Apoptotic Death of Inflammatory Cells in Human Atheroma
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Abstract—Although the accumulation of cholesterol and other lipidic material is unquestionably important in atherogenesis, the reasons why this material progressively accumulates, rather than being effectively cleared by phagocytic cells such as macrophages, are not completely understood. We hypothesize that atheromatous lesions may represent “death zones” that contain toxic materials such as oxysterols and in which monocytes/macrophages become dysfunctional and apoptotic. Indeed, cathepsins B and L, normally confined to the lysosomal compartment, are present in the cytoplasm and nuclei of apoptotic (caspase-3–positive) macrophages within human atheroma. The possible involvement of oxysterols is suggested by experiments in which cultured U937 and THP-1 cells exposed to 7-oxysterols similarly undergo marked lysosomal destabilization, caspase-3 activation, and apoptosis. Like macrophages within atheroma, intralysosomal cathepsins B and L are normally present in the cytoplasm and nuclei of these oxysterol-exposed cells. Lysosomal destabilization, cathepsin release, and apoptosis may be causally related, because inhibitors of cathepsins B and L suppress oxysterol-induced apoptosis. Thus, toxic materials such as 7-oxysterols in atheroma may impair the clearance of cholesterol and other lipidic material by fostering the apoptotic death of phagocytic cells, thereby contributing to further development of atherosclerotic lesions. (Arterioscler Thromb Vasc Biol. 2001;21:1124-1130.)

Key Words: atherosclerosis ■ apoptosis ■ lysosomal enzymes ■ oxysterols ■ plaque instability

The most obvious characteristic of atherosclerotic lesions is an abundance of cholesterol and other lipidic material that, in advanced lesions, is often referred to as “gruel.” It is not fully understood, however, why this material persists rather than being cleared by the professional phagocytes such as monocytes/macrophages that typically abound in such lesions. Our investigations have centered on the idea—certainly not original with us—that components of these atherosclerotic lesions may be cytotoxic and, in particular, cause dysfunction and death of phagocytic cells recruited to the site. Our particular focus has been on oxidized lipids, including oxidized LDL (oxLDL) and associated oxysterols. These are known to be abundant in atherosclerotic lesions and to be toxic to a variety of vascular cells.1–6 Furthermore, increased plasma concentrations of 7β-hydroxycholesterol (7β-OH) are related to enhanced risk for cardiovascular diseases.7 Among the oxysterols, 7β-OH and 7-ketocholesterol (7-keto) are major components of oxLDL.8

Apoptosis is a prominent feature of atherosclerotic lesions and may be associated with instability and remodeling of atheroma plaques.9,10 Previous investigations have suggested that destabilization of lysosomes might be an important early event in apoptosis triggered by a variety of agonists. This has led us to the idea that minor leakage of lysosomal proteases and other hydrolytic enzymes into the cytosol of cells may actually be an initiating event in apoptosis.11–15 Two lysosomal cysteine proteases, cathepsins B and L, have been identified as caspase-processing enzymes and inducers of apoptosis.16,17

Therefore, in the present investigations, we examined macrophages within human atherosclerotic plaque for evidence of the release of lysosomal enzymes, in particular cathepsins B and L. We also sought to determine whether the release of cathepsins might coincide with the occurrence of typical signs of apoptosis in these cells. Finally, we tested the effects of one potential class of toxins, 7-oxysterols, on macrophage cell lines to determine whether the features found in macrophages within atherosclerotic lesions might be reproduced by one known toxic component of the atherosclerotic plaque. Overall, the results support the concept that the interior of atheroma lesions may be a “death zone” in which various cytotoxic plaque components cause the dysfunction and, ultimately, death of phagocytes that would otherwise be capable of clearing the debris and resolving the lesion.

Methods

Immunohistochemistry
Segments from normal human mammary arteries (n=4), atherosclerotic coronary arteries (n=5), and thoracic aortas (n=5) were collected from clinical autopsy cases. For visualization of cathepsins B and L, serial sections were overlaid with goat polyclonal anti-human cathepsins B– and L–reactive primary antibodies (Santa Cruz Biotechnology, Inc), respectively, followed by an alkaline phosphatase–conjugated swine anti-goat antibody. The alkaline phosphatase activity was visualized with 4-benzoylamino-2,5-dihydroxybenzenediazonium chloride (Sigma Chemical Co).
Immunoreactivity of the active form of caspase-3 was detected by incubating the samples with polyclonal rabbit anti-human active caspase-3 (Pharmingen) followed by a goat anti-rabbit peroxidase conjugated antibody (DAKO Corp). The peroxidase activity was visualized by the use of 3-aminophenyl-ethylcarbazole (Sigma).

To determine the relationship between caspase-3 and lysosomal cathepsins within the same atherosclerotic lesions, double immunostaining of cathepsins B and L and caspase-3 was performed. The immunodetection of macrophages with monoclonal anti-CD 68 antibodies (DAKO) was carried out as previously described. As a negative control, the primary antibody was omitted. In all cases, endogenous peroxidase was blocked by preincubation with 3% H2O2. To avoid nonspecific antibody adsorption, preimmune serum of the same origin as the secondary antibody was used before primary antibody application. All antibodies were diluted with 1% BSA.

**Cells and Culture Conditions**

Cells from 2 human monocytic cell lines, U-937 and THP-1, were cultured in RPMI-1640 culture medium containing 10% FCS. For the experiments, cells were directly exposed to either 7β-OH or 7-keto (0 to 56 μmol/L), dissolved in ethanol for 6 to 48 hours or pretreated with 75 μg/mL E64 [trans-epoxysuccinyl-l-leucylamido-(4-guanidino)butane, Sigma] for 14 hours, and then exposed to 7β-OH and 7-keto for another 24 hours. A selective inhibitor of cathepsins B and L, Z-Phe-Ala-fluoromethylketone (Z-FAFMK) (Enzyme Systems Products), was applied (50 μmol/L) for 24 hours together with 7β-OH and 7-keto in some experiments. The solvent, ethanol (maximum 0.4%), had no toxic effect. Cell membrane integrity was determined by trypan blue dye exclusion test, lactate dehydrogenase (LDH) assay, and propidium iodide (PI) staining.

**Assessment of Apoptosis**

For morphological assessment, cultured cells were stained with Wright-Giemsa and examined by light microscopy as previously described. The cells were also examined by transmission electron microscopy as previously described. Caspase-3 activity of the cultured cells was assayed by spectrofluorometry. The cells were incubated with 20 μmol/L Ac-DEVD-AMC (Pharmingen) at 37°C for 2 hours. The fluorescence intensity of the liberated 7-amino-4-methylcoumarin was measured at excitation 380/emission 435 nm.

Apoptosis of both cultured cells and sections from normal and atherosclerotic human arteries was detected by the terminal deoxynucleotidyl transferase (TUNEL) technique according to the manufacturer’s instructions (Oncor Inc). Apoptotic and necrotic cells were simultaneously detected by flow cytometry by use of an annexin V–PI kit according to the manufacturer's instructions (Roche Diagnostics GmbH).

**Determination of Lysosomal Stability**

Lysosomal stability was assessed by the acridine orange (AO) vital uptake technique as described previously. The intensity of AO-induced red fluorescence was measured by static cytofluorometry (50 cells per sample) or by FACScan (Becton-Dickinson) flow cytometer (10 000 cells per sample) fitted with Lysis II software.

**Assessment of Cathepsin B and L Activity**

Control and E64-treated cells were collected by centrifugation and washed twice with PBS. Cell pellets were lysed with 0.1% Triton X-100, and the activities of the lysosomal cysteine proteinases, cathepsins B and L, were measured with Z-Arg-Arg-7-amido-4-methylcoumarin and Z-Phe-Arg-7-amido-4-methylcoumarin as the respective substrates.

**Statistics**

Statistical comparisons were made by use of the Mann-Whitney U test. Differences were considered significant at values of P≤0.05.

**Results**

**Arterial Cathepsins B and L and Caspase-3 Immunohistochemistry**

Normal human arteries contain few or no cathepsin B– and L–positive cells. In atherosclerotic lesions, there is a lesion-dependent enhanced expression of cathepsin B (Figure I, see online at http://atvb.ahajournals.org), mainly in macrophages and smooth muscle cells in the lesions (cathepsin L showed the same pattern). In support of a possible causal relationship between lysosomal leakage and cellular apoptosis, in the same plaques the presence of immunoreactive cathepsins B and L (Figure I, middle) colocalized with apoptotic macrophages, as assessed by both CD68 and TUNEL staining (Figure I, top). Interestingly, immunoreactive cathepsins B and L were seen not only in the cytoplasm of arterial macrophages and in extracellular spaces but also in the nuclei of some apoptotic macrophages (Figure I, bottom right). This finding is in accord with earlier reports that translocation of cathepsin B may participate in apoptosis by proteolytic attack on nuclear contents. By double immunohistochemistry, both cathepsins B and L were found colocalized with caspase-3–positive areas in the lesions, especially in advanced fibrous plaques (Figure I, bottom).
Lysosomal Destabilization and 7-Oxysterol Cytotoxicity

The in vivo findings reported above support the general concept that the contents of atherosclerotic plaque might be cytotoxic, perhaps by engendering lysosomal instability, release of lysosomal material such as cathepsins, and subsequent apoptosis. As a model system for these events, we used the human monocytic cell lines U937 and THP-1, exposing them to varying doses of the oxysterols 7β-OH and 7-keto.

Lysosomal integrity was assayed by static and flow cytofluorometry after AO staining. AO is a lysosomotropic weak base and a metachromatic fluorophore that accumulates in the lysosomal compartment as a result of proton trapping. In normal cells it induces red granular lysosomal fluorescence on green light excitation. Cells with ruptured lysosomes, on the contrary, appear “pale” (diminished red fluorescence due to fewer intact lysosomes). Lysosomal destabilization caused by 7β-OH and 7-keto (28 μmol/L) was clearly time-dependent and occurred before apoptotic cell death. The number of pale cells increased to more than 3 times (7β-OH) and 2 times (7-keto) that of untreated control cells after 12 hours of oxysterol exposure, and 80% (7β-OH) and 95% (7-keto) of the cells were still intact after 24 hours of exposure to 28 μmol/L of 7-oxysterols (Figure 2, bottom row). The trypan blue exclusion test and LDH assay gave similar results. These cytotoxic effects were specific for oxysterols, also were seen in THP-1 cells under the same conditions (data not shown).

Cellular plasma membranes remained intact during the first 12 hours of oxysterol exposure, and ~80% (7β-OH) and 95% (7-keto) of the cells were still intact after 24 hours of exposure to 28 μmol/L of 7-oxysterols (Figure 2, bottom row). The trypan blue exclusion test and LDH assay gave similar results. These cytotoxic effects were specific for oxysterols, inasmuch as native cholesterol (28 to 56 μmol/L) or <0.4% ethanol had no effect on either cell membrane integrity or growth over a 48-hour period. Almost identical results were obtained with both cell lines.

Role of Cathepsins B and L in Apoptosis Induced by 7-Oxysterols

The expression and localization of lysosomal cathepsins were examined in U937 cells after exposure to 7-oxysterols. Normally, the cells display a granular immunoreactivity of cathepsins B and L, reflecting the lysosomal localization of these proteases. In contrast, oxysterol treatment caused enhanced cathepsin B and L immunoreactivity, no longer of a granular nature and dispersed throughout the cytoplasm of the cells. The apoptotic cells also showed pronunciad cathepsins B and L, which localized with the condensed and shrunken nuclei (Figure 4). These findings correspond well to the

Figure 2. A time sequence of lysosomal destabilization and apoptotic/necrotic cell death induced by 7-oxysterols. U937 cells were exposed to 7β-OH or 7-keto (28 μmol/L) for different periods of time and stained with either AO (top row) or annexin V plus PI (bottom row). The fluorescence intensity was viewed in FL3-H (AO), FL1-H (annexin V), and FL2-H (PI). The results are from flow cytometry, and the letter “P” in the histograms represents the percentage of pale cells (reduced numbers of lysosomes) in the samples. The values for the upper right (UR), lower right (LR), and lower left (LL) squares of each histogram in the bottom row represent percentage of necrosis, apoptosis, and normal cells, respectively. The histograms are representative of ≥3 independent experiments.
above observations in human atheroma, indicating that release of lysosomal cathepsins and their dispersion throughout the cell is associated with apoptotic cell death in these lesions. The activities of cathepsins B and L were significantly increased after 6 hours of exposure to 7-oxysterols and then gradually decreased (as assayed after 12 and 24 hours). The early increase of the lysosomal enzymes may represent induction of autophagocytotic repair, whereas the later decline may be due to autodegradation when the enzymes are released to the cytosol during the apoptotic process.

To determine whether the release of lysosomal cathepsins and apoptotic cell death might be causally associated, we investigated the effects of E64 (inhibits mainly the cysteine proteases cathepsins B, H, and L)\(^ {21} \) and the selective inhibitor of cathepsins B and L, Z-FAFMK, on 7-oxysterol-induced apoptosis. Because E64 is relatively membrane-impermeable, a high concentration (75 \( \mu g/mL \)) was applied. Neither inhibitor caused any changes in cell growth or viability, but cathepsin B activity was reduced to \( \approx 30\% \) (E64) and 50\% (Z-FAFMK), and cathepsin L activity to \( \approx 12\% \) (E64) and 40\% (Z-FAFMK) of that of control cells. Both E64 and Z-FAFMK provided remarkable protection against oxysterol-induced apoptosis and necrosis. The inhibition of oxysterol-induced cell death by E64 was stronger than that of Z-FAFMK, which may be a result of the more extensive inhibition of cathepsins B and L by the former. As shown in Figure 5, both E64 and Z-FAFMK significantly reduced the numbers of apoptotic cells, as well as the activation of caspase-3. These results may lend further support to the idea that leakage of lysosomal cysteine proteinases may play a crucial role in caspase-3 activation. Similar activation of caspase-3 and its prevention by E64 also were observed in Figure 3.
THP-1 cells exposed to 7β-OH (data not shown). Importantly, we did conduct control in vitro assays of the activity of caspase-3 (using both active caspase-3 and lysates of 7β-OH–treated cells). The results showed that unlike caspase inhibitors Z-VAD, neither E64 nor Z-FAFMK had direct inhibitory effect on caspase-3 activity.

**Discussion**

The experiments reported above were focused on the question of why atheromatous lesions not only persist but increase in size despite the presence of cells, such as macrophages, that should ordinarily be capable of catabolizing the material within these lesions. Our overall hypothesis was that cytotoxic substances within atheroma lesions (such as, in this case, oxysterols) might not only suppress the catabolism of plaque material by macrophages but also predispose these cells, which are constantly recruited to atherosclerotic lesions, to apoptotic death. In fact, it was reported earlier that unlike caspase inhibitors Z-VAD, neither E64 nor Z-FAFMK had direct inhibitory effect on caspase-3 activity.

Figure 4. Cathepsin B immunoreactivity in control and 7-OH–treated cells. Control cells (top) display mainly a punctate pattern of immunoreactive cathepsin B, reflecting a lysosomal location. 7β-OH (28 μmol/L, 24 hours)–treated cells show a disappearance of this punctate immunoreactivity, with enhanced and dispersed cathepsin B immunoreactivity throughout whole cells, especially around and in apoptotic cell nuclei (bottom). Magnification ×1000.

Figure 5. Protective effect of E64 and Z-FAFMK on apoptosis and caspase-3 activation induced by 7-oxysterols in U937 cells. Different groups of cells were collected and stained with Wright-Giemsa or analyzed for caspase-3 activity, respectively. Note the more evident apoptotic morphology (left) in 7β-OH–treated cells compared with E64/7β-OH and Z-FAFMK/7β-OH groups (magnification ×200). Top right, Percentages of apoptotic cells after different treatments (mean±SEM, n=3). *P<0.01 vs control cells. **P<0.05 vs inhibitor-treated cells. ***P<0.05 vs control and inhibitor-treated cells. Bottom right, Caspase-3 activity in U937 cells (mean±SEM, n=6). *P<0.01 vs other groups.

We therefore elected to determine whether the cytotoxic effects that one of these components of oxLDL, 7-oxysterols, might have on the human monocytic cell lines U937 and THP-1 are similar to those observed in macrophages within human atherosclerotic lesions. Indeed, we find that micromolar concentrations of both 7β-OH and 7-keto induce apoptotic and necrotic death of these cultured cells, in agreement with an earlier report.9 Furthermore, as was the case for apoptotic macrophages within atherosclerotic lesions, we find that apoptosis induced by 7β-OH and 7-keto is associated with lysosomal rupture, apparent release to the cytosol of cathepsins B and L, and activation of caspase-3. The possibility that released lysosomal enzymes might initiate the apoptotic process is strengthened by our observations that E64 (which mainly inhibits lysosomal cathepsins B, H, and L) and also the nonlysosomal proteases papain and calpain) and Z-FAFMK (a relatively selective inhibitor of cathepsins B and L) increase cell survival and partially suppress both apoptosis and activation of caspase-3 after exposure to 7-oxysterols. E64 also has been found to prevent cell death in other cell culture models,27,28 as well as neuronal death in an ischemic animal model.29 Z-FAFMK effectively protects against apoptosis induced by P53 and several cytotoxic agents.30 A
Matrix degradation by proteolytic enzymes secreted from macrophages has been suggested to be an important factor in atherosclerotic plaque disruption. Enzymes involved in the process include serine proteases, cysteine proteases, and matrix metalloproteinases. Here, we report, for the first time, the importance of the cysteine protease cathepsin B in matrix degradation and its association with matrix metalloproteinases. We, here, report, for the first time, the occurrence of extensive immunoreactivity of cathepsins B and L in the nuclei of apoptotic cells within the atherosclerotic plaque and 7-oxysterol–treated apoptotic U937 cells. The mechanism involved in the increase of cathepsins B and L immunoreactivity in apoptotic nuclei is not yet clear. The importance of the cysteine protease cathepsin B in matrix degradation and its association with matrix metalloproteinases was recently demonstrated. The authors reported that, in addition to direct participation in tissue destruction, cathepsin B enhances the activity of matrix metalloproteinases by destroying their inhibitors. These findings, together with our results, indicate that lysosomal cathepsins may play an important role in the formation and destabilization of atherosclerotic plaques.

It should be emphasized that the concentrations of oxysterols used in the present experiments are substantially lower than those reported to exist in human atherosclerotic plaque. Therefore, there is good reason to suspect that cells that enter into atheroma will be damaged or killed by these and similar toxic plaque materials. If the interior of atheroma lesions does, indeed, represent a death zone for inflammatory cells such as monocytes/macrophages, this has implications for the overall disease process. First, this may lead to the accumulation of large numbers of dead and dying macrophages that ultimately release degradative enzymes, resulting in matrix degradation. Second, it is conceivable that products of these intoxicated cells will serve to attract further inflammatory cells to the lesion, thereby amplifying the inflammatory component and, perhaps, contributing to the overall growth of the plaque. Finally, these observations suggest one possible reason for the evident impotence of lesion-associated macrophages in clearance of the plaque material itself. These findings may open new perspectives for understanding the progression of atherosclerotic disease and, perhaps, generate new molecular targets for pharmaceutical control of plaque development.

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13. Roberg K, Johansson U, Ollinger K. Lysosomal release of cathepsin D does, indeed, represent a death zone for inflammatory cells than those reported to exist in human atherosclerotic plaque. Therefore, there is good reason to suspect that cells that enter into atheroma will be damaged or killed by these and similar toxic plaque materials. If the interior of atheroma lesions does, indeed, represent a death zone for inflammatory cells such as monocytes/macrophages, this has implications for the overall disease process. First, this may lead to the accumulation of large numbers of dead and dying macrophages that ultimately release degradative enzymes, resulting in matrix degradation. Second, it is conceivable that products of these intoxicated cells will serve to attract further inflammatory cells to the lesion, thereby amplifying the inflammatory component and, perhaps, contributing to the overall growth of the plaque. Finally, these observations suggest one possible reason for the evident impotence of lesion-associated macrophages in clearance of the plaque material itself. These findings may open new perspectives for understanding the progression of atherosclerotic disease and, perhaps, generate new molecular targets for pharmaceutical control of plaque development.

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