Role of smooth muscle cell death in advanced coronary primary lesions: implications for plaque instability

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Abstract

Objective: Instability of coronary atheroma leads to the onset of acute coronary syndromes including myocardial infarction and death, as well as to the progression of the arteriosclerotic disease. As yet, the underlying factors and mechanisms causing plaque rupture are not completely understood. Since a low content of smooth muscle cells (SMCs) apparently plays a key role, the question points to the events leading to the loss of intimal SMCs. Methods: We compared coronary atherectomy specimens from 25 patients with unstable angina to those from 25 patients with stable angina. Transmission electron microscopy was used to identify intimal cell population, to detect stage and cell type of apoptosis, and to differentiate between apoptosis and necrosis. Results: Plaques associated with unstable angina contained more macrophages/lymphocytes and significantly less SMCs ($P<0.01$), compared with stable angina plaques. Specific cell death forms, apoptosis and necrosis, were present in all coronary atheroma. As key findings, both the proportion of SMCs undergoing apoptosis and the frequency of cytoplasmic remnants of apoptotic SMCs (matrix vesicles) were significantly increased in unstable versus stable angina lesions ($P=0.002$ and $P=0.002$). In addition, cellular necrosis was more frequent in the first coronary atheroma group ($P=0.02$). Positive correlations were found between the frequency of apoptotic cells and necrosis ($r=0.41, P=0.04$), and that of matrix vesicles and necrosis ($r=0.63, P=0.001$) only in plaques with unstable angina, but not in those with stable angina. Conclusions: Our data demonstrate that high cell death due to apoptosis and necrosis is a basic in situ feature found in advanced coronary primary lesions associated with unstable angina, possibly explaining their low density of (viable) SMCs. Thus, antagonization of intimal cell death should be considered in order to stabilize the intimal plaque texture of coronary atheroma with the ultimate goal to prevent plaque rupture. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Plaque rupture and subsequent thrombosis are key events in the onset of acute coronary syndromes and in the progression of the underlying arteriosclerotic disease [1–3]. Therefore, a better understanding of the mechanisms and factors that allow the endovascular border to become vulnerable is of outstanding importance. However, our knowledge of the underlying pathogenic elements that (1) predispose to or even (2) elicit the rupture of coronary atheroma is still incomplete. A body of intense investigational work on human vulnerable lesions shows that a low density of smooth muscle cells (SMCs) and collagen, as well as an increased frequency of inflammatory cells, associated with a considerable tissue degrading activity, are basically implicated in the breakdown of the plaque fibrous cap [1–8]. Based on the concept that apoptosis (programmed cell death) of SMCs plays an important role in vascular wall remodeling, recent studies on human
plaque tissue have focused on apoptosis in different intimal settings, such as restenotic lesions [9], saphenous vein grafts [10], early and advanced atherosclerotic lesions [11–13]. Indeed, apoptotic self-elimination of SMCs may represent a beneficial adaptive response with the ultimate result of less obstructive vascular lesions. However, SMC apoptosis may also contribute to a weakened intimal plaque texture and to the reduced elaboration and deposition of extracellular matrix proteins, both leading to plaque instability. An additional, not yet answered question, in particular in plaque rupture, addresses to a possible cross-talk between apoptosis and necrosis (as the accidental form of cell death).

The objective of the present report was to identify stage and cell type of intimal apoptosis, to differentiate apoptosis from necrosis, and to relate these cell death forms to the intimal cell population. To this end, we analyzed coronary atherectomy specimens from a total of 50 patients by transmission electron microscopy, and compared lesions associated with unstable angina to those associated with stable angina. Our data demonstrate that increased apoptosis of SMCs and necrosis are important pathogenic mechanisms involved in the vulnerability of human coronary atheroma, coincident with low SMC and high inflammatory cell density.

2. Methods

2.1. Patients and arterial specimens

The present study is a retrospective analysis of plaque tissue retrieved by percutaneous directional atherectomy [14–16] from coronary target lesions of symptomatic individuals. Consecutive patients were classified for their clinical disease activity by obtaining a detailed angina history and by reviewing the medical record for evidence of unstable angina as defined by Braunwald [17]. The diagnosis/classification stable vs. unstable angina was made clinically and before angiography. Directional atherectomy was performed on 50 patients (mean age 60.3±9.8 years, range 35–78 years; eight female, forty-two male) with either unstable angina (group 1, n=25) or stable angina (group 2, n=25) attributed to the presence of primary atheromatous lesions (angiographic stenosis degree >75%). Tissue samples were obtained from thirty-eight left anterior descending, nine right, and three circumflex coronary arteries. Unstable angina was classified according to the Braunwald criteria [17] as follows: Class I (n=7), class II (n=9), class III (n=9). Clinical circumstances categorized in class A (secondary unstable angina) were not observed, whereas class B (primary unstable angina) was seen with seventeen patients, and class C (unstable angina within 2 weeks post infarction) was seen with eight patients. Macroscopic thrombi were detected within the plaque material removed from six lesions associated with acute coronary syndrome. These lesions were categorized as IB, IIB, IIC, IIIB (2) and IIC. Restenotic lesions were excluded from the present study. Medication of the patients included nitrates and aspirin in all patients, lipid-lowering therapy in 41 of 50 (82%), β-blockers in 36 of 50 (72%) and calcium antagonists in 16 of 50 patients (32%), and thereby did not significantly differ between both patient groups. All patients were treated with heparin 30–60 min before and throughout the atherectomy procedure. Informed consent for the analysis of tissue samples was obtained from all patients prior to revascularization. The investigation conforms with the principles outlined in the World Medical Association Declaration of Helsinki [18].

2.2. Fixation and transmission electron microscopy (TEM)

Immediately after percutaneous atherectomy, all specimens were fixed in phosphate buffered 3.5% glutaraldehyde (pH 7.4). After 2 h, the tissue was placed in 1% glutaraldehyde. Additional fixation was performed for 2 h in 1% OsO4 in phosphate buffer, followed by a thorough rinse of the tissue in buffer. Samples were then dehydrated twice through graded concentrations of alcohol and propylene oxide. After additional incubation in a mixture of propylene oxide and araldite (1:1), the blocks were embedded in a mixture of araldite, (2-dodecen-1-yl)succinic anhydride and accelerator. Tissue sections (1.0 µm) were cut from the araldite blocks and stained with standard hematoxylin for light microscopy. Ultrathin sections were contrasted with uranyl acetate dihydrate and 1% lead nitrate and were viewed at 80 kV using a Philips CM 10 electron microscope.

2.3. Ultrastructural analysis by TEM

Ultrastructural analysis is extremely helpful in the evaluation of apoptosis, in distinguishing apoptosis from necrosis, and even in detecting apoptotic cell death without nuclear condensation or DNA fragmentation, as recently reported [9,19,20]. TEM micrographs were evaluated quantitatively and qualitatively. A primary magnification of 3600× was used.

Non-overlapping images of randomly selected intimal regions were photographically enlarged an additional 2.3 times to a final magnification of 8300×. For each lesion studied, eight photographs (17×21 cm each) were taken and 15 to 32 plaque cells were classified as SMCs, macrophages or lymphocytes. Ultrastructural recognition of altogether more than 900 plaque cells was performed according to ultrastructural features, as previously reported [21–23]. The identification of apoptotic cells and their cytoplasmic remnants, and cellular necrosis was based upon their specific morphological criteria as defined in several reports [9,13,24–28]. Morphometric evaluation
was performed as recently described [9]; also compare Section 2.4.

2.4. Assessment of cell density

Hematoxylin stained histological sections allowed the detection of cellular nuclei from intimal regions; adjacent medial areas of the vessels were not analyzed. Assessment of cell density was performed by a computer-assisted morphometry system (VFG-1 graphic card) to count stained cell nuclei per area (0.04 mm²) and to calculate the final cell density [9]. The image of the microscope (Optiphot-2; Nikon) was relayed by a miniaturized video camera/downstream monitor (KP-C-553-CCD; Hitachi). Ten randomly-selected intimal areas, each encompassing 0.04 mm², were assessed per tissue sample. Tissue analysis and subsequent computer-assisted evaluation were performed by two different investigators blinded to patient categorization and plaque feature data.

2.5. Statistical analysis

For data analysis, the SPSS/PC+ software package for Windows 5.02 (Microsoft) was used. Group differences in densities of plaque cells, necrosis, apoptotic cells and their cytoplasmic remnants, were evaluated by the Mann–Whitney rank-sum test. Correlation coefficients were determined by Pearson’s test. All probability values were two-tailed and corrected for ties. Values of \( P<0.05 \) were considered significant. Group data are given as mean±S.D.

3. Results

The present study analyzes tissue specimens of primary coronary origin from patients presenting unstable angina (group 1) and stable angina (group 2), and compares them with respect to cell density, cell type and frequency of cell death, specifically SMC apoptosis and necrosis. Cellular composition of SMCs and inflammatory cells, such as macrophages and lymphocytes, was different in both plaque groups. As a key finding, the cell pool of group 1 lesions contained significantly less SMCs compared to that of group 2 lesions (75±22% vs. 90±14%, \( P=0.01 \)). Also, macrophages and lymphocytes were more frequently found in plaques associated with unstable vs. stable angina (20±18% vs. 7±11%, \( P<0.01 \); 5±7% vs. 3±6%, \( P=0.21 \)), whereas the average cellularity of both plaque types was similar (290±151 vs. 327±164 cells/mm², \( P=0.53 \)).

Based on the key finding of sparse SMC density with unstable angina, this report studied SMC loss, morphologically reflected by SMC death. Programmed cell death, or apoptosis of SMCs was found in all lesions. Fig. 1 compares representative examples of SMCs undergoing apoptosis with viable SMCs. Most importantly, morphometric evaluation demonstrated a 2-fold (\( P=0.005 \)) higher proportion of SMCs undergoing apoptosis for group 1 vs. group 2 lesions. Conversely, the proportion of viable SMCs was significantly lower (\( P=0.001 \)) in plaques associated with unstable compared to stable angina (Fig. 2). Also, macrophages undergoing apoptosis were more frequently seen in plaques associated with unstable vs. stable angina (4±2% vs. 1±1% of the intimal cell pool, \( P=0.01 \)).

Membrane-bound remnants of apoptotic cells, or matrix vesicles, reflective of final stages of apoptosis [13,24] revealed a variable size and pattern (Fig. 3) and were consistently seen in all lesions. These apoptotic remnants were present in extracellular matrix areas, isolated or in membrane contact to SMCs and macrophages, respectively, as well as in the cytoplasm of both cell types. Quantitatively, their density was markedly higher (\( P<0.0001 \)) for group 1 vs. group 2 lesions (Fig. 4). In contrast to the features of apoptosis, typical characteristics of necrotic events were destroyed cell membranes and disintegrated extracellular matrix (Fig. 3d). Necrotic cells, whose morphology did not allow a classification of their previous cell type, were frequently found adjacent to macrophages, indicating local inflammation. Quantitatively, necrosis was more frequent in lesions associated with unstable vs. stable angina (19±16 vs. 10±11 necrotic cells/total cells, \( P=0.04 \)).

Regarding a possible cross-talk between the two different cell death types in coronary atheroma, a significant correlation was evident (i) between the frequency of apoptotic cells and that of necrosis (\( r=0.41, P=0.04 \)), and (ii) between the frequency of apoptotic remnants and that of necrosis (\( r=0.63, P=0.001 \)). Most interestingly, this correlation was only found for lesions associated with unstable angina, but not for the stable angina group (Figs. 5 and 6). Thus, increased necrosis that goes parallel with increased levels of apoptotic cells and apoptotic remnants in unstable angina may explain the loss of (viable) SMCs as a characteristic feature in coronary atheroma complicated by plaque rupture.

4. Discussion

The present study compares extent and type of intimal cell death in atherectomy-derived primary coronary lesions associated with unstable versus stable angina and relates them to plaque composition. As expected, macrophages and lymphocytes indicating inflammatory events revealed a significantly larger portion in group 1 vs. group 2 plaques (25 vs. 10%), whereas overall intimal cellularity was similar. These data are confirmed by several ex vivo and post mortem studies that show the prevalence of macrophages in vulnerable lesions with focal localization in ruptured and eroded zones, irrespective of the dominant plaque architecture [3–8]. Also, apoptosis of macrophages was observed to a degree of 1–4% of the intimal cell pool,
Fig. 1. Transmission electron microscopy (TEM) of primary coronary lesions illustrating ultrastructural features of viable SMCs (a) compared with apoptotic SMCs (b and c) and necrotic SMCs (d). (a) Two viable SMCs of beginning intermediate phenotype that show intact nuclei with a granular pattern of chromatin organization (N) and that are surrounded by a distinct basement membrane (B); ×4100. (b) High-power magnification of two small apoptotic SMCs in a lesion with unstable angina. The dark, electron-dense appearance indicative of cross-linking events of cytoplasmic proteins leading to SMC shrinkage, condensed clumped chromatin lining the nuclear envelope (N), and increased activity and outpouching of membrane segments (A) are characteristic of SMC apoptosis; ×10,600. (c) Shrinkage of apoptotic SMC and detached anchorage from surrounding extracellular matrix are indicated by condensed cytoplasm and a de novo pericellular territory (arrowheads). The multi-lamellated basal laminae (B) encircling the apoptotic SMC suggests repetitive loss of SMC/matrix adhesion and reconstitution of B synthesis; ×7600. (d) Three necrotic cells in a lesion with unstable angina (same lesion as in b). Typical findings of cellular necrosis are almost lost cell membranes, dispersed cytoplasmic organelles (arrows) and degraded pericellular matrix (arrowheads); ×7000.
ly, Han et al. [11] found maximal apoptosis in macro-

abundant in coronary and peripheral plaque tissue [11,12]. Interestingly, recently shown by in vitro work, we still lack information on the further cytoplasmic pathway of apoptotic debris.

using immunohistochemical TUNEL labeling that defined an apoptotic index between 10 and 46% for human coronary and peripheral plaque tissue [11,12]. Interestingly, Han et al. [11] found maximal apoptosis in macro-

the more with acute coronary syndromes. This finding that was coincident with a higher frequency of macrophages in vulnerable coronary lesions, points to an increased intimal turn-over of macrophages with the onset of unstable angina. In this context, recent work on human carotid plaques demonstrated proliferative activity to be largely restricted to macrophages, but not to SMCs [29].

Though SMCs represented the predominant intimal cell type in both lesion groups, their density was lower, when lesions were associated with unstable angina, as this is a widely accepted feature of vulnerable plaques [3,5,6]. In addition, most importantly, our data also demonstrate a significantly lower proportion of viable SMCs and, correspondingly, a higher proportion of apoptotic SMCs in the intimal cell pool present in unstable angina lesions (Fig. 2). The programmed form of SMC death was consistently observed in all coronary atheroma specimens. Our finding of an increased percentage of SMCs that undergo apoptosis with unstable angina (Fig. 2) is accompanied by an increased frequency of apoptotic remnants (Fig. 4). Therefore, apoptosis could principally explain the loss of viable SMCs. However, the present report demonstrates that, in addition to apoptosis, accidental cell death events were also more frequently seen in group 1 versus group 2 lesions. Taken together, our data on coronary plaque composition clearly identify apoptosis and necrosis to be involved as key mechanisms in predisposing and/or eliciting plaque rupture via SMC depletion.

When we regard our data on apoptosis in more detail, apoptotic SMCs showed a markedly higher proportion in unstable versus stable angina plaque tissue with 24 and 12%, related to the total intimal cell pool, respectively (Fig. 2). These findings are confirmed by recent reports using immunohistochemical TUNEL labeling that described an apoptotic index between 10 and 46% for human coronary and peripheral plaque tissue [11,12]. Interestingly, Han et al. [11] found maximal apoptosis in macro-

phage-rich areas, however, did not differentiate between arteriosclerotic lesions associated with unstable and stable angina. Also, one may critically question the specificity of the TUNEL test in detecting apoptosis and in differentiating it from the accidental type of cell death [30]. To overcome these limitations, the present study exclusively used transmission electron microscopy that is able to detect apoptotic events devoid of DNA fragmentation and definitely discriminate between apoptosis and necrosis [9,19,20].

Although we observed high, yet different levels of apoptosis for each lesion type, mean cell density remained similar, and self-elimination or regression of the atheroma may be anticipated as a consequence, was not documented. Also, as known, proliferation of SMCs in coronary plaques is low [31,32]. Therefore, one may speculate that apoptosis, particularly that of SMCs, may be reversible at early stages or reveal an incomplete late course, as recently reported for dermal tissue cells [12]. Indeed, our data give evidence for this hypothesis by the ultrastructural scenario specifically observed for apoptotic SMCs (Fig. 1b and c). These cells reveal typical features of apoptosis and, notably, an intense loss of pericellular adherence, a phenomenon called anoikis [33]. Most interestingly, this discriminant pattern of SMC apoptosis frequently found in human coronary plaque tissue is accompanied by the presence of surrounding multilayered, basal lamina cages (Fig. 1c), suggesting repetitive alternating episodes of cellular recovery, including basal lamina synthesis, and apoptosis, indicated by the loss of cell adhesion.

The higher level of apoptosis in group 1 vs. group 2 lesions is supported by a significantly larger frequency of matrix vesicles reflecting the final stage of apoptosis (Fig. 4). Also, as illustrated in Fig. 3, neighbouring SMCs and macrophages were engaged in binding and internalizing the apoptotic remnants. These findings have several implications. Apparently, intimal SMCs bear a similarly developed capacity to engulf apoptotic debris as macrophages that are prototypes of phagozytosing cells. Indeed, SMCs found in human arteriosclerotic lesions express specific integrins [34] such as the vitronectin receptor (αvβ3) and the thrombospondin receptor (CD36) that are adequate binding structures for apoptotic bodies as known from experimental work with human peritoneal macrophages [30,35,36]. As a consequence, competition for the cellular binding sites between apoptotic debris and other potential ligands, such as the proliferation-promoting thrombospondin, the extracellular matrix components fibronectin, vitronectin, tenascin, osteopontin or other proteins with RGD sequences [36,37] may impede with SMC–matrix interactions and, thereby, modify intimal tissue texture. Though the engulfment phase of apoptotic structures is completed within hours [24–26] and mediated in part by exposure of phosphatidylserine [36,38], as recently shown by in vitro work, we still lack information about the further cytoplasmic pathway of apoptotic debris.

Fig. 2. Proportion of viable and apoptotic smooth muscle cells (SMCs) in the intimal cell pool (=100%). Group 1 denotes 25 primary coronary lesions with unstable angina, group 2 denotes 25 primary coronary lesions with stable angina. All values are given as mean±S.D. The sum of viable and apoptotic SMCs results in SMC proportions of 75±22% vs. 90±14% of the intimal cell pool for group 1 vs. group 2.
Fig. 3. Transmission electron microscopy (TEM) of primary coronary lesions illustrating ultrastructural features of apoptotic remnants (matrix vesicles) found in different locations. (a) The variable, often speckled appearance of these vesicles (arrows) is due to gradual differences in the organization and the electron density pattern of their content, and suggests ongoing degradation processes. Membranes surrounding the inclusions are presumably intact, since the adjacent extracellular matrix appears normal; $\times$3300. (b) Large matrix vesicle with a partially fragmented surrounding membrane (arrows), apparently being phagocytosed by a macrophage; $\times$5200. (c) SMC that is surrounded by a broad basement membrane and contains three matrix vesicles (arrows). The engulfed structures fill more than a third of the cellular cross-section and are clearly demarcated from cytoplasm by their membrane ‘package’; L=lipid droplet; $\times$5700. (d) Two matrix vesicles (arrows) adjacent to a viable SMC that is surrounded by an intact basement membrane (arrowheads) and extracellular matrix. Both vesicles are encircled by a distinct membrane and reveal a granular filling pattern that clearly differs from the intact SMC (M=mitochondria; ER=endoplasmic reticulum membranes; N=nucleus). An attachment site between the matrix vesicle and the SMC is noted by an open arrow. This may represent an early event in the SMC engulfment process; $\times$8200.
The residual, smaller portion of viable SMCs that was detectable in unstable angina lesions (Fig. 2) is apparently not capable to compensate or even to eliminate the accumulated output of the apoptosis machinery.

Other important findings of the present report concern the normal pattern of the extracellular matrix and the paucity of cell debris adjacent to apoptotic structures (Fig. 3). This suggests an effective sealing of the putative caustic contents of apoptotic structures [27], as commonly defined for apoptosis versus inflammatory necrosis. However, importantly, this also implies a focal destructive activity in case of a lost integrity of the membrane surrounding the apoptotic cell or its cytoplasmic remnants. Indeed, our data reveal a positive correlation between the frequency of apoptosis and that of necrosis which becomes apparent with increased levels of apoptosis as found in unstable angina lesions (Figs. 5 and 6). Based on the concept of a cross-talk between apoptosis and necrosis, plaque areas rich in apoptotic structures may reflect a focally labile tissue architecture and, therefore, should be considered to predispose to plaque fissures and rupture. Conversely, locally effective proteinases, such as interstitial collagenase, gelatinase ($M_r$ 92 000), stromelysin or matrilysin [3,6,39–41], may not only induce the degradation of specific extracellular matrix components, but also digest the membranes of nearby apoptotic structures with the ultimate result of non-selective ‘boostering’ of tissue degradation processes.

The present study quantifies the extent of all apoptosis events that are implicated in chronic coronary arteriosclerosis of patients with unstable versus stable angina. By necessity, this is a momentaneous and not a sequential insight into the lesional composition. Therefore, these data do not allow to conclude for the rate or the spatiotemporal pattern of intimal apoptosis. Also, this report does not specifically focus on the pathways and mechanisms of apoptosis. However, with regard to the different extent of SMC apoptosis (24 vs. 12%) observed in unstable compared to stable angina lesions [9], one may question, whether there are two (or even more) different pathways of apoptosis. Basically, one pathway may specifically regulate intimal cell density in chronic arteriosclerosis, apparently by a complex framework of protooncogenes (BCL-2, BCL-X, BAX), cell cycle regulators (E2F1, RB, p53) and growth factors (PDGF, IGF), as shown by recent reports [13,20,42–47]. In addition to this pathway that is responsible for the basal extent of apoptosis found in primary coronary lesions, a second or even more pathways of apoptosis are postulated leading to plaque vulnerability and rupture, as demonstrated by the present study. Specific cytokines, such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) and/or interleukin-1β (IL-1β) that are produced by activated macrophages [3] have recently been shown to lead to a severe loss of cellular integrity and, finally, to predispose to apoptosis in vitro [48].

In summary, high apoptosis and necrosis in human
coronary primary lesions, clinically associated with acute coronary syndromes, indicate the presence of one or more intimal factors that induce intimal cell death. Therefore, implications of our findings point to the identification of these factors and to the modulation of endogenous apoptosis to increase the regional density of viable SMCs with the ultimate goal of preventing plaque rupture.

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References


