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

Mark M. Kockx, Guido R.Y. De Meyer, Norbert Buysens, Michiel W.M. Knaapen, Hidde Bult, Arnold G. Herman

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. 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. Apoptosis is also present in human atherosclerotic plaques 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. 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 al have found that the 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. 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), M0/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-amin-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 against BAX. This resulted in a complete disappearance of the staining. This indicates that the antibodies used for the α-smooth muscle actin stain (first stain) did not interfere with the secondary antibody used to detect BAX (second stain).

**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 in situ nick translation (ISNT) were used. For the TUNEL technique, the ApopTag kit (Oncor) was used with minor modifications. For the ISNT technique, the sections were rinsed in a buffer (50 mmol/L Tris-HCl, 5 mmol/L MgCl₂, and 0.0005% BSA, pH 7.5) for 10 minutes, dried, and then incubated at 37°C for 1 hour with the same buffer containing 0.01 mmol/L dATP, dCTP, and dGTP (Sigma) and 0.01 mmol/L biotin-16-dUTP (Boehringer Mannheim) with 20 U/mL of the Klenow fragment of DNA polymerase I (Boehringer Mannheim). Incorporated biotin-16-dUTP was demonstrated by incubating the sections with a monoclonal antibody against biotin (Dako) at a dilution of 1:40 for 30 minutes. The antibody was visualized by a goat anti-mouse peroxidase (Jackson) at a dilution of 1:125 for 45 minutes.

In both the TUNEL and the ISNT techniques, the labeled antibody was visualized by 3-amin-9-ethylcarbazole. Sections were lightly counterstained with hematoxylin and mounted in glycerin jelly. Negative controls included omission of terminal deoxynucleotidyl transferase or the Klenow fragment from the labeling mixture. Tonsils were used as a positive control.

To identify cell types undergoing apoptosis, double staining was performed by combining TUNEL and immunohistochemistry for RAM-11 and α-smooth muscle actin.

**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.

**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.
Lipid Values at Start of Experiment (Week 0) and After a 26-Week Cholesterol Supplement of 0.3% (Week 26), Followed by a 26-Week Cholesterol Withdrawal Period (Week 52)

<table>
<thead>
<tr>
<th></th>
<th>Week 0</th>
<th>Week 26</th>
<th>Week 52</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total cholesterol</strong></td>
<td>41±5</td>
<td>912±141*</td>
<td>...</td>
</tr>
<tr>
<td>26w chol wd</td>
<td>43±6</td>
<td>1245±148*</td>
<td>28±2</td>
</tr>
<tr>
<td><strong>Free cholesterol</strong></td>
<td>11±1</td>
<td>298±52*</td>
<td>...</td>
</tr>
<tr>
<td>26w chol</td>
<td>12±1</td>
<td>375±33*</td>
<td>6±1</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td>64±56</td>
<td>94±14</td>
<td>...</td>
</tr>
<tr>
<td>26w chol wd</td>
<td>91±12</td>
<td>105±45</td>
<td>65±9</td>
</tr>
<tr>
<td><strong>Phospholipids</strong></td>
<td>91±6</td>
<td>410±48*</td>
<td>...</td>
</tr>
<tr>
<td>26w chol wd</td>
<td>96±8</td>
<td>525±42*</td>
<td>59±4</td>
</tr>
<tr>
<td><strong>HDL cholesterol</strong></td>
<td>22±2</td>
<td>20±3</td>
<td>...</td>
</tr>
<tr>
<td>26w chol wd</td>
<td>22±3</td>
<td>20±2</td>
<td>15±2</td>
</tr>
<tr>
<td><strong>LDL cholesterol</strong></td>
<td>8±1</td>
<td>321±105†</td>
<td>...</td>
</tr>
<tr>
<td>26w chol</td>
<td>7±1</td>
<td>396±140*</td>
<td>6±1</td>
</tr>
<tr>
<td><strong>VLDL cholesterol</strong></td>
<td>11±1</td>
<td>218±89‡</td>
<td>...</td>
</tr>
<tr>
<td>26w chol wd</td>
<td>20±8</td>
<td>412±46*</td>
<td>8±2</td>
</tr>
</tbody>
</table>

The values (mg/dL) of the serum lipids were not different between 26w chol and 26w chol wd groups at week 0 or week 26. Values after 26 weeks of cholesterol supplement that were different from week 0 and week 52 are indicated.

*P<0.001, †P<0.05, and ‡P<0.01 vs week 0 and week 52 by paired Student t test (2 time points).

Nova (Reichert-Jung). They were stained for 30 minutes at 40°C with uranyl acetate and for 15 minutes at 20°C with lead citrate in an Ultrostainer 2168 (LKB). The sections were examined in a Jeol-1200 EX transmission electron microscope at 80 kV. Photographs were made with electron microscopy film (4489 Estar Thick Base, Kodak).

**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 a-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 a-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 a-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-
Expression 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
macrophages.

Both apoptotic smooth muscle cells (Figure 4E) and
of TUNEL with RAM-11 or
a
tochemical staining of adjacent sections and double stainings
of TUNEL with RAM-11 or
a
 reportedly increased after cholesterol withdrawal. The percentage
of the α-smooth muscle actin (α-SMC actin)–immunoreactive
area is significantly higher in the superficial layer and
increased after cholesterol withdrawal. However, the area of
α-SMC actin per cell remained constant (see Figure 7). Cell rep-
lication, as demonstrated by the percentage Ki-67–labeled
nuclei, is higher in the superficial layer at 26 weeks. The replication
rate is almost absent after the cholesterol withdrawal
period. The percentages of apoptotic cell nuclei (TUNEL tech-
ique) were not different in the different layers of the plaques.
After the cholesterol withdrawal period, apoptotic cell death as
detected by DNA in situ end-labeling was absent. *P<0.05,
**P<0.01, and ***P<0.001 vs before 26-week cholesterol with-
drawal (Mann-Whitney U test).

All endothelial cells, a significant proportion of macro-
phage-derived foam cells, and many smooth muscle cells,
particularly in superficial layer of the plaque, expressed
BAX. The smooth muscle cells in the adjacent media did not
express BAX. A stain and washout technique for α-smooth
muscle actin and BAX demonstrated that the lipid-laden
smooth muscle cells expressed BAX (Figure 4F and 4G); BCL-2 could not be detected. By combining the BAX
immunohistochemical stain with a PAS stain (Figure 4H), we
demonstrated that 47% of all BAX immunoreactive cells in
the plaques were smooth muscle cells; the others were
macrophages. Interestingly, 35% of all TUNEL-labeled 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).

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 with-
drawal 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 α-smooth muscle actin (Figure 2D, chol wd).
A superficial band of α-smooth muscle actin–expressing
smooth muscle cells was often present and was reminiscent of
a 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, α-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 choles-
terol 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\(^{19}\) and subsequently confirmed by other authors.\(^{20-34}\) The atherosclerotic plaques in the present...
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. 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. 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. 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. 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.

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. Stary 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 immuno- 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.

Plaque areas in the different parts of the aorta (ascending, arch, thoracic, abdominal proximal [abd prox], and abdominal distal [abd dist]). In none of the different localizations could a significant decrease of the plaque area be demonstrated after cholesterol withdrawal (unpaired Student t test).

Figure 6. Plaque areas in the different parts of the aorta (ascending, arch, thoracic, abdominal proximal [abd prox], and abdominal distal [abd dist]). In none of the different localizations could a significant decrease of the plaque area be demonstrated after cholesterol withdrawal (unpaired Student t test).

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. The occurrence of DNA fragmentation 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 expression and are believed to be one of the most sensitive subpopulations of the brain for ischemic cell death. In contrast to Isner et al., 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.
protein and mRNA.\textsuperscript{39} It is not clear from the present study which factor is a BAX heterodimer in the smooth muscle cells and macrophages, since BCL-2 was not detectable in either cell type. However, it is possible that BCL-2–related proteins like BCL-xL\textsuperscript{40} or McI-1 are involved.

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.\textsuperscript{45} Geng et al\textsuperscript{46} 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.\textsuperscript{47} 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\textsuperscript{4} and collagen breakdown. Macrophages are reported to be present in unstable atherosclerotic plaques and at sites of plaque ruptures.\textsuperscript{3,4,48} 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 angiological 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.

The detection of macrophage infiltration in atherosclerotic plaques in vivo would be the ideal technique for evaluating the beneficial effect of lipid-lowering drugs on plaque stability. An interesting approach could be the thermal detection of cellular infiltrates in living atherosclerotic plaques by a thermistor.\textsuperscript{49} Understanding the molecular mechanism of plaque progression, combined with modern medical imaging techniques, could help to explain the benefit of lipid-lowering therapy on plaque stabilization.

Acknowledgments

Dr De Meyer is a research associate of the Flemish Fund for Scientific Research. Dr Kockx is a holder of a fund for fundamental clinical research of the Flemish Fund for Scientific Research. The authors want to acknowledge the technical support of Johannes Muhring, Ludo Zonnekeyn, Rita VandenBossche, and Christel Van Campenhout.

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

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_Circ Res._ 1998;83:378-387
doi: 10.1161/01.RES.83.4.378

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:

http://circres.ahajournals.org/content/83/4/378

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