Oxidized Low-Density Lipoprotein Is Associated With Apoptosis of Vascular Smooth Muscle Cells in Human Atherosclerotic Plaques

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Background—Cytotoxic oxidized LDL (oxLDL) has been shown to promote apoptosis in cultured vascular smooth muscle cells (VSMCs). We investigated the localization of oxLDL and its association with apoptosis and the expression of apoptosis-related proteins in early and advanced atherosclerotic lesions.

Methods and Results—Atherosclerotic plaques (n=23) from patients undergoing aortic, carotid, or femoral arterial surgery were studied. In early lesions, oxLDL was located predominantly in the superficial intima and in the media just beneath the internal elastic lamina. Medial VSMCs staining positive for oxLDL showed expression of BAX, a proapoptotic protein of the BCL-2 family. Apoptosis, as detected by DNA in situ terminal deoxynucleotidyl transferase end-labeling (TUNEL), was not present in these early lesions. In advanced plaques, areas of the intima positive for oxLDL showed lower α-smooth muscle actin immunoreactivity (P<0.01) and higher BAX immunoreactivity (P<0.05). Furthermore, these areas showed an increased number of apoptotic VSMCs (P<0.01). Western blot analysis revealed that oxLDL increases BAX expression in cultured human coronary VSMCs.

Conclusions—We conclude that in early atherosclerotic lesions, oxLDL-positive VSMCs express BAX, which increases the susceptibility of these cells to undergo apoptosis. This could be important in our understanding of the transition of early lesions into advanced atherosclerotic plaques, which are characterized by regions of cell death. In advanced plaques, oxLDL-positive areas of the intima show higher BAX immunoreactivity and TUNEL-positive VSMCs, and this may contribute to plaque instability and rupture. (Circulation. 2000;102:2680-2686.)

Key Words: apoptosis ■ atherosclerosis ■ cells ■ muscle, smooth ■ lipoproteins

The role of cell loss and apoptotic cell death in the pathophysiology of atherosclerosis has received renewed attention, and several studies have demonstrated the presence of apoptosis in human and experimental atherosclerotic plaques.1-4 Plaques that are prone to rupture have a thin fibrous cap, with fewer vascular smooth muscle cells (VSMCs) and a dense infiltration of macrophages.3-7 VSMCs are important in maintaining the tensile strength of the fibrous cap, because these are the only cells in the cap that can synthesize collagen fibers types I and III.8 Although apoptosis has been detected in human plaques, the mechanisms triggering this apoptosis and its contribution to VSMC loss are unclear.

Oxidative modification of LDL is believed to be involved in atherogenesis, and uptake of oxidized LDL (oxLDL) by macrophages and VSMCs leads to the formation of foam cells, which accumulate lipid droplets.9-11 OxLDL has also been shown to be cytotoxic for and to promote apoptosis of cultured VSMCs.12-14 Although various apoptosis-related factors have been demonstrated to be associated with VSMC apoptosis,15-18 there has been no investigation of a potential association of oxLDL with VSMC apoptosis in animal or human atherosclerotic lesions.

In the present study, the localization of oxLDL and its correlation with proapoptotic protein expression and apoptotic cells was studied in early and advanced human atherosclerotic plaques.

Methods

Atherosclerotic specimens (n=23) were obtained from patients undergoing carotid endarterectomy (n=13), aortic valve replacement (n=4), and femoral arterial surgery (n=6). Mean age of the patients (14 men and 9 women) was 72.5±7.6 years. Specimens were fixed in 4% formaldehyde, paraffin-embedded, sectioned at 5-μm thickness, and mounted on VectaBond precoated (Vector Laboratories) slides. Every first section was stained with hematoxylin and eosin to

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assess general morphology. We used the definitions of the American Heart Association Medical/Scientific Statement to confirm that each specimen contained advanced atherosclerotic plaques accompanied by early lesions.

**Immunohistochemistry**

Monoclonal antibodies were α-smooth muscle (SM) actin from Sigma Immunochemicals; HAM-56 (anti-macrophage) from Dako; and BAX and BCL-2 from Santa Cruz Biotechnology, Inc. Polyclonal anti-BAX antibodies were from Santa Cruz Biotechnology, Inc and from Pharmingen. The monoclonal antibody against oxLDL (DLH3) was prepared as reported previously. Sections were preincubated with 5% serum, then incubated with primary antibody followed by biotinylated secondary antibody, avidin-biotin–alkaline phosphatase complex (Vectastain ABC-AP Kit), and visualized with Vector Red (Vector Laboratories). Specificity was checked by omitting the primary antibody and substituting nonimmune serum or by preincubating the antibody with BAX protein for 2 hours. Counterstaining was performed with hematoxylin or methyl green.

For colocalization of oxLDL and BAX, samples were first stained for BAX by the alkaline phosphatase substrate system, then stained for oxLDL by the peroxidase substrate system with the metal-enhanced diaminobenzidine (DAB) as a brown/black chromogen (Boehringer Mannheim).

**In Situ 3' End-Labeling of DNA Fragments (TUNEL)**

To identify apoptotic cells, the in situ cell death detection kit from Boehringer Mannheim was used, with minor modifications. Sections were incubated with 3% citric acid or TE buffer to eliminate free calcium, pretreated with proteinase K and with 0.3% H₂O₂ in methanol before exposure to labeling mixture. Incorporated fluorescein-dUTP was detected with anti–fluorescein peroxidase antibody and DAB. Sections were counterstained with methyl green.

For colocalization of oxLDL and BAX, samples were first stained for BAX by the alkaline phosphatase substrate system, then stained for oxLDL by the peroxidase substrate system with the metal-enhanced diaminobenzidine (DAB) as a brown/black chromogen (Boehringer Mannheim).
volunteers by sequential density ultracentrifugation (1.019 to 1.063 g/mL) in the presence of EDTA and oxidized with 5 \( \mu \)mol/L of CuSO\(_4\) at 37°C for 2 hours. Confluent VSMCs, with or without a 24-hour prior serum deprivation, were treated with 200 \( \mu \)L/mL of native LDL or oxLDL in serum-free medium for 24 hours, and total cell lysates were extracted with 1% NP-40 buffer. Protein samples (50 \( \mu \)g) were separated by 12% SDS-PAGE, transferred to a polyvinylidine difluoride membrane, and incubated with anti-BAX antibody or with anti-\( \alpha\)-SM actin antibody overnight. Detection was with horseradish peroxidase–conjugated secondary antibody and enhanced chemiluminescence (ECL, Amersham). HeLa cells were used as a positive control.

Quantification and Statistical Analysis

Twelve different regions, each 284 \( \times \) 213 \( \mu \)m, from each atherosclerotic specimen were quantitatively analyzed with a color image analysis system (KS 400: Kontron Elektronik GmbH). These regions were equally distributed in the intima and media with and without significant oxLDL staining. The lipid core was omitted from analysis because of its acellular characteristics. For each region, immunohistochemical data were described quantitatively by use of the percent immunoreactive areas for \( \alpha\)-SM actin and BAX. TUNEL-stained nuclei surrounded by a cage of PAS-positive material, consistent with VSMCs undergoing apoptosis, were counted in all regions. The percentage of TUNEL-positive VSMCs was calculated by dividing the number of TUNEL-positive VSMCs by the total number of nuclei.

Data are expressed as the mean\( \pm \)SEM. After 1-way ANOVA, the Scheffé \( F \) test was used for the comparison of \( \alpha\)-SM actin and BAX immunoreactivity and the TUNEL-positive VSMCs in the different regions. Differences with a value of \( P<0.05 \) were considered statistically significant.

Results

Early Lesions

Early lesions were composed primarily of VSMCs that strongly expressed \( \alpha\)-SM actin independently of the presence of oxLDL (Figures 1A and 2). In early lesions, oxLDL immunoreactivity was pronounced in the superficial intima and in deeper layers, particularly in the media just beneath the internal elastic lamina (Figure 1B and 1C). Macrophages were localized almost exclusively in the superficial intima (Figure 1D). They were very rare in the deeper intima and absent in the media, irrespective of the presence of oxLDL (Figure 1D). VSMC-derived foam cells positive for oxLDL were present in the media (Figure 1C).

The intima showed scarce BAX-positive cells. Medial VSMCs just beneath the internal elastic lamina in regions staining positive for oxLDL showed higher expression of BAX than regions without oxLDL staining (Figures 1E and 3, \( P<0.01 \)). Double staining for oxLDL and BAX showed colocalization of intracellular oxLDL and BAX in some medial VSMCs just beneath the internal elastic lamina (Figure 1F). There were no VSMCs positive for BCL-2 in early lesions. In early lesions, apoptotic cell death as demonstrated by TUNEL could not be detected (not shown).

Advanced Plaques

Advanced plaques showed a fibrous cap, a shoulder part, and a lipid core, and most lipid cores were largely acellular, indicating that cell death must have occurred.

In general, oxLDL was present predominantly in the intima, particularly in areas close to the lipid core and in the cellular debris dispersed in the lipid cores. Medial VSMCs showed no significant presence of oxLDL (Figures 4A and 5A4). Swollen collagen fibers, in a fibrous cap or in the shoulder part, contained oxLDL (Figures 4A and 5A3).

\( \alpha\)-SM actin immunoreactivity in the intima of advanced plaques was lower than in early lesions (Figure 2, \( P<0.01 \)). Areas of the intima and of the lipid core that were positive for oxLDL showed little or no \( \alpha\)-SM actin staining (Figure 4B). Intimal regions positive for oxLDL showed significantly lower \( \alpha\)-SM actin immunoreactivity than oxLDL-negative intima and than medial regions (Figure 2 and Figure 5). The absence of \( \alpha\)-SM actin immunoreactivity in areas of the intima positive for oxLDL is consistent with VSMC loss in these areas.

Many intimal VSMCs and some medial VSMCs, which did not stain strongly for oxLDL, showed morphological features of foam cells (Figure 5B2 and 5B4). Macrophages were...
present predominantly in regions of the intima positive for oxLDL (Figure 5C1 and 5C3). Areas of the intima and media with little or no oxLDL contained no macrophages (Figure 5C2 and 5C4).

The advanced plaque showed higher expression of BAX in the intima and media than did the early lesions negative for oxLDL (Figures 1G through 1J and 3, \( P < 0.01 \)). Medial regions of the advanced plaque positive for oxLDL showed higher BAX expression than those negative for oxLDL (Figures 1J and 3, \( P < 0.05 \)). Furthermore, there was a trend toward increased BAX in intimal regions of the advanced plaque positive for oxLDL compared with those negative for oxLDL (Figures 1H and 3, \( P = 0.07 \)). The intimal and medial regions of advanced plaques positive for oxLDL also showed higher BAX expression than those of early lesions positive for oxLDL (\( P < 0.05 \)). However, unlike early lesions, areas of the intima and media negative for oxLDL still contained VSMCs expressing BAX (Figure 1G and 1I). There were also VSMCs coexpressing oxLDL and BAX in advanced plaques (data not shown), as described in early lesions (Figure 1F).

In general, TUNEL-positive cells were localized in areas close to lipid cores (arrows in Figure 6A through 6D). Double staining with the TUNEL technique and PAS revealed TUNEL-positive nuclei that were surrounded by a cage of PAS-positive basal lamina, indicating VSMCs undergoing apoptotic cell death, in the fibrous cap close to the lipid core (Figure 6A and 6B). Adjacent to these cells are cages of PAS-positive material that contained clusters of small vesicles (Figure 6A, open arrowhead) or PAS-positive empty cages of thickened basal lamina (Figure 6A, closed arrowhead), consistent with remnants of apoptotic VSMCs. TUNEL-positive nuclei and PAS-positive material were detected in the shoulder part positive for oxLDL (Figure 6C and 6D), but not in the medial layer (Figure 6E). A comparison of the percentage of TUNEL-positive VSMCs from 4 different regions of advanced plaques showed that the intima with oxLDL had significantly higher TUNEL positivity (Figure 7, \( P < 0.01 \)). Double staining with TUNEL and \( \alpha \)-SM actin also identified apoptotic VSMCs in the shoulder part positive for oxLDL (Figure 6F). There were no VSMCs positive for BCL-2 in advanced atherosclerotic lesions.

Although most lipid cores were acellular, some lipid cores contained \( \alpha \)-SM actin immunoreactivity in some cell components and some areas surrounding cholesterol crystals. This is consistent with the remnants of VSMC-derived foam cells (Figure 8A). In these regions, BAX immunoreactivity was also detected (Figure 8B), suggesting that VSMC death occurred, at least in part, through the apoptotic process. Thus, some regions of the lipid core also contained foci of TUNEL-positive nuclei surrounded by a cage of PAS-positive basal lamina, indicating VSMCs undergoing apoptotic cell death within the lipid core (Figure 8C).
nuclei (black) surrounded by a cage of PAS-positive basal lamina, indicating apoptotic VSMCs (C). Bars=50 μm.

Figure 8. Lipid core stained for α-SM actin (A) and BAX (B) and double-stained with TUNEL and PAS (C). Some cell components and some areas surrounding cholesterol crystals show α-SM actin immunoreactivity and increased TUNEL-positive VSMCs (A). B is a serial section of A showing small fragments positive for BAX (B). Some regions of lipid core also contained foci of TUNEL-positive

Figure 7. Quantification of percentage of TUNEL-positive VSMCs in 4 different regions of advanced plaques (mean±SEM). *P<0.01 vs 3 other regions of advanced plaques.

Western Blot Analysis

As shown in Figure 9, BAX expression was very low in nonquiescent cells and was significantly increased by 24 hours of serum deprivation. OxLDL, but not native LDL, caused a marked increase in BAX expression. Thus, BAX expression in cells exposed to oxLDL was 2.1±0.2-fold higher than in cells exposed to native LDL (n=4, P<0.01). These results were confirmed with 2 different antibodies, and experiments were performed ≥3 times with each antibody.

Discussion

We demonstrate that oxLDL in early atherosclerotic lesions is localized not only in the intima, where it is associated with macrophage infiltration, but also in medial VSMCs, where it colocalizes with BAX, a proapoptotic protein. In advanced atherosclerotic plaques, intimal regions positive for oxLDL show poor α-SM actin immunoreactivity, increased BAX expression (compared with the intima of early lesions), and TUNEL-positive VSMCs. These data strongly suggest that oxLDL is involved in triggering apoptosis of VSMCs, leading to formation of the lipid core. Thus, the lipid core, one of the regions that stain strongly positive for oxLDL, shows BAX expression and TUNEL-positive VSMCs.

In atherosclerotic plaques, VSMCs maintain the tensile strength of the fibrous cap; thus, plaques that are prone to rupture have a thin fibrous cap with fewer VSMCs and a dense infiltration of macrophages. Macrophage infiltration is associated with apoptosis, whereas lesions consisting only of VSMCs present very little apoptosis. Kockx et al recently identified apoptotic VSMCs in advanced plaque but not in early lesions, consistent with our findings. Our data indicate that oxLDL localization is associated with decreased α-SM actin immunoreactivity and increased TUNEL-positive VSMCs, as well as macrophage infiltration. Infiltrating macrophages may destabilize plaque by secreting or activating metalloproteinases that digest matrix. Alternatively, VSMCs could be programmed to die because of the effect of oxLDL or macrophage-derived factors such as TNF-α.

The mechanism of the relationship of oxLDL with the apoptotic process remains uncertain. Previous data have shown that oxLDL injures VSMCs. Highly oxidized LDL, unlike lightly oxidized LDL, stimulates VSMC apoptosis. Apoptosis can be induced by lipid peroxides, a component of oxLDL, in cultured VSMCs through caspase (CPP-32 protease) activation and BCL-2 protein downregulation. In a recent study, colocalization of caspase 3 expression and TUNEL positivity was detected in human atherosclerotic plaques.

Uptake of oxLDL by macrophages and VSMCs leads to the formation of foam cells, which accumulate lipid droplets. In this study, a significant fraction of VSMCs in deeper layers of early lesions showed intracellular oxLDL immunoreactivity; moreover, VSMC-derived foam cells positive for oxLDL were localized mainly in the media just beneath the internal elastic lamina. Furthermore, extracellular oxLDL accumulation was found in the deeper layer of early lesions. Colocalization of the proapoptotic protein BAX and oxLDL in these VSMCs of early lesions strongly suggests that these cells subsequently undergo apoptosis, leading to cellular loss and formation of the lipid core. Reduced cellularularity has been described in deeper layers of fatty streaks, coexisting with areas of cholesterol accumulation, and VSMC-derived foam cells tend to appear in the deeper layer of early atherosclerotic lesions.

Areas of positive oxLDL staining correlated with increased BAX expression in both early lesions and advanced plaque; however, BAX immunoreactivity was significantly greater in oxLDL-positive areas of advanced plaque than in oxLDL-positive areas of early lesions. This suggests that a greater number of VSMCs in the advanced plaque are committed to the apoptotic process and may explain the finding that VSMCs derived from the atherosclerotic plaque, but not from normal media, die when brought into culture. It is of note that medial VSMCs in both early lesions and advanced plaques showed colocalization of oxLDL and BAX without the presence of adjacent macrophages. This suggests that BAX expression in these VSMCs was probably independent of macrophage-derived factors and more likely resulted from the effects of oxLDL. In our study, BCL-2 was not detected...
in early lesions or in advanced plaques, consistent with previous reports.27,34

In early lesions, apoptotic cell death as demonstrated by TUNEL was absent. The overall number of TUNEL-positive nuclei in the advanced plaques was relatively low compared with previous reports,1,11,16,22,25 and foci of TUNEL-positive nuclei and nuclear fragments could be found mainly around the lipid cores. In this study, the TUNEL assay was performed according to the modified method of Kockx et al15,24 to remove the small calcium-containing vesicles that can be involved in nonspecific nucleotide binding. To identify VSMCs undergoing apoptosis, double staining with the TUNEL assay and PAS was performed.27 A feature of VSMCs in atherosclerotic plaques is that they are surrounded by cages of PAS-positive basal lamina (pancake-like VSMCs).35 Clusters of TUNEL-negative cytoplasmic remnants enclosed by basal lamina were present especially in the area close to the lipid core. These matrix vesicles are involved in the granulovesicular degeneration of apoptotic VSMCs.37,38 In some lipid cores, α-SM actin and TUNEL-positive VSMCs were identified, providing evidence that VSMC apoptosis is involved in lipid core formation.

In summary, our data indicate that oxLDL is localized not only in macrophage-rich intimal areas in early atherosclerotic lesions but also in medial VSMCs that coexpress BAX, a proapoptotic protein. The colocalization of oxLDL and BAX in these medial VSMCs in early lesions predisposes these to undergo apoptosis and most likely contributes to the formation of the lipid core, which is characterized by oxLDL accumulation, BAX expression, and TUNEL-positive VSMCs. Thus, our findings indicate that the lipid core may be derived, in part, from these medial regions of early lesions that are positive for both oxLDL and BAX. In advanced plaques, the higher BAX immunoreactivity and TUNEL-positive VSMCs in the intima positive for oxLDL may be involved in plaque instability and rupture.

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