Cell Composition, Replication, and Apoptosis in Atherosclerotic Plaques After 6 Months of Cholesterol Withdrawal

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Abstract—Unstable human atherosclerotic plaques are characterized by a thin fibrous cap that contains few smooth muscle cells (SMCs) and numerous foam cells of macrophagic origin. Apoptosis of SMCs in the fibrous cap could destabilize the plaque and promote plaque rupture. In an experimental approach, we have studied apoptotic cell death and related proteins in atherosclerotic plaques of cholesterol-fed rabbits and examined the effects of cholesterol withdrawal. The induced atherosclerotic plaques at the thoracic aorta were composed of both fibromuscular tissue and foam cells. The presence of SMCs overlying macrophage accumulation was reminiscent of the structure of human atherosclerotic plaques. The plaques showed signs of cell replication and apoptotic cell death (1.8±0.5% terminal deoxynucleotidyl transferase end-labeling [TUNEL]-positive nuclei). Cell replication was confined mostly to the macrophages, whereas 34% of the TUNEL-labeled cells were SMCs. Both the macrophages and SMCs in the plaques expressed BAX, a proapoptotic protein of the BCL-2 family. After 6 months of cholesterol withdrawal, the thickness of the plaques in all localizations of the aorta was unchanged, but apoptosis was nearly absent (<0.1% of nuclei). Moreover, macrophages disappeared from the plaques, whereas the SMCs that remained present lost their lipid accumulation and strongly reduced their BAX expression. These changes were associated with a reduction of cell replication and increased deposition of fibrillar collagen fibers in the plaques, which pointed to plaque stabilization. In conclusion, the cell composition but not the thickness of atherosclerotic plaques was profoundly altered after a 6-month cholesterol withdrawal period. These changes were associated with a strong reduction of cell replication and apoptotic cell death. Moreover, the expression of the proapoptotic factor, BAX, was reduced in the remaining cells, which were mainly SMCs. These findings could help to explain the benefit of lipid-lowering therapy on plaque stabilization. (Circ Res. 1998;83:378-387.)

Key Words: atherosclerosis ■ apoptosis ■ smooth muscle cell ■ BAX ■ terminal deoxynucleotidyl transferase nick end-labeling

Macrophage accumulation is linked to plaque destabilization and can lead to acute coronary syndromes.1–4 In a previous study involving the occurrence of cholesterol-induced atherosclerotic plaques of the rabbit aorta, we were struck by the high cell replication of macrophages and the presence of apoptotic cell death in the plaques.5 Apoptosis is also present in human atherosclerotic plaques6–12 and is pronounced in regions with macrophage infiltration, whereas lesions consisting only of smooth muscle cells exhibit very little apoptosis. Most of these studies detect apoptotic cells by the terminal deoxynucleotidyl transferase nick end-labeling (TUNEL) technique, which uses DNA fragmentation as a marker of apoptotic cell death.

DNA fragmentation is a rather late stage of apoptotic cell death. Apoptosis occurs in at least 2 stages.13 After a signal, which may be either intrinsic or extrinsic to the cell, the cell enters a committed phase. This is terminated in cell-autonomous fashion by a transition to a final execution phase. The latter, which includes DNA fragmentation, is brief and decisive. Bennet et al14 have found that most smooth muscle cells derived from atherosclerotic plaques but not those from the media die when brought in culture. This suggests that the smooth muscle cells of the atherosclerotic plaques but not the medial smooth muscle cells are committed to die. In the present study, cell replication and the execution and commitment phases of apoptotic cell death were quantified in experimental atherosclerotic plaques after 26 weeks of cholesterol feeding and after a period of durable normalization of the serum cholesterol levels. The execution phase of apoptosis was detected by DNA in situ end-labeling.15–18 Changes in the expression of proteins of the BCL-2 family were used to detect the commitment phase.

Materials and Methods
Male New Zealand White rabbits (2.8 to 3.5 kg) were fed a diet supplemented with low-dose cholesterol (0.3%) for 26 weeks.

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Ten randomly selected animals were killed after this period (26w chol group): the other animals were fed a normal diet for another 26 weeks (26w chol wd group). After this period, these animals were also killed for study. A control group of 10 animals was included. Five control animals were killed after 26 weeks and 5 were killed after 52 weeks. Serum was stored at −20°C, and serum total cholesterol, free cholesterol, serum triglyceride, phospholipid, LDL, HDL, and VLDL levels were determined. For histological examination, the complete aorta was dissected and fixed in situ in 4% formalin. Transversal sections (5 μm thick) of paraffin-embedded tissues were mounted on slides precoated with 3-aminopropyltriethoxysilane (Sigma). Masson’s trichrome stain, sirius red hematoxylin, and Verhoeff’s elastic stain were used on all sections. A Sudan IV (Scarlet Red Michaelis, BDH) fat stain was used on frozen sections of formalin-fixed segments.

Immunohistochemistry

The following primary monoclonal antibodies were used: α-smooth muscle actin (Sigma Chemical Co), MO/RAM-11 (anti-rabbit macrophages) and BCL-2 (Dako), BAX (rat monoclonal, Pharmingen), and Ki-67 (MIB-1, Immunotech).

All antibodies were diluted in PBS. The monoclonal antibodies were detected by an indirect peroxidase antibody conjugate technique. The sections were incubated with a goat anti-mouse peroxidase antibody (Jackson) and a rabbit anti-rat antibody (Dako) for 45 minutes. For demonstration of the complex, 3-amino-9-ethylcarbazole was used as a chromogen. The specificity of the immunohistochemical reactions was checked by omitting the primary antibody and substituting the antibody by an unrelated antibody at the same concentration.

To demonstrate the colocalization of BAX and α-smooth muscle actin, we used a stain and washout technique that was recently developed in our laboratory. A classical double immunohistochemical stain was difficult to interpret because of the color overlap and the problem of mixed colors. Therefore, we first stained the sections with an antibody against α-smooth muscle actin and made digital photomicrographs. This was followed by an antigen-retrieval step (trypsin digestion and citrate buffer treatment in a microwave oven), which was necessary to destroy the residual antibodies of the first stain and retrieve the antigen for the immunohistochemical stain for BAX. The same cells that were photographed for α-smooth muscle actin were relocated in the section, and digital pictures were taken. Controls for this stain and washout technique included omitting the primary antibody and substituting the antibody by an unrelated antibody at the same concentration.

DNA In Situ End-Labeling

After deparaffinization and rehydration, tissue sections were incubated with 3% citric acid. This step removes all small calcium-containing vesicles that can be responsible for aspecific binding of the nucleotides. Both TUNEL and ISNT were used. For the TUNEL technique, the ApopTag kit (Oncor) was used with minor modifications. For the ISNT technique, the sections were incubated with a goat anti-mouse peroxidase antibody (Jackson) and a rabbit anti-rat antibody (Dako) for 45 minutes. For demonstration of the complex, 3-amino-9-ethylcarbazole was used as a chromogen. The specificity of the immunohistochemical reactions was checked by omitting the primary antibody and substituting the antibody by an unrelated antibody at the same concentration.

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Quantification

The images were analyzed using a color image analysis system (PC Image Color, Foster Findlay Associates). The atherosclerotic plaques were divided into rectangular areas located in the superficial and deep part of the plaques. The area and percentage of immunoreactive areas within each rectangle were measured. The segmentation of the immunoreactive area was performed by interactive selection of the gray level zone corresponding with the brown color of the immunoreactive regions.

In the atherosclerotic plaques of the cholesterol-fed rabbits, 20 rectangular areas per transversal section were counted along both the superficial and the deep parts of the plaques. The areas were randomly chosen. If the intimal thickness exceeded 200 μm, then a luminal rectangle (base, 90 μm along the endothelial cell layer; height, 100 μm into the intima) and a deep rectangle (base, 90 μm along the internal elastic lamina; height, 100 μm into the plaque) were measured. If the intimal thickness was <200 μm, the intima was split into a superficial and a deep half. The side along the endothelial and the internal elastic membrane was again 90 μm. The mean percentage of the total plaque area per transversal section that was covered by the 20 rectangular areas was 15±2%.

For each region, the total area, the total number of nuclei, the numbers of Ki-67 and DNA in situ end-labeled nuclei, and the percentage of immunoreactive areas for RAM-11, α-smooth muscle actin, and BAX were measured. The latter variables were expressed as percentage of the total area.

The plaque area of the different parts of the aorta (ascending, arch, thoracic, abdominal proximal, and distal) was measured by tracing the internal elastic lamina and the luminal circumference.

Statistical Analysis

Cell Number per Area, Cell Replication, and Apoptosis of Atherosclerotic Plaques

The total number and the number of labeled nuclei per fixed cross-sectional area in superficial and deep layers was calculated. The data for the deep versus the superficial layer were statistically evaluated using the Wilcoxon signed rank test. To compare the 26w chol group with the 26w chol wd group, the Mann-Whitney U test was applied.

Plaque Area and Thickness in Different Parts of the Aorta

The 26w chol and 26w chol wd groups were compared using an unpaired Student t test.

Serum Lipid Values

The values between the 2 groups (26w chol versus 26w chol wd) were compared using the unpaired Student t test. To compare the values between the time points (week 0, week 26, and week 52), ANOVA (3 time points) or the paired Student t test (2 time points) was applied.

The SPSS package for Windows (SPSS Inc) was applied for these purposes. A 5% level of significance was selected.

Transmission Electron Microscopy

The fragments for transmission electron microscopy were fixed for 2 hours in 1% (vol/vol) glutaraldehyde in 0.1 mol/L sodium cacodylate buffer (pH 7.4). They were postfixed for 30 minutes in 1% (vol/vol) osmium tetroxide in 0.1 mol/L sodium cacodylate buffer (pH 7.4). After dehydration in an ethanol gradient, they were embedded in LX-112 (Ladd Research Industries). Selection of the zones most representative of the lesions was made on 2-μm sections oriented in a transverse plane (perpendicular to the bloodstream) and stained with toluidine blue. Sections (50 nm thick) were cut on an Ultratome...
Results

Serum Lipid Values

The details of the serum lipid values at the start of the experiment, after 26 weeks of cholesterol supplement, and after the cholesterol withdrawal period are shown in the Table. The diet with a cholesterol supplement of 0.3% induced a pronounced hypercholesterolemia at week 26, which was completely reversed after 26 weeks of cholesterol withdrawal.

Atherosclerotic Plaques of Cholesterol-Fed Rabbits at 26 Weeks (n=10)

The induced atherosclerotic plaques at the thoracic aorta were composed of both fibromuscular tissue and foam cells. The presence of smooth muscle cells overlying macrophage accumulation was reminiscent of the structure of human atherosclerotic plaques (Figure 1). However, an important difference was the absence of plaque rupture and thrombosis.

The atherosclerotic plaques showed a clear distinction between a superficial region composed of numerous foam cells and a deep layer that was fibrous and contained few cells. In the superficial layer, the majority of lipid was located within the cytoplasm of the cells (foam cells) (Figure 2A), and interstitial collagen fibers were nearly absent in this layer (Figure 2B). Most of these foam cells were of macrophagic origin, as indicated by their cytoplasmic immunoreactivity for α-smooth muscle actin (Figure 2C). The smooth muscle cells were filled with lipid vacuoles, and foam cells of smooth muscle origin were also present in this layer (Figure 2D).

The deeper layer contained areas of extracellular lipid and interstitial collagen fibers, as demonstrated by sirius red polarization microscopy (Figure 2B).

The nuclear density and the percent RAM-11 and α-smooth muscle actin immunoreactive areas were higher in the superficial layer (Figure 3). Although the size of the plaques decreased toward the bifurcation, the composition and distribution of the cells in the plaques was rather consistent.
Cell Replication

Within the superficial region, 11.2% of the nuclei demonstrated immunoreactivity for Ki-67 (Figure 3). This indicates that the cell replication in this region is high. A double immunohistochemical stain for Ki-67 and RAM-11 (Figure 4A) or α-smooth muscle actin (Figure 4B) demonstrated that 95% of the Ki-67–labeled cells were macrophages and 5% were smooth muscle cells.

The deeper layer of the atherosclerotic plaques was cell poor, with low replication rates, which were significantly different from the superficial layer. This deeper layer contained macrophages, cellular remnants, and smooth muscle cells with a decreased α-smooth muscle actin content. These cells were negative for RAM-11.

Apoptotic Cell Death/BAX Expression

Both the superficial and the deep layer of the atherosclerotic plaques showed apoptotic nuclei, as visualized by TUNEL and ISNT. The percentages of labeled nuclei in the deep layer were not different from the percentages in the superficial regions (Figure 3). The labeled nuclei were either condensed or enlarged. A significant fraction of the TUNEL-labeled nuclei and nuclear remnants could not be stained by RAM-11 or α-smooth muscle actin, which could reflect a loss of specific markers during the execution phase of apoptosis. A feature of smooth muscle cells but not of macrophages in atherosclerotic plaques is that they are surrounded by cages of thickened basal lamina.5,8 Basal laminae and basement membranes can be stained by a periodic acid–Schiff’s (PAS) stain. By combining the TUNEL technique with a PAS stain, we could detect TUNEL-labeled nuclei and nuclear fragments that were not enclosed by PAS-positive laminae, indicating apoptosis of macrophages (Figure 4C), and labeled nuclei that were enclosed by a cage of thickened basal laminae, which points to smooth muscle cell apoptosis (Figure 4D). Moreover, clusters of TUNEL-negative cytoplasmic remnants, which were enclosed by thickened basal lamina, were present, which can be considered as cytoplasmic remnants of apoptotic smooth muscle cells. By use of this technique, we have found that 34% of all TUNEL-labeled cells were smooth muscle cells and 66% were macrophages.

The cellular origin of the TUNEL-labeled nuclei was also studied by immunohistochemical double stains. Immunohis-
BAX. A stain and washout technique for BAX. The smooth muscle cells in the adjacent media did not particularly in superficial layer of the plaque, expressed phage-derived foam cells, and many smooth muscle cells, macrophages.

A superficial band of a and expressed BAX. The smooth muscle cells were also BAX immunoreactive, whereas only 8% of all TUNEL-labeled macrophages expressed BAX.

The arteries of the age-matched control animals (n=5) were without intimal thickening and did not show TUNEL-labeled nuclei. The endothelial cells expressed BAX, whereas the smooth muscle cells of the media did not (Figure 4L).

Transmission Electron Microscopy
Transmission electron microscopy of the plaques showed an uninterrupted layer of endothelial cells (Figure 5A). Underneath the endothelial cells, numerous foam cells were present. The foam cells were crowded with large nonmembrane-bound vacuoles, presenting ramified lamellipodia, containing lysosomes and nondescript cellular debris, all characteristics of monocyte-derived macrophages. The smooth muscle cells contained large dilated profiles of rough endoplasmic reticulum, medium-sized optically empty vacuoles not bounded by a membrane, few filaments, few pinocytotic vesicles, and a fragmented basal lamina (Figure 5A), all stigmata of cells with the synthetic phenotype and fat accumulation. The thickened basal laminae surrounding these smooth muscle cells are the ultrastructural equivalent of PAS-positive cages of the smooth muscle cell (see Figure 4D and 4H). Smooth muscle cells showing dropping off and fragmentation of cytoplasm and condensation of the chromatin, which are ultrastructural characteristics of apoptosis, could be demonstrated. The deep layer contained cross-banded collagen fibers among irregular moderately electron-dense granular material and smooth muscle cells.

Atherosclerotic Plaques in Rabbits After 26 Weeks of Cholesterol Supplement Followed by 26 Weeks of Cholesterol Withdrawal (n=10)
The lipid values showed a normalization after the cholesterol withdrawal period (Table). The cell composition of the atherosclerotic plaques after a period of cholesterol withdrawal was profoundly altered (Figure 2A, chol wd; Figure 2B, chol wd). The distinction between a superficial foam cell–rich layer and a deep fibrous region was lost. Most plaques were transformed into fibrous collagen-rich plaques, although residual fat deposits could still be detected. The thickness of the plaques was not significantly different from the plaque thickness of the 26-week group, and the areas of the plaques in the different parts of the aorta were not decreased (Figure 6). The cell density was decreased (Figure 7). Macrophages (as demonstrated by a RAM-11 stain) disappeared almost completely from the plaques (Figure 2C, chol wd; Figure 3). Most of the remaining cells were smooth muscle cells. The smooth muscle cells were spindle-shaped and expressed a-smooth muscle actin (Figure 2D, chol wd). A superficial band of a-smooth muscle actin–expressing smooth muscle cells was often present and was reminiscent of the fibrous cap present in human atherosclerotic plaques.

Cell Replication
The plaques showed a strong reduction in nuclei that were labeled by the antibody against Ki-67. This indicates a strong reduction of cell replication within the plaques (Figure 3).

Apoptotic Cell Death/BAX Expression
The plaques showed also a strong reduction in the nuclei that were labeled by the TUNEL and ISNT techniques (Figure 3). Moreover, a strong reduction in the expression of BAX was noticed and quantified (Figures 4I, 4J, 4K, and 7). This was a consequence of both the disappearance of the BAX—expressing macrophages and a loss of the BAX expression in the smooth muscle cells. BCL-2 could not be detected. We have calculated RAM-11, a-smooth muscle actin, and BAX expression per cell before and after cholesterol withdrawal (Figure 7). Interestingly, the BAX immunoreactive area expressed per cell was significantly decreased after cholesterol withdrawal.
The arteries of the age-matched control animals (n=5) did not develop atherosclerotic plaques. The smooth muscle cells in the media of these control animals remained strictly negative for BAX (Figure 4L). The endothelial cells of the aorta in these control animals showed BAX expression that was independent of the age of the animals.

**Transmission Electron Microscopy**

The plaques were covered by an uninterrupted layer of endothelial cells, which had become flatter. The plaques were mainly composed of smooth muscle cells that showed a contractile phenotype. Few lipid vacuoles remained in the cytoplasm of the cells. Between the smooth muscle cells, numerous cross-banded collagen fibers were present (Figure 5B).

**Discussion**

The present study demonstrates that a cholesterol withdrawal period of 6 months does not induce a regression of experimentally induced atherosclerotic plaques. This finding was reported by Anitschkow and subsequently confirmed by other authors. The atherosclerotic plaques in the present

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**Figure 4.** Double immunohistochemical stain for cell replication and macrophages and TUNEL/PAS, BAX/PAS, and BAX expression. A, Atherosclerotic plaque at 26 weeks. Double immunohistochemical stain for Ki-67 (brown-labeled nuclei) and RAM-11 for macrophages (red). Two labeled nuclei are shown that express RAM-11 in their cytoplasm, indicating replication in the macrophage population. B, Double immunohistochemical stain for Ki-67 (nuclei with brown color) and α-smooth muscle actin (red). Four labeled nuclei are shown in the superficial layer of an atherosclerotic plaque at 26 weeks. Two of these labeled nuclei are present in cells that express α-smooth muscle actin, indicating that low levels of smooth muscle cell replication are detectable in the plaques. The other labeled nuclei that belong to cells that are negative for α-smooth muscle actin are probably replicating macrophages. C, TUNEL+PAS. A TUNEL-labeled nucleus that is not surrounded by a cage of PAS-positive material is present. This points to apoptotic cell death of a macrophage, because macrophages are not surrounded by PAS-positive basal lamina. D, TUNEL+PAS. A TUNEL-labeled nucleus is present that is surrounded by a cage of PAS-positive basal lamina. This points to apoptotic cell death of a smooth muscle cell in the superficial region of the plaque. E, TUNEL+α-smooth muscle actin. Atherosclerotic plaque at 26 weeks. A TUNEL-labeled nucleus present in a cell that expresses α-smooth muscle actin. This is another argument for the presence of smooth muscle cell apoptosis in the plaques at 26 weeks. F and G, Stain and washout technique for α-smooth muscle actin/BAX. Panel F demonstrates lipid-laden smooth muscle cells in an atherosclerotic plaque at 26 weeks that are immunoreactive for α-smooth muscle actin. The same section was subsequently stained for BAX. Panel G demonstrates the same lipid-laden smooth muscle cells showing a strong granular cytoplasmic immunoreactivity for BAX. H, BAX+PAS. A BAX immunoreactive smooth muscle cell that is surrounded by a cage of PAS-positive basal lamina is present. I to L, BAX immunohistochemical stain of the superficial region (SL) of an atherosclerotic plaque at 26 weeks (I), the deep region (DL) of the same atherosclerotic plaque and the adjacent media (MED) (J), atherosclerotic plaque after 26 weeks of cholesterol withdrawal (K), and the thoracic aorta of an age-matched control animal that has received a normal diet for 52 weeks (L). Bars=30 μm (A to H) and 60 μm (I to L).
study were induced by giving rabbits a cholesterol supplement of 0.3% for 6 months. This resulted in atherosclerotic plaques that contained both foam cells of macrophagic origin, smooth muscle cells, and collagen fibers. The presence of smooth muscle cells overlying macrophage accumulation was reminiscent of the structure of human atherosclerotic plaques.

The plaques after 26 weeks of cholesterol supplement fulfill some criteria of unstable human atherosclerotic plaques, since most of the superficial area was occupied by foam cells of macrophagic origin, and interstitial collagen fibers were absent or scarce in the superficial region of the plaque.1,35 However, an important difference was the absence of plaque rupture and thrombosis. Before the cholesterol withdrawal period, the plaques showed a high cell replication, which was mainly limited to the macrophage population, and demonstrated apoptotic cell death. Moreover, both the macrophages and the smooth muscle cells expressed BAX, a proapoptotic protein of the BCL-2 family.36–39 This protein was only weakly expressed in the adjacent media and was absent in the aorta of age-matched normocholesterolemic rabbits.

Although the plaques did not regress and even tended to extend into the arch and thoracic and abdominal aorta after the cholesterol withdrawal period, the plaques showed numerous qualitative changes. The distinction between a superficial foam cell–rich layer and a deep fibrous region was lost. Most plaques were transformed into fibrous collagen-rich plaques that showed few residual fat deposits, confirming the study of Aikawa et al.28 Birefringent polarizing fat, which at 26 weeks is mostly globular and intracellular, is present after the cholesterol withdrawal period as needlelike crystals in an extracellular position in the deep layer of the plaques. This corresponds to deposition of interstitial collagen fibers and is the ultrastructural hallmark of plaque stabilization.

Figure 5. Transmission electron micrograph of the superficial layer fibrous cap of an atherosclerotic plaque at 26 weeks and at 26 weeks + cholesterol withdrawal period. A, At 26 weeks, the endothelial cells (ec) are cuboidal and show a pronounced rough endoplasmic reticulum. Beneath the endothelial layer, the smooth muscle cells contain numerous lipid vacuole myeloid bodies and a clear rough endoplasmic reticulum. The intercellular space contains a basal lamina–like material (1 arrow), but cross-banded collagen fibrils are rare or absent. Bar=1 μm. B, At 26 weeks + 26-week cholesterol withdrawal period, the ec are flattened. The smooth muscle cells show a contractile phenotype with abundant microfilaments. The intercellular space contains numerous collagen fibrils (2 arrows) with transverse bands that show a periodicity of 68 nm. This corresponds to deposition of interstitial collagen fibers and is the ultrastructural hallmark of plaque stabilization. Bar=1 μm.
plaques increased during plaque progression.40 However, we and others have not observed a complete fibrous transformation of the superficial layers and the edges of the plaques when the cholesterol supplement was not stopped.5,40

Most of the changes after 6 months of cholesterol withdrawal are the result of pronounced changes in the cell composition, cell replication, and apoptosis within the plaques during the cholesterol withdrawal period. A strong reduction or even disappearance of the macrophages from the plaques was a major feature. The mechanism and complete time course by which the macrophages disappear from the plaques was beyond the scope of the present study. Stary41 has demonstrated that the foam cells of macrophagic origin in atherosclerotic plaques of cholesterol-fed monkeys disappeared by cell death in the first weeks after cholesterol withdrawal, whereas death of smooth muscle cells became infrequent. In the present study, it is possible that the macrophages disappeared by apoptosis in the early period after cholesterol withdrawal. However, further studies with tissues collected and examined at several time points after the cholesterol withdrawal could reveal interesting data concerning the fate of the disappearing macrophages. After the 26-week cholesterol withdrawal period, the plaques are mainly composed of smooth muscle cells; cell replication and apoptotic cell death are no longer detectable in these plaques. This is another argument for the hypothesis that the macrophages are responsible for the induction of apoptotic cell death and cell replication in the plaques. A double immunohistochemical stain demonstrated that 95% of the Ki-67–labeled cells were macrophages, whereas only 5% of the labeled cells were smooth muscle cells. These data are comparable to the findings of Rekhter and Gordon42 in human atherosclerotic plaques. A major difference is the absence of neovascularization and thrombosis in the present study. In the study of Rekhter and Gordon, a significant fraction of the proliferating cell nuclear antigen–labeled cells were endothelial cells. These authors found an overall proliferative activity of 2% in the plaques, which is less than the values we found in the plaques at 26 weeks. The difference in the percentage of labeled cells in the present study could be a consequence of the denominator used to calculate the fraction of labeled nuclei. In a previous study involving human atherosclerotic plaques in human saphenous vein grafts and carotid atherosclerotic plaques, we found that cell replication was mainly present in the foam cells of macrophagic origin.8 Regions around the necrotic core and in the cap of unstable atherosclerotic plaques contained high percentages of Ki-67–labeled nuclei. However, it remains to be proven whether these cells are dividing or arrested in the G0 phase of the cell cycle.

Apoptotic cell death was studied by the detection of DNA fragmentation by use of the TUNEL technique. Low but reproducible values were detected in the plaques. The values of 0.5% to 2% were in the same range as those found and reported in atherosclerotic plaques of cholesterol-fed rabbits in a previous study by our group.5 The occurrence of DNA cleavage in a cell is also called the final or executive phase of the apoptotic cascade. This phase is short (6 hours) and irreversible. The short duration of this phase could explain the low values that we have found in the plaques. The execution phase is preceded by a phase of commitment. During this phase, the cells increase their susceptibility for apoptosis. During this phase, the cell increases different proapoptotic factors; however, the cells remain TUNEL-negative. The increased BAX expression can be considered as a marker of the commitment phase of apoptosis. The length of this phase is not known. There exists a correlation between the BAX/BCL-2 ratio and the susceptibility for cell death. For example, Purkinje cells of the cerebellum show a very high BAX expression9 and are believed to be one of the most sensitive subpopulations of the brain for ischemic cell death.51 In contrast to Isner et al,12 we could not detect BCL-2 in the normal media or the plaques. Other authors have also reported that normal arteries of mice contain little or no BCL-2.
Another indication that smooth muscle cells in the plaques can be committed for apoptotic cell death is coming from the caspase 8 system by Fas-Fas ligand interaction. Geng et al found that smooth muscle cells in atherosclerotic plaques showed increased Fas expression, which could be another argument that smooth muscle cells in the plaques are committed for apoptosis. The commitment phase can be followed by the final execution phase if other factors are also present. The TUNEL technique detects only the final execution phase of apoptosis. This could explain the fact that numerous cells are BAX-positive in the plaque but that only a small fraction is TUNEL-positive. The relationship between BAX and TUNEL is obvious for the smooth muscle cells but less clear for the macrophages: 35% of all TUNEL-labeled smooth muscle cells were also BAX immunoreactive before the lipid lowering, whereas only 8% of all TUNEL-labeled macrophages were BAX immunoreactive. After a period of cholesterol withdrawal, BAX expression in the atherosclerotic plaques was greatly decreased. This was a consequence of both the disappearance of the macrophages and a loss of the lipid accumulation in the smooth muscle cells. Interestingly, the BAX-immunoreactive area expressed per cell was significantly decreased after cholesterol withdrawal. This indicates that after the cholesterol withdrawal period, the remaining smooth muscle cells decrease their susceptibility to undergo apoptotic cell death.

The changes in the cell composition of plaques after cholesterol withdrawal is in agreement with the study of Shiomi et al. The authors found a significant decrease in the macrophage and lipid deposits and an increase in the collagen content in atherosclerotic plaques of Watanabe heritable hyperlipemic rabbits treated with pravastatin. Moreover, pravastatin treatment suppressed the decrease in the smooth muscle cell area during lesion progression. This indicates that the benefit of lipid lowering in human atherosclerotic plaques could be plaque stabilization caused by decreasing the macrophage content, resulting in less smooth muscle cell death and collagen breakdown. Macrophages are reported to be present in unstable atherosclerotic plaques and at sites of plaque ruptures. A major benefit of lipid lowering could be the reduction of the macrophage infiltration in the plaque, a phenomenon that is not always associated with a reduction of the plaque thickness.

A problem in the evaluation of lipid-lowering trials is that the clinical symptoms and angiographical data are not always related to the changes that occur in the plaques. In the present experimental study, we have determined that the thickness and area of the plaques does not change after cholesterol withdrawal. Moreover, we have found that the lesions tended to increase in the aortic arch and thoracic and abdominal aortas. This indicates that an angiographical study performed on these animals would possibly not have shown a beneficial effect of the cholesterol withdrawal.
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