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Review

Smooth muscle cell apoptosis in arteriosclerosis

Manuel Mayr[a], Qingbo Xu[b,*]

[a]*Institute for Biomedical Aging Research, Austrian Academy of Sciences, Rennweg 10, 6020 Innsbruck, Austria*
[b]*Department of Cardiological Sciences, St George's Hospital Medical School, Cranmer Terrace, London SW17 0RE, UK*

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Abstract

Arteriosclerosis, a paradigmatic age-related disease, encompasses (spontaneous) atherosclerosis, restenosis after percutaneous transluminal coronary angioplasty, autologous arterial or vein graft arteriosclerosis and transplant arteriosclerosis. In all types of arteriosclerosis, vascular smooth muscle cell (SMC) accumulation in the intima is a key event, but abundant evidence also indicates the importance of SMC apoptosis in the development of arteriosclerosis. Because SMC proliferation and apoptosis coincide in arteriosclerotic lesions, the balance between these two processes could be a determinant during vessel remodeling and disease development. Various stimuli, including oxidized lipoproteins, altered hemodynamic stress and free radicals, can induce SMC apoptosis in vitro. As risk factors for arteriosclerosis, these stimuli may also lead to vascular cell apoptosis in vivo. The presence of apoptotic cells in atherosclerotic and restenotic lesions could have potential clinical implications for atherogenesis and contributes to the instability of the lesion. Based on the progress in this field, this review focuses on the mechanism and impact of SMC apoptosis in the pathogenesis of arteriosclerosis and highlights the role of biomechanical stress in SMC apoptosis.

Keywords: Aging; Apoptosis; Atherosclerosis; Smooth muscle cells; Signaling; Mechanical stress

1. Introduction

Cell death is accomplished via necrotic or apoptotic pathways that can be discriminated

* Corresponding author. Tel.: +44-8725-2817; fax: +44-8725-2812.
E-mail address: q.xu@sghnas.ac.uk (Q. Xu).

Abbreviations: Akt: Protein kinaseB; ApoE −/−: Apolipoprotein E deficient; EC: Endothelial cell; Fas: CD95; FasL: Fas ligand; IGF-1: Insulin-like growth factor 1; JNK: c-jun NH_2-terminal protein kinases = SAPK; LDL: Low-density lipoproteins; MAPK: Mitogen-activated protein kinases; NO: Nitric oxide; oxLDL: Oxidized low-density lipoproteins; PS: Phosphatidylserine; PKC: Protein kinase C; ROS: Reactive oxygen species; SAPK: Stress-activated protein kinases = JNK; SMC: Smooth muscle cell; TNFα: Tumor necrosis factor-α; TUNEL: Terminal deoxynucleotide transferase-mediated dUTP-biotin nick end labeling

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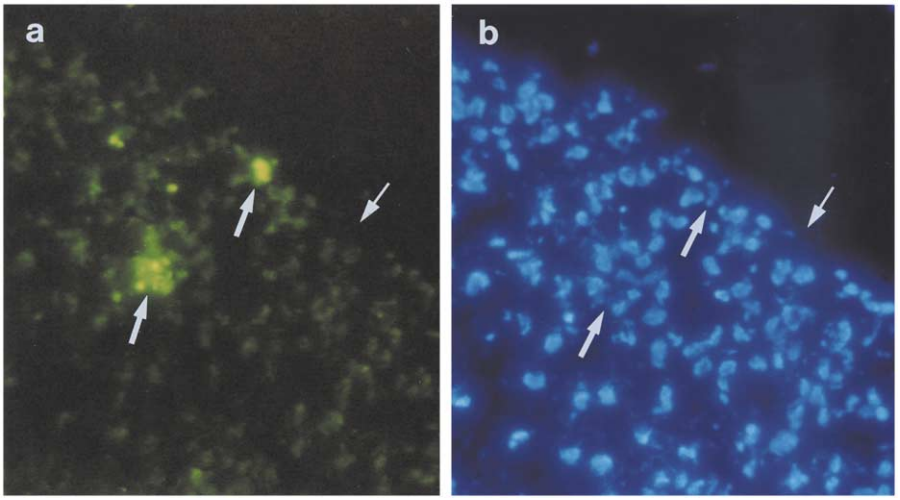

Fig. 1. Apoptotic cells in atherosclerotic lesions. TUNEL (a) and Hoechst 33258 (b) stained sections from rabbits receiving cholesterol-enriched diets for 16 weeks. Arrows indicate endothelial surface, and fat arrows point to examples of positive-stained cells, original magnification 250×.

on the basis of morphological studies and the propensity for necrotic, but not apoptotic, cells to induce an inflammatory response. Cells usually die by apoptosis, often described as "silent death" or "programmed cell death", because this process is controlled by intrinsic cellular mechanisms. However, in case of severe injury, cells may instead undergo necrosis, a passive death resulting in cellular lysis. Apoptosis encompasses at least two stages, including the early stage of signal initiation and the later stage of final execution (Earnshaw, 1995) characterized by DNA fragmentation, alterations of nucleus morphology (chromatin condensation and nucleus fragmentation), organelle relocalization, and cell fragmentation without leakage of cytosolic macromolecules. Primary necrosis (or oncosis) is characterized by cellular swelling, organelle alterations, rupture of plasma membrane, and finally cell lysis and leakage of the cellular components. Under both circumstances, dead cells are rapidly cleared from the body. But the balance of apoptosis and necrosis determines the biological response of the phagocytes, excessive necrosis leading to inflammation and immunity while predominance of apoptosis, e.g. during normal tissue turnover, stimulates tolerance to the self-displayed peptides (Green, 2000). Thus, each death process can have long-term consequences by altering the behavior of the phagocytes.

The atherosclerotic lesion is defined by arterial intimal and SMC proliferation, lipid accumulation, and connective tissue deposition (Ross, 1993; Steinberg and Witztum, 1990). Arteriosclerosis is characterized by SMC hyperplasia or hypertrophy and matrix protein accumulation in the intima and/or media with or without lipid deposition, resulting in thickening and stiffness of the arterial wall. Vascular SMCs as a main cell component of arteriosclerotic lesions are believed to be crucial in the development of vascular diseases. Although most studies concerning the role of SMCs emphasize cell proliferation, vascular SMC apoptosis in atherosclerotic lesions, neointima of restenosis, and vein graft arteriosclerosis has been

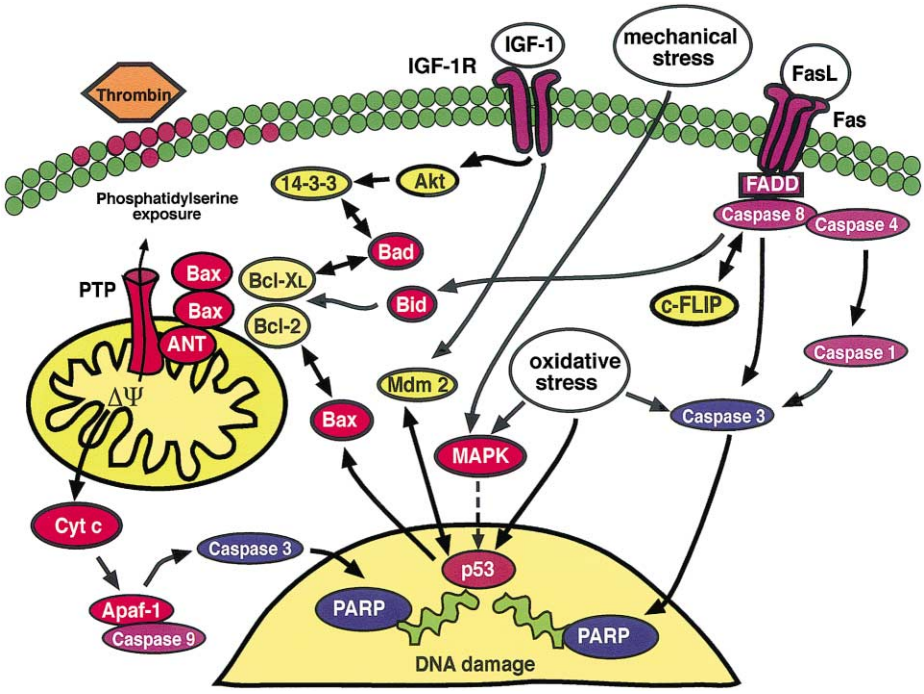

Fig. 2. Signaling pathways in SMC apoptosis. Exogenous signaling pathways involve death receptors like tumor necrosis factor receptor (TNF-R) and Fas (CD95). Trimerization of death receptors by death receptor ligands such as TNFα and FasL recruits caspase 8 via cytoplasmatic death domain and adapter proteins like FADD. Caspase 8 self-activates and cleaves the effector caspase 3 or Bid, a pro-apoptotic member of the Bcl-2 family linking the exo- with the endogenous death pathway by antagonizing Bcl-2 and Bcl-xL. These anti-apoptotic Bcl-2 family members are located at the outer mitochondrial membrane, bind the pro-apoptotic protein Bax and prevent the opening of the mitochondrial permeability transition pores (PTP), a crucial event during apoptosis resulting in loss of mitochondrial membrane potential ($\Delta\psi$) and cytochrome *c* (Cyt *c*) release. Cyt *c*, in concert with the adapter protein Apaf-1 and caspase 9, activates caspase 3 mediating downstream events like PARP cleavage. Stimuli such as mechanical or oxidative stress act through this mitochondrial pathway, for example, by p53 activation and Bax expression. Additionally, survival signals like IGF-1 are capable of modulating cell death. In response to IGF-1, Akt inhibits mitochondrial translocation of Bad, another pro-apoptotic Bcl-2 family member, releasing Bax from anti-apoptotic Bcl-2 family members. Survival factor withdrawal results in dephosphorylation and release of Bad from the cytoplasmatic binding protein 14-3-3. Abbreviations: ANT, adenine nucleotide translocator; Apaf-1, apoptotic protease activating factor-1; FADD, Fas-associated death domain protein; c-FLIP, FLICE inhibitory protein; IGF-1R, insulin-like growth factor-1 receptor; PARP, poly(ADP-ribose) polymerase.

reported repeatedly (Isner et al., 1995, Bennett and Boyle, 1998; Haunstetter and Izumo, 1998; Kockx, 1998). Therefore, studies on SMC apoptosis could help us understand the molecular mechanisms of vascular diseases.

2. Apoptosis in atherosclerosis

Atherogenesis is a slowly progressive disease (Stary, 1989). Depending on their size

and composition, lesions are usually divided into fatty streaks, early stages of lesions, and advanced stages of atherosclerosis called plaques. The three major cellular components of human atherosclerotic plaques are SMCs, which dominate the fibrous cap; macrophages, which are the most abundant cell type in the lipid-rich core; and lymphocytes, which have been mainly ascribed to the fibrous cap (Ross, 1993).

Recent studies demonstrate the presence of apoptotic cells in atherosclerotic lesions (Fig. 1; Geng and Libby, 1995; Bennett et al., 1995; Han et al., 1995; Kockx, 1998). Apoptotic cells in atherosclerotic lesions were restricted to the intima of the diseased arteries. The media contained levels of apoptosis similar to that in normal vessels. Both macrophages and SMCs, the two major cell types in the atherosclerotic intima, bear markers of apoptosis. Apoptotic SMCs localized mainly in the fibrous cap of the atheroma, whereas macrophages underwent apoptosis in the vicinity of the necrotic core (Kockx and Herman, 2000). Increased apoptosis in plaque SMCs is due to increased sensitivity to death induced by death receptors and the tumor suppressor gene p53, and failure to endogenous protection through IGF-1 (insulin-like growth factor 1), which activates Akt (protein kinase B) and inhibits cytochrome *c* release of mitochondria (Fig. 2; Mc Carthy and Bennett, 2000).

SMC apoptosis is linked closely to interactions with macrophages. The ratio of monocytes to SMCs in lesions is about 2:1. The local environment and macrophage priming of SMCs is required for SMCs to respond to apoptotic stimuli (Kockx and Herman, 2000). Monocyte maturation into macrophages is also necessary for effective killing. Differentiation of monocytes is accompanied by a decrease of death receptors and an increase of ligands like tumor necrosis factor α (TNFα) and Fas ligand (FasL). Furthermore, macrophages express high levels of inducible nitric oxide (NO) synthase. NO primes the SMCs to die either by DNA damage activating p53 or by increasing expression of the death receptor ligands like TNFα or FasL leading to caspase activation. It has been shown that p53 is able to induce the translocation of Fas (CD95) from the cytosol to the cell surface (Bennett et al., 1998). Fas^+ SMCs localize in the intima of the plaques, whereas the medial SMCs express the Fas antigen less prominently (Geng et al., 1997). Fas co-localizes with TUNEL-positive SMCs in areas that contain CD3+ T-cells and CD68+ macrophages, suggesting a role of the FasL death-signaling pathway in the induction of SMC apoptosis and downregulation of vascular immune responses. Overexpression of adenovirus encoding FasL accelerated SMC apoptosis in vivo, inhibited intimal hyperplasia and allowed the adenovirus-harboring cells to evade immune destruction (Sata et al., 1998). Despite Fas expression, many cells resist Fas-induced apoptosis. Similarly, SMCs show a marked heterogeneity to Fas-induced apoptosis. Soluble FasL and agonistic anti-Fas antibodies can only trigger SMC apoptosis in vitro after pretreatment with cycloheximide or cytokines to stimulate Fas expression. Sensitivity is determined by differences in surface Fas expression as well as below receptor levels (Chan et al., 2000). Additionally, soluble FasL and agonistic anti-Fas antibodies might be less efficient in clustering the cell-surface receptors than membrane-bound FasL after adenovirus transfection.

SMCs derived from plaques or restenosis are more sensitive to p53-mediated apoptosis (Bennett et al., 1997). In contrast, human medial SMCs from normal coronary arteries are resistant to apoptosis by either p53 or death receptors/cytokines. p53 is the most commonly mutated tumor suppressor gene in human cancers. As the guardian of the

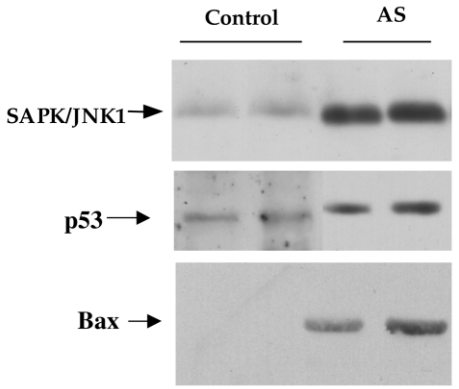

Fig. 3. Elevated SAPK/JNK, p53 and Bax proteins in atherosclerotic lesions. Normal intima and media (control) from chow-fed rabbits and atherosclerotic intima (AS) from cholesterol-fed rabbits (16-week diet) were dissected, frozen in liquid nitrogen and homogenized with polytron homogenizer. Protein extracts (50 μg/lane) were separated on 10% SDS-polyacrylamide gel, transferred onto membrane and probed with the antibody against SAPK/JNK1, p53 or Bax. Immunocomplexes were visualized by a Western blot detection kit.

genome, it exerts at least two main functions to prevent uncontrolled cell proliferation: First, it mediates G_1 cell cycle arrest to allow the repair of damaged DNA, and second, it induces apoptosis to eliminate cells with seriously damaged genomes (Agarwal et al., 1998; Chao et al., 2000). A number of genes have been identified as transcriptional targets regulated by p53, including the cyclin-dependent kinase inhibitor, $p21^{WAF-1}$ (Hsieh et al., 2000) and the pro-apoptotic gene, Bax (Brady and Gil-Gomez, 1998). Consistent with these data, adenoviral transfer of p53 accelerated carotid atherosclerotio plaque composition in apolipoprotein E deficient (apoE −/−) mice. Intimal cross-section areas were unchanged between both groups, but p53 gene transfer caused extensive cell loss and thinning of the fibrous cap, resulting in a more destabilized plaque (Von der Thüsen et al., 2000). Additionally, plaque-derived SMCs are not protected by IGF-1 due to decreased expression of IGF-1 receptors and expression of IGF-binding proteins that competitively inhibit IGF-1 binding to IGF-receptors (Mc Carthy and Bennett, 2000). In cardiac myocytes, IGF-1 induces Mdm2 and downregulates p53 by formation of Mdm2–p53 complexes, which attenuated the susceptibility of myocytes to stretch-mediated apoptosis (Leri et al., 1999).

Stress-activated protein kinases (SAPKs) or c-jun NH_2-terminal protein kinases (JNKs) are thought to be crucial in transmitting transmembrane signals required for cell differentiation and apoptosis in vitro and in vivo (Tournier et al., 2000; Hu et al., 1997). Immunofluorescence analysis revealed abundant and heterogeneous distribution of pan- and phosphorylated-SAPK/JNK, which were mainly localized in cell nuclei of the lesional cap and basal regions (Metzler et al., 2000). SAPK/JNK protein levels were two- to three-fold higher in protein extracts from atherosclerotic lesions than in vessels of chow-fed rabbits. Interestingly, increased SAPK/JNK in lesions was co-localized or coincided with high levels of transcription factor p53, as identified by double-labeling and immunoprecipitation (Fig. 3; Metzler et al., 2000). Thus, SAPK/JNK, together with p53, persistently

hyperexpressed and activated in lesions, may play a key role in mediating cell apoptosis during the development of atherosclerosis e.g. by stimulating Bax expression in SMCs.

3. Apoptosis in restenosis

Interestingly, apoptotic cell death is positively correlated to cell replication. Proliferating SMCs show more apoptotic cell death than non-proliferating SMCs (Bennett and Boyle, 1998). Vascular restenotic lesions with high replication rates also demonstrate more apoptotic nuclei (Han et al., 1995). Inverse correlations were observed between the density of SMCs and the frequency of apoptotic cell death (Bauriedel et al., 1998). A decrease in apoptosis may contribute to restenotic hyperplasia by prolonging the life span of intimal cells. The presence of p53 is pivotal in the regulation of apoptosis in SMC growth, probably through the Bax pathway (Aoki et al., 1999; Ihling et al., 1999). Antisense strategy against p53 results in abnormal SMC growth and increased lesion formation (Matsushita et al., 2000).

A rapid onset of apoptosis in medial SMCs was observed within hours after balloon injury, coinciding with a downregulation of Bcl-xL (Perlman et al., 1998; Pollman et al., 1999). This first wave of apoptosis seems to be associated with the redox-sensitive activation of stress-activated protein kinases. Acute induction of cell death is followed by a second wave of apoptosis in which p53 might play a crucial role. Post-angioplasty coronary restenosis is an accelerated form of arteriosclerosis associated with increased p53 production (Speir et al., 1994). In humans, prior cytomegalovirus infection inactivating p53 accelerates development of post-atherectomy coronary restenosis (Zhou et al., 1996; Tanaka et al., 1999). While early death might induce a greater wound healing process to overcome initial cell loss, apoptosis at later stages seems to counteract cell proliferation and control lesion formation. In a rat carotid injury model, p53 gene transfer decreases neointimal formation by promoting apoptosis (Scheinman et al., 1999). The transcription factor p53 is associated with upregulation of Bax (Fig. 3) and Bcl-xL, pro- and anti-apoptotic members of the Bcl-2 family. SMC survival is determined at a number of signaling levels, among which the ratio of members of the Bcl-2 family determines cell fate. Extracellular stimuli to anti-apoptotic Bcl-xL include growth factors and cell–matrix interactions. Pollman et al. (1998) demonstrated that inhibition of neointimal cell Bcl-xL expression induces apoptosis and regression of vascular disease, which demonstrates that the upregulation of anti-apoptotic genes, such as Bcl-2 and Bcl-xL, plays a fundamental role in preserving intimal viability during formation of hypercellular intimal lesions. It is noteworthy that balloon injury to vessels with pre-existent neointimal lesions resulted in medial cell death, but relative preservation of neointimal cell viability. A possible explanation for this observation might be the increased Bcl-xL expression as demonstrated in neointimal SMCs providing protection against apoptosis.

4. Apoptosis in vein graft arteriosclerosis

Autologous vein grafts remain the only surgical alternative for many types of vascular reconstruction. However, the patency rate is limited due to obliterative stenosis that

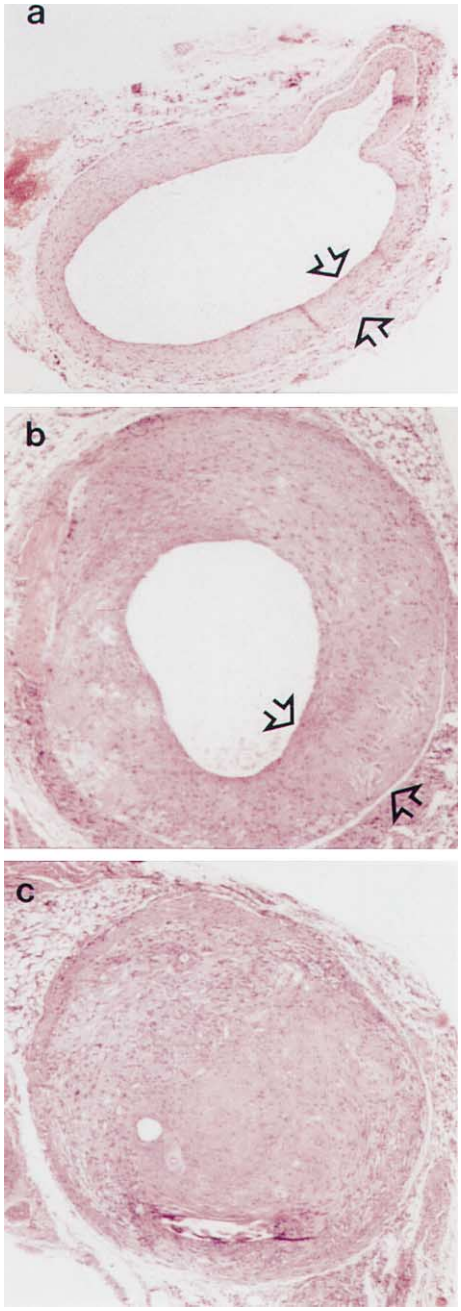

Fig. 4. Hematoxylin-eosin- (HE-) stained sections of mouse vein grafts. Under anesthesia, vena cava veins of apoE +/+ (a) or −/− (b,c) mice were removed and isografted into carotid arteries of apoE +/+ (a) or −/− (b,c), respectively (Dietrich et al., 2000). Animals were sacrificed 8 weeks after surgery, and the grafted tissue fragments fixed in 4% phosphate-buffered (pH 7.2) formaldehyde, embedded in paraffin, sectioned, and stained with HE. Arrows indicate lesions, original magnification 40×.

occludes 50% of the grafted vessels within 5 years after the operation. Although veins do not develop spontaneous arteriosclerosis, accelerated arteriosclerosis occurs rapidly in venous bypass grafts, which bear increased biomechanical forces due to alterations in blood pressure, i.e. vein (0–30 mmHg) versus artery (120 mmHg). Thickening of the thin vessel wall is a response to the new high-pressure environment that the grafted vein must withstand. The pathogenesis of this disease is poorly understood, and no successful clinical intervention is available. Vein grafts become occluded when abnormal cell proliferation in the smooth muscle layer produces extra tissue in the inner lining of the vessel, a process called neointimal hyperplasia (Vlodaver and Edwards, 1971). Moreover, neointimal cells also express an unusual amount of adhesion molecules, cytokines and growth factors that attract atherosclerotic deposits (Motwani and Topol, 1998). Arteriosclerosis, the major cause of late vein graft occlusion, yields lesions that, in this context, are prone to rupture and embolization. Typical plaques occurring in vein grafts have poorly developed fibrous caps, their necrotic core and secondary dystrophic calcified deposits develop adjacent to the lumen rather than deep underneath the surface, as in typical native atherosclerosis. Similar to native arteries, secondary thrombosis may be initiated by plaque rupture.

Recently, we established the first mouse model of vein graft arteriosclerosis by grafting autologous jugular veins or vena cava to carotid arteries (Fig. 4; Zou et al., 1998). The availability of genetically modified mouse models provides a new investigative tool that will result in a clearer understanding of the molecular mechanisms in vein graft disease. In many respects, the morphological features of this murine vascular graft model resemble those of human venous bypass graft disease.

Using this model, we demonstrated that one of the earliest events after grafting veins to arteries is cell death, in which mechanical stress is a critical initiator of SMC apoptosis (Mayr et al., 2000). This early onset of cell death is followed by massive mononuclear cell infiltration, but occurs before SMC proliferation, peaks at 4 weeks after the surgical procedure and declines thereafter (Mayr et al., 2000). Importantly, apoptotic/necrotic cells, as determined by TUNEL assay, were significantly higher in veins grafted into apoE $-/-$ mice than wildtype mice, although a similar number of proliferating cell nuclear antigen positive cells in both types of lesions was found (Dietrich et al., 2000). Interestingly, vascular SMCs cultivated from aortas of apoE $-/-$ mice showed a high rate of spontaneous apoptosis/necrosis and a higher rate of cell death stimulated by the NO donor sodium nitroprusside, H_2O_2 and oxidized low-density lipoproteins (oxLDL) (Dietrich et al., 2000). Thus, the pathogenic mechanism of vein graft arteriosclerosis involves increased intimal cell death initiated by biomechanical stress and amplified by hypercholesterolemia, which leads to continuous recruitment of blood mononuclear cells to constitute atheromatous lesions.

We hypothesize that cell death evokes an inflammatory response, followed by SMC proliferation. The mechanism by which monocytes/macrophages are continuously recruited to the neointima of the vessel wall may involve chemokine-like substances released by dead cells (Horino et al., 1998; Nishiura et al., 1998) contributing to the induction of inflammatory responses in the vessel wall (Miwa et al., 1998; Zou et al., 2000). The molecular mechanisms of SMC proliferation in vivo remain to be elucidated. Given the fact that SMC accumulation in the intima occurs

between 4 and 8 weeks post-operatively (Hu et al., 1999), we postulate three possibilities: (1) Phenotypic selection; the phenotype of neointimal SMCs differs from medial SMCs, increased resistance against apoptosis being one of their striking features (Pollman et al., 1999). Elevated biomechanical forces result in apoptosis of a large portion of SMCs. The small remaining population of SMCs can sustain the arterial blood pressure and may expand rapidly to form neointima. (2) Inflammatory response; monocytes/macrophages in vein grafts can release a panel of cytokines and growth factors, such as tumor growth factor-β1 and fibroblast growth factor, which lead to SMC apoptosis or proliferation (Bryant et al., 1999). (3) Concomitantly, a marked loss of SMCs has been observed in early lesions of human vein grafts (Kockx et al., 1994). Thus, cell death at early stages of vein graft disease could influence SMC migration, proliferation and accumulation in the intima of vein grafts and exacerbate lesions at later time points.

5. SMC heterogeneity and apoptosis

Populations of SMC are phenotypically distinct within a given blood vessel and also between blood vessels of the same vascular bed. From a morphological point of view, one can discriminate at least two phenotypes of vascular SMCs, namely, the epitheloid and the spindle-shaped cells, coinciding with the more functional in vivo classification of synthetic and contractile cell types, respectively (Gittenberg-de Groot et al., 1999). In general, there is a preference of the synthetic type in the intima and the contractile type in the media of adult vessels. Interestingly, the epitheloid phenotype, often referred to as a non-muscle phenotype because it lacks contractile differentiation markers, is more prone to apoptosis. When culture conditions are sub-optimal, the spindle-shaped cell type can alter to the epitheloid phenotype. Whether such a transition is also possible in vivo remains to be determined. However, in vivo, sub-populations of SMCs within the same tissue express different levels of caspase 3 and show different sensitivities to apoptosis (Chan et al., 2000). Intimal thickening is linked to re-expression of fetal markers, indicating dedifferentiation of SMCs. This re-expression of fetal markers is accompanied by increased apoptosis (Kockx et al., 1994; Slomp et al., 1997).

6. oxLDL-induced apoptosis

Among a wide variety of biological properties, oxLDL exhibits a dramatic cytotoxic effect on several vascular cell types, including SMCs, ECs and macrophages (Escargueil-Blanc et al., 1997). The cytotoxic effect depends on concentration and oxidative modification. High concentrations of oxLDL are pro-apoptotic, whereas low concentrations are mitogenic for vascular SMCs. Regarding the degree of oxidation, Siow et al. provided evidence that moderately oxLDL, with its high lipid hydroperoxide content, seems to be more cytotoxic than mildly or highly oxLDL with its secondary lipid oxidation products, such as aldehydes, ketones, and lipid alcohols (Siow et al., 1999; Napoli et al., 2000). In contrast, protein modifications do not seem to be necessary for LDL-induced cytotoxicity. Interestingly, oxLDL activates p53, but also downregulates IGF-1 and IGF-1 receptors,

whereas native LDL increases IGF-1 expression (Scheidegger et al., 2000), a potent survival factor for vascular SMCs. Binding of IGF-1 to its receptor stimulates phosphatidyl inositol-3 and Akt kinase inhibiting Bad and caspase 9 activation.

In vitro, Bcl-2 did not prevent cell death triggered by oxLDL, but rather induced a shift from apoptosis to necrosis, therefore altering the balance between apoptosis and necrosis by oxLDL (Meilhac et al., 1999). Bcl-2, located predominantly in the outer mitochondrial membrane, the endoplasmic reticulum, and the nuclear membrane, is a prototypic anti-apoptotic protein, but its efficiency depends on the cause and mechanisms of cell death. Three mechanisms are proposed to explain the anti-apoptotic effect of Bcl-2: antioxidant-like effects, inhibition of calcium release by the endoplasmic reticulum and prevention of caspase activation by inhibition of mitochondrial cytochrome *c* release. A sustained calcium rise is a common trigger for both types of cell death and might be a lethal hit. In addition to its inhibitory effect on apoptosis, Bcl-2 can inhibit transactivation of p53-regulated genes (Zhan et al., 1999) including p21 and Bax.

In principle, apoptotic cells are rapidly engulfed and cleared by phagocytic cells (Green, 2000), a possible reason for underestimation of apoptosis in vivo. Recognition and uptake of apoptotic cells is mediated, at least in part, by bridging phosphatidylserine (PS) to receptors on macrophages. PS is exposed predominantly on the surface of apoptotic, but not necrotic, cells. Other potential candidates for PS recognition include CD36, CD68, and CD14. However, these scavenger/pattern-recognition molecules do not appear to discriminate between PS and other anionic phospholipids. For example, CD36 is a well-established scavenger receptor for oxLDL. oxLDL deposits might interfere with the clearance of apoptotic cells, especially within the necrotic core of atherosclerotic plaques (Sambrano and Steinberg, 1995). Additionally, apoptotic cells are known to be under oxidative stress and oxidized phospholipids are present on their surface (Mallat et al., 1999). Similar to PS, these oxidized phospholipids function as ligands to mediate macrophage recognition and facilitate engulfment of apoptotic bodies. Autoantibodies to oxLDL cross-react with oxidized phospholipids on apoptotic, but not viable, cells inhibiting their uptake by phagocytes (Shaw et al., 2000). These data suggest that hypercholesterolemia has direct and indirect influences on cell death. First, high concentrations of oxLDL are cytotoxic, while low concentrations of oxLDL stimulate cell proliferation. Second, oxLDL interferes with the scavenger receptor CD36 and competitively inhibits the recognition of apoptotic cells by macrophages. Third, autoantibodies to oxLDL bind to oxidized phospholipids masking an important antigen that facilitates macrophage recognition of apoptotic cells.

7. Mechanical stress-induced apoptosis

The vessel wall is an integrated functional component of the circulatory system and subject to continuous remodeling in response to hemodynamic or biomechanical stress, a process that contributes to the development of arteriosclerosis (Xu, 2000). In vivo, the vessel wall is exposed to two main hemodynamic forces: shear stress, the dragging frictional force created by blood flow, and mechanical stretch, a cyclic strain stress created by blood pressure (Davies, 1995; Patrick and McIntire, 1995). Shear stress stimulates

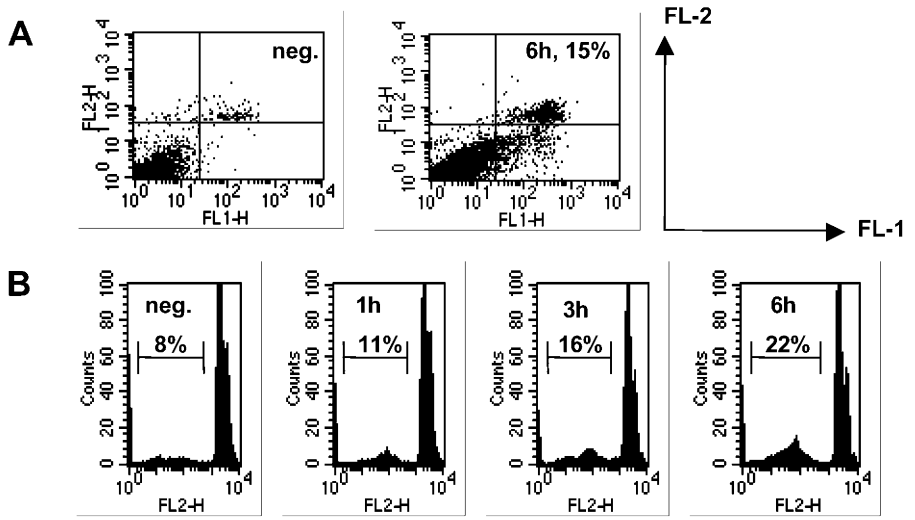

Fig. 5. SMC apoptosis after mechanical stress. SMCs were cultured on elastomer-bottomed plates and subject to cyclic strain stress by application of computer-controlled vacuum. 18 h after the onset of mechanical stress (6 h, 15% elongation), cells were stained by annexin/propidium iodide (PI) double-staining to detect PS exposure on the cell surface (Mayr et al., 2000). Rat arterial SMCs showed a marked increase in annexin V-FITC$^+$/PI$^-$ and annexin V-FITC$^+$/PI$^+$ cells in the lower and upper right quadrants, respectively, reflecting early and late stages of apoptosis (A). In addition to changes in the membrane asymmetry, nuclear changes like DNA fragmentation could be demonstrated by PI staining of cellular nuclei (B). Human arterial SMCs were treated with mechanical stress (15% elongation) for 0, 1, 3 and 6 h, harvested 24 h later, and stained with PI for DNA content after cell lysis (Mayr et al., 2000). FL2-histograms indicate the percentage of nuclei in the sub-G1 population representing DNA fragmentation. Note the time-dependent increase of percent apoptotic cells induced by mechanical stress.

endothelial cells (EC) to release NO (Rubanyi et al., 1986) and prostacyclin (Bhagyalakshmi and Frangos, 1989), resulting in vessel relaxation and protection of vascular cells, whereas cyclic strain stress, the pulsatile deformation of the vessel wall, influences both ECs and SMCs (Vinters and Berliner, 1987). Lack of hemodynamic forces triggers apoptosis in vascular ECs (Kaiser et al., 1997), hinting at mechanical forces as essential stimuli for the maintenance of blood vessels.

Apoptosis is involved in the regression of human umbilical vessels and the ductus arteriosus during neonatal remodeling (Kim et al., 2000). Upregulation of pro-apoptotic Bax and Bcl-xS was observed at vascular sites where SMCs undergo apoptosis on hemodynamic changes during birth. Evidence of SMC apoptosis in the neonate has also been found at the branching points of great arteries with exposure to disturbed blood flow, whereas SMC viability was preserved in areas with regular flow pattern. While in physiological remodeling, decreased flow and perturbation of flow patterns contribute to SMC apoptosis, enhanced wall tension and loss of matrix interactions seem to be major triggers of cell death under pathological circumstances. The microvascular rarefaction or disappearance of microvessels in hypertension might be one example for consequences of

increased biomechanical wall stress. Reminiscent of the situation in the neonate, SMCs derived from those vessels showed an increased expression of Bax compared to normotensive animals, indicating higher susceptibility to apoptosis (Hamet et al., 1996). Indeed, increased SMC apoptosis could be demonstrated in spontaneously hypertensive rats compared to normotensive animals (DeBlois et al., 1997; Vega et al., 1999).

When mouse, rat and human arterial SMCs cultured on a flexible membrane were subjected to cyclic strain stress, apoptosis was observed in time- and strength-dependent manners. All three types of SMCs showed apoptotic death, as confirmed by TUNEL, propidium iodide and annexin V staining (Fig. 5; Mayr et al., 2000). To study the signal pathways leading to apoptosis, activities of p38 mitogen-activated protein kinases (MAPK) were determined. Mechanical stress results in p38 MAPK activation (Li et al., 2000). SMC lines stably transfected with a dominant negative Rac, an upstream signal transducer, or overexpressing MAPK phosphatase-1 (Li et al., 1999, 2000), a negative regulator for MAPKs, completely inhibited mechanical stress-stimulated p38 activation, and abolished mechanical stress-induced apoptosis (Mayr et al., 2000; Li et al., 2000). Mechanical stress-induced apoptosis is, at least in part, mediated by p38 MAPK pathways responsible for transducing signals leading to apoptosis. There is evidence that p38 MAPKs can phosphorylate p53 (Bulavin et al., 1999), leading to its activation (Milne et al., 1995). In cardiac myocytes, mechanical stress can induce cell death by directly or indirectly activating p53, which decreases the Bcl-2-to-Bax protein ratio in the cell (Leri et al., 1998). Since p53 is an essential molecule in both cell proliferation and apoptosis, activation of p53 may be an important factor in the development of arteriosclerosis.

8. Characteristics of SMC apoptosis

As described above, SMCs derived from atherosclerotic plaques are more sensitive to apoptosis in vitro. Similar to other cells, SMCs undergo apoptosis in response to a broad array of stress via exo- and endogenous pathways, e.g. Fas/FasL binding or exposure to free radicals, involving death receptors or mitochondria, respectively (Fig. 2). However, differences in SMC apoptosis exist. For example, TNFα, interleukin-1β or interferon-γ alone cannot effectively induce SMCs apoptosis (Geng et al., 1997). Cultured SMCs undergo apoptosis in response to combinations of various cytokines, growth factor deprivation, and treatment with NO donors or oxLDL (Dietrich et al., 2000; Xu et al., 1997). Moreover, exposure to certain pharmacological agents, i.e. calcium channel blockers, causes SMC apoptosis (Stead et al., 2000), and pyrrolidinedithiocarbamate and *N*-acetylcysteine, two widely used antioxidants (Tsai et al., 1996), prevent EC apoptosis but induce SMC apoptosis.

Inhibition of the protein kinase C (PKC) by calphostin C causes a transient increase in SMC apoptosis, returning to baseline levels after 1 week of exposure (Leszczynski et al., 1994). Induction of apoptosis via a PKC-dependent mechanism is prevented by supplementing the culture medium with serum, a striking similarity with the regulation of apoptosis by the c-myc-dependent pathway. Rat SMCs express alpha, beta, delta, epsilon and zeta isozymes of PKC (Leszczynski et al.,

1996). Selective depletion of PKCα by anti-sense oligodeoxynucleotides attenuated SMC proliferation but did not result in increased apoptosis, indicating that another PKC isoform is likely to be involved in SMC apoptosis.

The importance of PKC in SMC apoptosis is underlined by the finding that PKC seems to convert the reactive oxygen species- (ROS-) induced signals from necrotic cell death to activation of the apoptotic cell death program. Phorbol 12-myristate 13-acetate, a PKC activator, increases NAD(P)H oxidase activity in SMCs. Vascular tissues are rich sources of ROS, including superoxide, peroxynitrite, hydrogen peroxide and NO. Increased levels of oxidatively modified proteins and lipoproteins, and advanced glycosylation end products have been identified in patients with vascular disease. The plasmalemmal NADH/NADPH oxidase and mitochondria are key sources of ROS in vascular tissues, and it has been established that SMCs and fibroblasts account for the majority of superoxide in the normal vessel wall (Griendling et al., 1994). Production of superoxide in the vessel wall has been shown to inactivate NO, leading to peroxynitrite formation and impaired endothelium-dependent vasodilatation, oxidize LDL, increase adhesion molecule expression resulting in monocyte infiltration, and activate matrix metalloproteinases, which are involved in vascular remodeling (Griendling et al., 2000). NAD(P)H-derived superoxide and hydrogen peroxide are intimately involved in SMC growth and apoptosis. Pretreatment with physiologically relevant doses of vitamin C protects SMCs against apoptosis induced by oxidative stress and lipid hydroperoxides by increasing levels of the key intracellular antioxidant glutathione (Siow et al., 1998) and expression of the anti-apoptotic mediator Bcl-xL (Siow et al., 1999).

In ECs, mechanical forces stimulate NAD(P)H oxidase activity. Laminar shear results in a transient activation, whereas oscillatory shear causes a sustained increase in NAD(P)H oxidase activity. Overall, ECs are more sensitive to ROS-mediated damage than SMCs (Griendling et al., 2000). Although ROS serve as second messenger in vascular cells to mediate short-term signaling events, the long-term effects of these radicals induce cellular damage and may potentiate lesion formation.

Extracellular adenosine may be part of an amplification mechanism for vascular SMC apoptosis (Peyot et al., 2000). Adenosine causes SMC apoptosis beyond a certain concentration and additionally enhances NO production. An initial event, such as hypoxia or free radicals may stimulate a first wave of adenosine release. Adenosine generated from ATP released from dead cells might, in turn, be cytotoxic to surrounding cells. A similar cascade of events might occur when FasL expressing SMCs (Chan et al., 2000) or peroxides emitted by apoptotic cells (Reznikov et al., 2000) kill their neighboring cells in a paracrine manner.

9. SMC apoptosis and plaque instability

Cell death in the vessel wall is considered a two-sided sword depending on the amount and cell type involved. On one hand, cell death contributes to cell homeostasis during vascular remodeling and may help to antagonize overwhelming proliferation. In particular, apoptosis of macrophages could be beneficial for plaque stability if less matrix metalloproteinases and inflammatory cytokines are released. On the other hand, loss of SMCs

can be detrimental, since they produce most of the interstitial collagen fibers important for the tensile strength of the fibrous cap (Kockx and Herman, 2000). Further weakening of the fibrous cap could be due to inflammatory reactions, which might be triggered by cell death in the vessel wall. Interferon-γ produced by activated T-lymphocytes inhibits SMC collagen production, facilitating fibrous cap rupture.

Although apoptosis is believed to evoke inflammation responses in tissues rarely (Granville et al., 1998), apoptotic cells release the S19 ribosomal protein dimer (Horino et al., 1998), a chemokine for macrophages/monocytes by molecular mimicry to complement C5a (Nishiura et al., 1998). Apoptotic blebs containing biologically active oxidized phospholipids stimulate endothelial E-selectin expression and induce monocyte–endothelial interactions, suggesting oxidized phospholipids as active principles important for chronic inflammation. The infiltrating cells might remove apoptotic bodies (Björkerud and Björkerud, 1996; Isner et al., 1995) and paracrine stimuli such as cytokine secretion during phagocytosis could enhance infiltration and initiate an overwhelming repair mechanism. Thus, cell death may be a primary force for the induction of inflammatory responses.

If phagocytes do not scavenge apoptotic bodies, they activate thrombin and promote plaque thrombosis. Plaque thrombogenicity is related directly to its extracellular tissue factor, the key element in the initiation of the extrinsic coagulation cascade pathway (Tedgui and Mallat, 2000). The acellular lipid-rich core, the most thrombogenic part of an atherosclerotic plaque, also exhibits the most intense tissue factor staining. Enhanced extracellular activity of tissue factor directly correlates to the presence of shed membrane microparticles bearing PS and tissue factor. To be active, tissue factor must reside in an appropriate phospholipid environment, such as that provided by plasma membranes containing PS, which increases the catalytic efficiency of the tissue factor/factor VIIa complex. During cell apoptosis, PS, a potent procoagulant activity, is redistributed from membrane leaflets facing the cytosol to the cell surface. Apoptotic cells are known to be involved in microparticle shedding into the extracellular space. A direct relationship was established between the degree of apoptosis in cultured cells and the proportion of released microparticles stemming from surface blebs. Until now, microparticles in the atherosclerotic plaque were thought to originate exclusively from macrophages and lymphocytes, but recent data demonstrated that SMCs are another source of tissue-factor-containing microparticles in atherothrombosis (Schecter et al., 2000). Additionally, oxidized membrane vesicles are released in the circulation of patients with cardiovascular disease and are linked to coagulation (Mallat et al., 1999; Tedgui and Mallat, 2000).

Vascular calcification is also a potent predictor of future cardiovascular events in asymptomatic patients (Wexler et al., 1996). Recent evidence indicates that calcification might be initiated by apoptotic bodies, because matrix vesicles derived from apoptotic SMCs can act as nucleating structures for calcium crystal formation in the vessel wall (Proudfoot et al., 2000). Notably, SMC apoptosis is increased in unstable rather than stable lesions, supporting the involvement of SMC apoptosis in lesion instability promoting plaque rupture and its clinical consequences (Bauriedel et al., 1998).

10. Perspective

In summary, the past several years have seen a dramatic increase in the number of

studies of cell apoptosis in cardiovasculature during the response to various stressors or risk factors for arteriosclerosis. These studies may lead to a new strategy for understanding the mechanisms of the pathogenesis and for prevention and treatment of cardiovascular diseases. However, several open questions remain with regard to the role of apoptosis in vascular tissue, i.e. is SMC apoptosis in the early stage of arteriosclerosis beneficial or harmful? Can arteriosclerotic lesions regress or rupture if SMC apoptosis is enhanced by gene transfer or drug therapy? We believe that further research will enhance our understanding of the mechanism of atherogenesis significantly and lead to efficient therapeutic intervention in this disease.

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