal thickening induced in the rat after endothelial denudation by means of a balloon catheter represents the most used experimental model for the development of the atheromatous plaque (for review, see refs. 1–5). It is well accepted that the main component of intimal thickening is represented by smooth muscle cells (SMC) which have migrated from the media.[6] Some of these cells remain quiescent and others undergo proliferation within the intimal thickening, thus increasing the overall cellularity of the tissue.[6,7] After a few weeks, replication is only seen at the luminal surface of the lesion and disappears when endothelial regeneration is achieved (eg, in the aorta), whereas it persists when this phenomenon does not take place (eg, in the carotid artery[6]). In both cases there is an important reduction of SMC number with time,[8] but the mechanism of this phenomenon has not been clearly explained. We show here that after endothelial lesion of the aorta the decrease of SMC number taking place between 15 and 45 days is essentially due to apoptosis. These apoptotic changes should be taken into consideration in the evaluation of the mechanisms regulating the development and the regression of experimental intimal thickening.

Materials and Methods

Experimental Procedures

Twenty-five male Wistar rats weighing 280 to 300 g were used for the experiments. Rats were preanesthetized with enflurane (Ethrane, Abott Laboratories, North Chicago, IL) and then anesthetized with an intraperitoneal injection of pentobarbital sodium (nembutal sodium, Abott Laboratories). Experimental intimal thickening was induced in the thoracic aorta by removal of the endothelium using an inflated embolectomy catheter according to Baumgartner and Studer[9] with minor modifications.[8] The animals were sacrificed by cervical dislocation after enflurane anesthesia 7, 15, 20, 30, and 45 days after the injury (n = 5 per group). Involuting rat prostate (2 days after

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castration) and normal colon mucosa, known to contain apoptotic cells, were used as positive control.

In Situ *End Labeling of Fragmented DNA and Immunohistochemistry*

The DNA fragmentation characteristic of apoptosis can be detected at the cellular level in tissue sections by incorporating biotinylated nucleotides into natural DNA breaks using a DNA polymerase.[10] Injured thoracic aortas were fixed in 4% buffered formaldehyde and then embedded in paraffin. *In situ* end labeling (ISEL) of fragmented DNA was performed on deparaffinized and rehydrated 5-μ-thick sections. Tissue sections were digested by incubation with 5 μg/ml proteinase K (Life Technologies, Basel, Switzerland) for 15 minutes at room temperature. Sections were then rinsed and incubated in buffer containing 0.01 mmol/L dATP, dCTP, dGTP (Promega, Madison, WI), 0.01 mmol/L biotin-16-dUTP (Boehringer Mannheim, Mannheim, Germany), and 8 U/ml DNA polymerase I (Klenow) large fragment (New England Biolabs, Beverly, MA). Endogenous peroxidase was blocked with 2% H_2O_2 in distilled water. Sections were then incubated with horseradish peroxidase-conjugated streptavidin (Dako, Glostrup, Denmark), revealed in diaminobenzidine-H_2O_2 and counterstained with hematoxylin.

Cell counts were performed using a Zeiss Vidas image analysis system (Kontron Elektronik, Eching, Germany) connected to a high sensitivity Burle CCD camera type FMC-4005 (AVT-Horn, Aalen, Germany). The number of ISEL-positive cells (labeled nuclei per total nuclei) was counted: 1) in the superficial portion of intimal thickening, ie, in the two layers of cells beneath the lumen; 2) in the remaining intimal thickening; and 3) in the underlying media. Only cells clearly located within the intimal thickening were counted. Cells which were on the luminal surface or in the lumen were discarded. Results are expressed as mean ± SEM. Student's *t* test was used to evaluate significance of differences between two groups. Pictures were taken on Kodak Ektachrome 64 tungsten color film (Eastman Kodak, Rochester, NY).

Immunoperoxidase stainings were performed on 4-μ-thick sections from paraffin-embedded blocks by means of avidin-biotin-peroxidase complex method as previously described.[11] We used two mouse monoclonal antibodies: anti-α smooth muscle (SM) actin (anti-α-SM-1,[12]) and anti-vimentin (Dako); and two rabbit polyclonal affinity purified antibodies: anti-desmin[8] and anti-human von Willebrand factor which reacts with the rat antigen (Sigma, St. Louis, MO).

Electron Microscopy

Tissue samples were fixed at room temperature in 2% glutaraldehyde (Merck, Darmstadt, Germany) in 0.1 mol/L sodium cacodylate. They were stained *en bloc* in uranyl maleate for 1 hour, post-fixed in 1% osmium tetroxyde, dehydrated, and embedded in Epon 812 (Fluka Chemie, Buchs, Switzerland). Thin sections were stained with uranyl acetate and lead citrate and examined in a Philips 400 electron microscope.

Results

ISEL of Fragmented DNA and Immunohistochemistry

In rat involuting prostate 2 days after castration, apoptotic cells were scattered in the acinar epithelium as previously described.[10,13] In large intestine, apoptotic cells were detected at the tips of villi. Normal rat aorta did not contain apoptotic cells (data not shown).

Seven days after endothelial denudation, a number of SMC were already present in the intima. Some apoptotic cells were detected by ISEL of fragmented DNA in the portion of the vessel immediately beneath the lumen (Table 1). At 15 days, thickness of the intima had greatly increased compared with 7 days. Several ISEL-positive SMC appeared almost exclusively in the superficial portion of intimal thickening and represented 11.5 ± 0.1% of superficial intimal thickening cells (Figure 1a). At 20 days, the percentage of ISEL-positive cells had slightly but significantly increased always in the superficial portion of intimal thickening and represented 14.3 ± 0.3% ($p < 0.001$ compared with 15 days). At 30 days, this percentage decreased to 5.2 ± 0.4% ($P < 0.005$ compared with 15 days, and $P < 0.001$ compared with 20 days). At 45 days (Figure 1b), the intimal thickening was reendothelialized and apoptotic cells had practically disappeared. In all tissues examined, apoptotic SMC were only exceptionally present in the deep portion of the intimal thickening and absent in the underlying media.

Table 1. *Percentage of SMC Showing ISEL of Fragmented DNA in Different Portions of the Aorta*

Days after Injury	Luminal Intimal Thickening	Remaining Intimal Thickening	Underlying Media
7	2.6 ± 0.4	<0.1	0
15	11.5 ± 0.1	<0.1	0
20	14.3 ± 0.3	0.4 ± 0.1	0
30	5.2 ± 0.4	<0.1	0
45	0	0	0

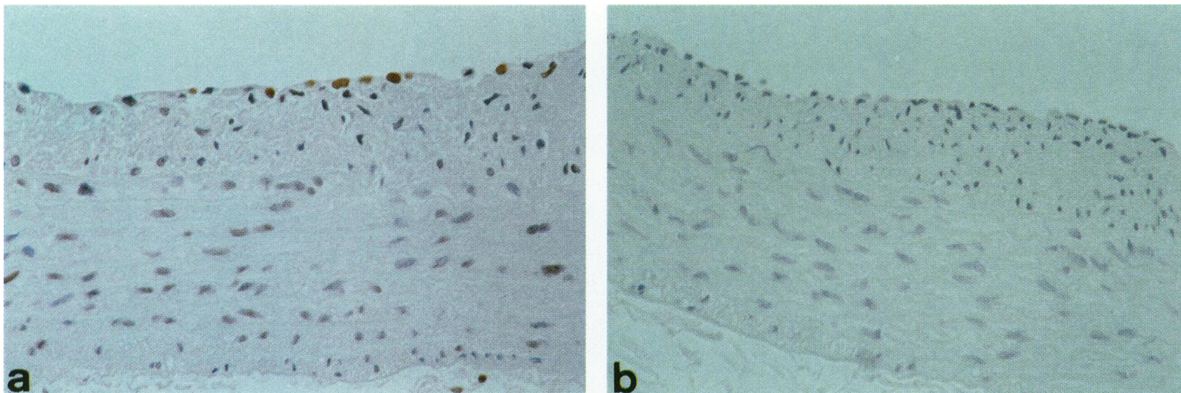

Figure 1. *Identification of apoptotic cells by ISEL of fragmented DNA in intimal thickening 15 days* (**a**) *and 45 days* (**b**) *after endothelial injury. Apoptotic SMC are detected in the superficial portion of the intimal thickening at 15 days* (**a**), *whereas no ISEL-positive cells are visible at 45 days* (**b**) (×*800*).

Immunohistochemical detection of von Willebrand factor revealed that 15 and 20 days after endothelial injury no endothelial cells were present at the surface of intimal thickening. Thirty days after endothelial lesion von Willebrand factor-positive cells were present at the luminal surface, particularly at the periphery of the thickening, and at 45 days the intimal thickening was covered by a continuous layer of endothelial cells. Interestingly, at that time scattered apoptotic cells were regularly noted among endothelial cells (data not shown), which suggests that the regenerated endothelial surface is remodeled through this phenomenon.

Immunohistochemical staining with vimentin and desmin antibodies showed that, as previously reported,[8,14] 15 days after endothelial denudation SMC of the intimal thickening contain practically only vimentin-positive intermediate filaments. At 45 days the proportion of desmin-positive SMC was similar to that present in the underlying media. At 15 days, the α-SM actin antibody stained weakly most SMC in the intimal thickening, whereas at 45 days it intensely labeled practically all SMC.[15,16]

Electron Microscopy

To our knowledge, no morphological report of damaged or dying SMC during the evolution of intimal thickening has been published. Thus we decided to examine by means of electron microscopy the presence of apoptotic SMC 15 and 45 days after endothelial lesion. At 15 days, as expected,[17] most SMC showed a well-developed endoplasmic reticulum and Golgi apparatus and less microfilament bundles compared with those of the normal aortic media (Figure 2). Close to the lumen, apoptotic cells were identifiable and frequently distributed in clusters (Figure 2); they were characterized by nuclear shrinking accompanied by margination and condensation of the chromatin, and the cytoplasm showed an important condensation and vacuolization. In SMC showing less advanced signs of apoptosis (Figure 3), dilatation of rough endoplasmic reticulum was evident and it was still possible to observe typical cytoplasmic microfilament bundles (Figure 3, inset).

Forty-five days after injury, the cellularity in intimal thickening had decreased. Intimal SMC showed a well-differentiated phenotype with numerous microfilament bundles distributed throughout the cytoplasm.[8] The re-endothelialization of the intimal thickening was clearly achieved. No SMC with apoptotic features were found.

Discussion

The possibility that cell death accounts for the regulation of SMC number within the arterial intimal thickening developing after endothelial injury was suggested in the first study of cellular kinetics in this experimental model,[6] but was never demonstrated. Our results support this hypothesis and show that SMC death is due to the occurrence of apoptosis in a well-defined portion of the intimal thickening. After endothelial injury, apoptotic changes appear clearly later than mitotic changes and reach a maximum 20 days after the lesion, when the volume of neointima begins to be caused by extracellular matrix deposition rather than by cell accumulation (for review see refs. 18 and 19). SMC apoptosis takes place mainly in the cells close to the lumen similar to what has been described for mitotic changes,[6] which suggests that highly replicative SMC undergo apoptosis. These observations are compatible with the possibility that rat (and possibly human) SMC are heteroge-

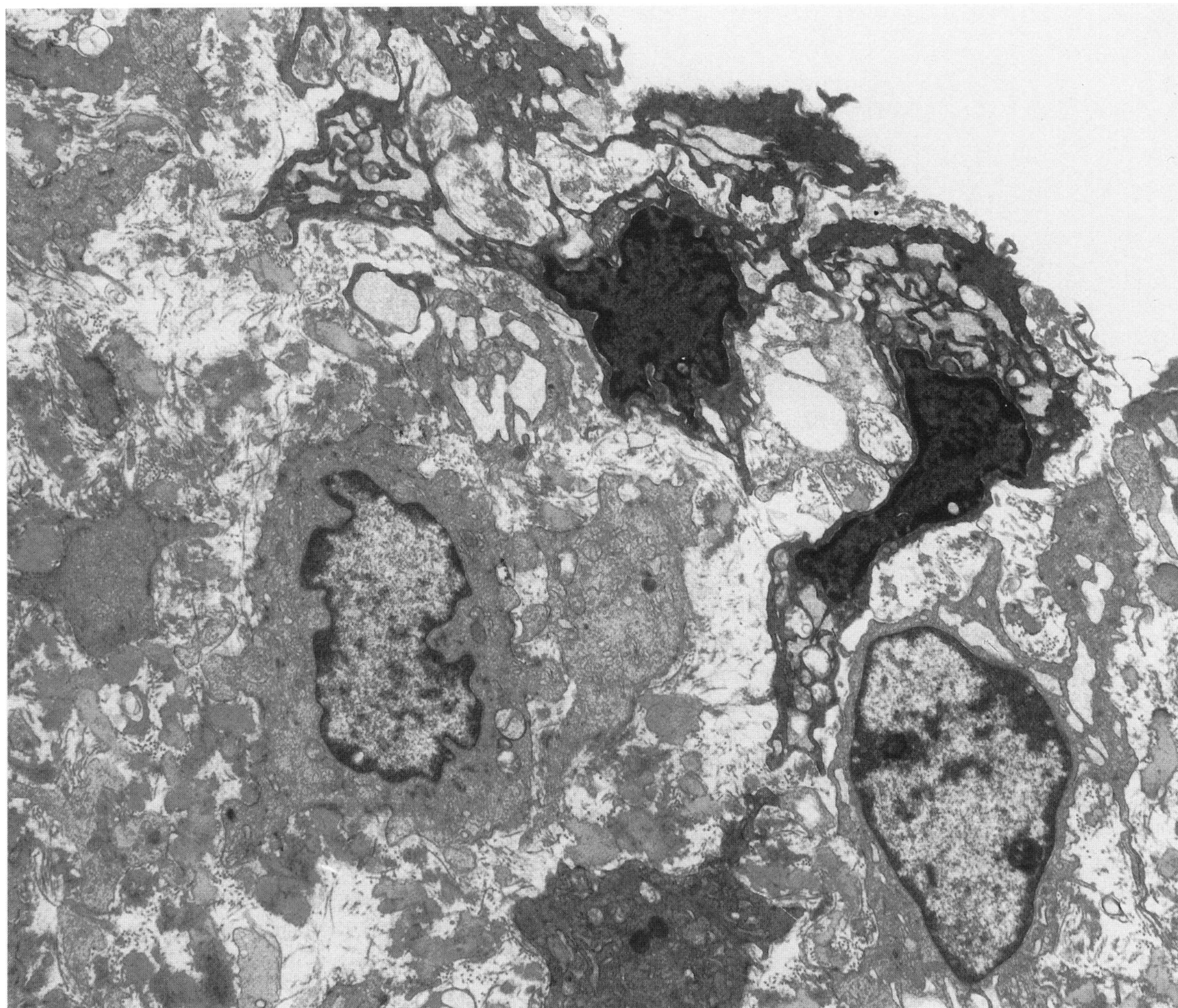

Figure 2. *Low magnification electron micrograph of a typical apoptotic cell cluster 15 days after endothelial injury. The cells are located close to the luminal surface and show an important nuclear and cytoplasmic condensation as well as cytoplasmic vacuoles* (×5,900).

neous as supported by different experimental approaches.[20,21]

It is noteworthy that apoptotic SMC display characteristic ultrastructural features,[22,23] that make them easily recognizable at electron microscopic examination even at low magnification. It would be of interest to investigate whether similar changes are present in human atheromatous plaques or restenotic lesions. A report of the presence of apoptotic SMC in occluded human saphenous vein aorto-coronary grafts obtained during re-intervention bypass grafting has been made.[24] In our model SMC apoptosis ceases when re-endothelialization is completed. In arteries such as the carotid, where after balloon-induced endothelial injury there is no complete re-endothelialization, it is known that mitotic cells remain present at the luminal surface for a long time,[6] and probably the same is true for apoptotic changes.

The question that remains is: what is the stimulus that leads to apoptosis during intimal thickening? Apoptosis could be due to exhaustion of highly replicating cells or to locally liberated yet unknown factors. c-*myc* has been shown to be directly involved in vascular SMC proliferation *in vivo* and *in vitro*.[25–27] Recently it has been reported that constitutive c-*myc* expression by SMC induces continuous cell proliferation as well as apoptosis.[28] Cultured human arterial SMC organized in three-dimensional aggregates display features of apoptosis particularly when they are cultivated in the presence of high concentrations of serum.[29] Whatever is the mechanism of apoptosis induction in arterial SMC, it appears that this phenom-

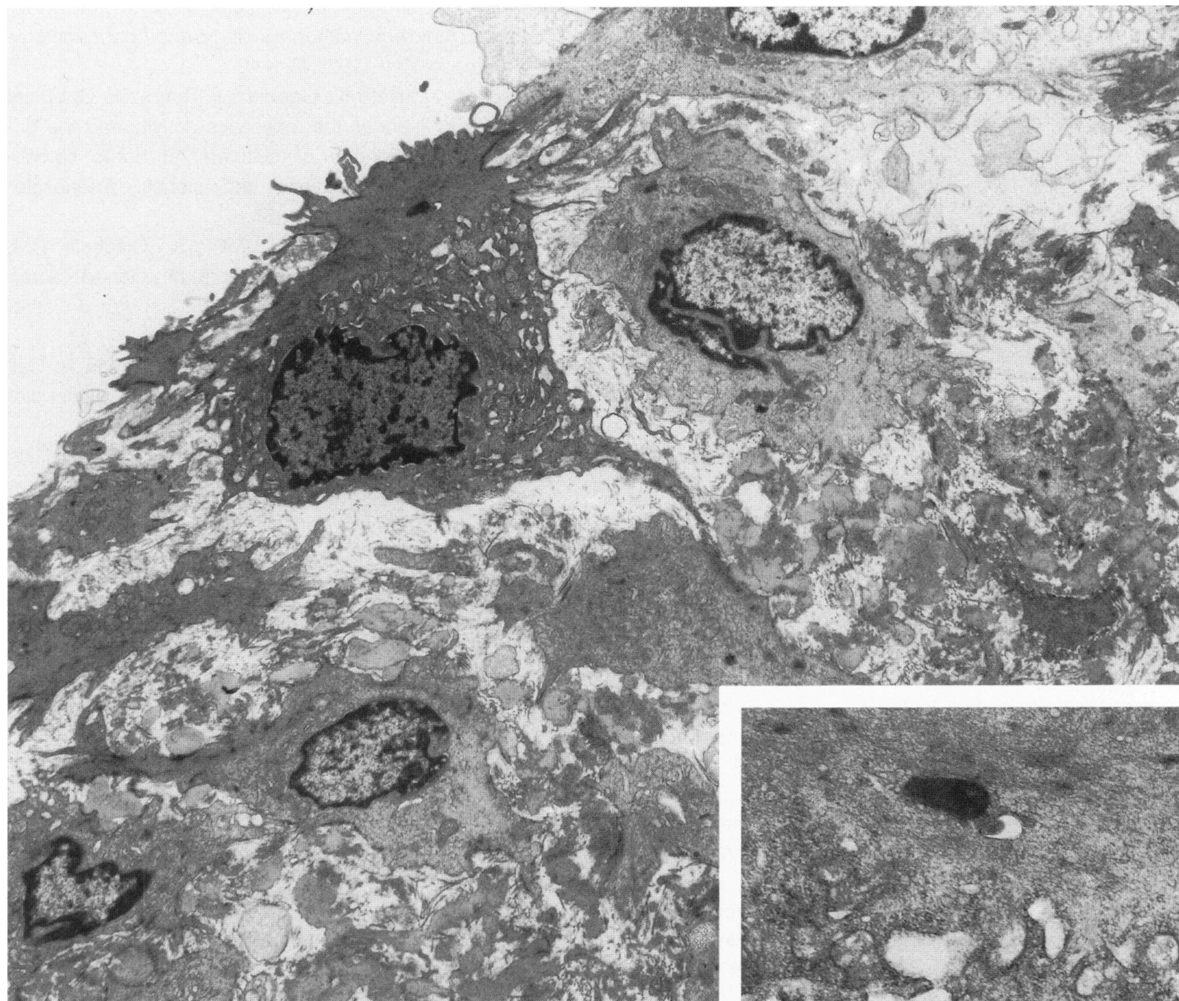

Figure 3. *Isolated cell at the luminal surface of the intimal thickening 15 days after endothelial injury shows signs of initial apoptotic changes such as nuclear and cytoplasmic condensation and cytoplasmic vacuoles. At higher magnification (inset), dilatation of endoplasmic reticulum and a typical microfilamentous bundle are recognizable (×5,900; inset, ×18,600).*

enon plays a role in the regulation of the cell mass during the evolution of endothelial denudation-induced intimal thickening. A better understanding of this phenomenon may shed some light on mechanisms involved in arterial stenotic changes.

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