Oxidative DNA Damage and Repair in Experimental Atherosclerosis Are Reversed by Dietary Lipid Lowering

Wim Martinet, Michiel W.M. Knaapen, Guido R.Y. De Meyer, Arnold G. Herman, Mark M. Kockx

Abstract—Increased oxidative stress is a major characteristic of hypercholesterolemia-induced atherosclerosis. The oxidative environment is mainly created by the production of reactive oxygen species, which are assumed to mediate vascular tissue injury. Oxidative DNA damage resulting from free radical attack remains, however, a poorly examined field in atherosclerosis. Male New Zealand White rabbits were fed a cholesterol-rich diet (0.3%) for 24 weeks. The induced atherosclerotic plaques showed elevated levels of the DNA damage marker 7,8-dihydro-8-oxoguanine (8-oxoG) as demonstrated by immunohistochemistry. 8-oxoG immunoreactivity was found predominantly in the superficial layer of the plaque containing numerous macrophage-derived foam cells but not in the media or in arteries of age-matched control animals. Alkaline single-cell gel electrophoresis revealed that the number of DNA strand breaks was significantly higher in the plaque as compared with control samples of normolipemic animals. These changes were associated with the upregulation of DNA repair enzymes (poly[ADP-ribose] polymerase-1, p53, phospho-p53 [phosphorylated at Ser392], and XRCC1 [x-ray repair cross-complementing 1]). DNA strand breaks normalized after 4 weeks of dietary lipid lowering. However, a significant reduction of 8-oxoG immunoreactivity was only observed after a prolonged period of lipid lowering (12 to 24 weeks). Repair pathways started to decline progressively when cholesterol-fed animals were placed on a normal diet. In conclusion, oxidative DNA damage and increased levels of DNA repair, both associated with diet-induced hypercholesterolemia, are strongly reduced during dietary lipid lowering. These findings may provide a better insight into the benefits of lipid-lowering therapy on plaque stabilization. (Circ Res. 2001;88:733-739.)

Key Words: DNA damage ■ DNA repair ■ apoptosis ■ oxidant stress ■ atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the arterial intima characterized by the formation of an atherosclerotic plaque.1 Accumulating evidence suggests that plaque formation is associated with the production of reactive oxygen species (ROS), which are currently assumed to induce oxidative tissue damage. Recent studies indicate that ROS can interact with isolated or cellular DNA to cause DNA strand breaks and/or base modifications.2-12 These oxidative modifications to DNA may cause mutations, preferentially at G-C base pair interactions.13 Repair of oxidative DNA damage is mediated by both base excision repair (BER) and nucleotide excision repair mechanisms.14-16 However, cells can respond differently to genotoxic species. Peroxynitrite-mediated DNA strand breaks, for example, may result in subsequent activation of the nuclear enzyme poly(ADP-ribose) polymerase-1 (PARP-1).17 The physiological role of PARP-1 is still unclear, but there are indications that PARP-1 functions as a DNA repair enzyme.17 Another form of cellular response to tissue injury is the upregulation of the tumor suppressor protein p53.18 In case of moderate DNA damage, p53 mediates a transient cell cycle arrest, allowing cells to repair their DNA before entering S phase. If the extent of DNA damage is severe and perhaps beyond the capacity of repair processes, the cell may undergo necrotic or apoptotic cell death. Pronounced activation of PARP-1 (as a result of extensive DNA strand breaks) induces an energy-consuming, futile repair cycle that eventually leads to cellular energy depletion and necrotic cell death.19 Apoptosis, on the other hand, may be triggered through p53-dependent upregulation of proapoptotic factors such as BAX and PAG608.18,20 Recently, we described apoptotic cell death in atherosclerotic lesions of cholesterol-fed rabbits on the basis of in situ indices for apoptosis such as terminal deoxynucleotidyl transferase nick end labeling (TUNEL) or in situ nick translation techniques.21 Apoptosis of macrophages was mainly found in regions showing signs of DNA synthesis/repair, as demonstrated by Ki-67 and proliferating cell nuclear antigen labeling. This was in contrast with the smooth muscle cell population showing apoptosis mainly in less cellular regions that are not associated with DNA synthesis/repair. Interestingly, plaque macrophages express inducible NO synthase and seem to contain large amounts of oxidized...
lipids and nitrotyrosine.22 These findings support the hypothesis that macrophages are subjected to high levels of oxidative stress. Because mitotic figures are rarely found in these cells, the high levels of DNA synthesis/repair may be a response to oxidative DNA damage.

In this study, we examined the distribution of oxidative DNA damage in rabbit plaques by immunohistochemistry and single-cell gel electrophoresis. Also, the extent of DNA damage before and after dietary lipid lowering was analyzed. Furthermore, we investigated whether DNA repair systems were upregulated in response to DNA damage and whether DNA damage/repair was reversed during cholesterol withdrawal.

Materials and Methods

Cholesterol-Induced Atherosclerosis

Male New-Zealand White rabbits (28 to 3.5 kg) were fed a diet supplemented with a low-dose cholesterol (0.3%) for 24 weeks (n=54). Seventeen randomly selected animals were euthanized after this period. The other animals received a normal diet and were euthanized after a cholesterol withdrawal period of 4 weeks (n=16), 12 weeks (n=6), or 24 weeks (n=8). Seven animals remained on the cholesterol diet for 48 weeks. A control group of 15 animals receiving a cholesterol-free diet was included as well. Ten control animals were euthanized after 24 weeks, and 5 animals were euthanized after 48 weeks. An overview of the different animal groups used in this study is presented in Figure 1. Serum was stored at −20°C, and total cholesterol, LDL cholesterol, and triglycerides were determined. Freshly isolated segments of the thoracic aorta were gently pressed against an agarose-coated slide to examine DNA strand breaks by the alkaline single-cell gel electrophoresis assay. The segments were then fixed in 4% formalin for histological examination. The remaining part of the thoracic aorta was snap-frozen in liquid nitrogen for total protein isolation. Transversal sections (5 μm thick) of paraffin-embedded tissues were mounted on 3-aminopropyltriethoxysilane–precoated slides. The experiments were approved by the ethical committee of the university.

Antibodies

The following mouse monoclonal antibodies were used for Western blotting and/or immunohistochemistry: anti–PARP-1 (clone C2-10) from Pharmingen, anti–7,8-dihydro-8-oxoguanine (clone N45.1) from the Japan Institute for the Control of Aging, anti–p53 (clone DO-7) and anti-macrophage (RAM-11) from DAKO, anti–phospho-AC-15) from Sigma, and anti–Ki-67 (clone MIB-1) from Immunotech. Goat anti-mouse peroxidase–conjugated secondary antibody was purchased from Jackson ImmunoResearch.

Immunohistochemistry

The immunohistochemical reactions were carried out by an indirect peroxidase antibody conjugate method as previously described.21 At least 5 sections per animal were examined. The images were quantified by using a color image analysis system (Soft Imaging System) as described elsewhere.21

Protein Isolation and Immunoblot Assays

Rabbit aorta segments (n=4) were pooled to reduce animal-to-animal variation and subsequently homogenized in a buffer containing 0.9% (wt/vol) NaCl, 20 mmol/L Tris-HCl pH 7.6, 1 mmol/L phenylmethanesulfonate fluoride, 0.01% (wt/vol) leupeptin, and 0.2% (vol/vol) Triton X-100. Equivalent amounts of protein (100 μg) from each animal group were loaded on a single 10% or 12.5% SDS-polyacrylamide gel. Western blotting of cell lysates to Hybond enhanced chemiluminescence membranes (Amersham Pharmacia Biotech) was performed according to standard procedures. Antibody detection was accomplished with enhanced chemiluminescence detection reagents (Amersham Pharmacia Biotech). Signals were visualized and quantified using a Lumi-imager (Roche Diagnostics). After visualization of the signals, the membranes were stripped and reprobed with a monoclonal antibody for β-actin.

DNA In Situ End Labeling

For the detection of oligonucleosomal DNA cleavage, a stringent TUNEL technique was used as previously described.21

Alkaline Single-Cell Gel Electrophoresis Assay

The alkaline comet assay or single-cell gel electrophoresis assay was performed according to De Boeck et al24 (Figure 2). To determine the

Figure 1. Overview of the animal groups used in this study. Control 24 wk and control 48 wk indicate control animals euthanized after 24 and 48 weeks, respectively; chol 24 and 48 wk, animals receiving low-dose cholesterol for 24 and 48 weeks; chol wd 4, 12, and 24 wk, animals receiving low-dose cholesterol for 24 weeks followed by a normal diet for 4, 12, and 24 weeks.

Figure 2. Principle of the alkaline comet assay. Normal microscope slides were coated with normal melting-point agarose and left to dry. Freshly isolated aorta segments were gently pressed against an agarose-coated slide. Cells that adhered to the slide were immediately covered with a second layer of low melting-point agarose. After gel solidification, cells were lysed in a high-salt solution that removed the cell content except for nuclear material. Slides were then submerged in an alkaline buffer to unwind genomic DNA from sites of strand breakage. A weak electric field drew negatively charged DNA toward the anode, with the amount of DNA able to migrate largely dependent on the size of DNA molecules and the number of broken ends. After electrophoresis, DNA was stained with ethidium bromide. Digitized comet images of an undamaged control cell (lymphocyte) and a damaged cell isolated from the thoracic aorta of a cholesterol-fed rabbit are shown.
Plasma Cholesterol, LDL Cholesterol, and Triglyceride Levels of Cholesterol-Fed and Normocholesteremic Rabbits Before and After the Different Cholesterol Withdrawal Periods

<table>
<thead>
<tr>
<th></th>
<th>Total Cholesterol</th>
<th>LDL Cholesterol</th>
<th>Triglycerides</th>
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<tbody>
<tr>
<td>Control 24 wk</td>
<td>&lt;50</td>
<td>&lt;50</td>
<td>131±40</td>
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<tr>
<td>Chol 24 wk</td>
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<td>589±98</td>
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<td>Chol wd 4 wk</td>
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<td>64±6*</td>
<td>65±10</td>
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<td>1327±138*</td>
<td>585±74</td>
<td>391±95</td>
</tr>
<tr>
<td>Control 48 wk</td>
<td>&lt;50</td>
<td>&lt;50</td>
<td>128±23</td>
</tr>
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Abbreviations are as in Figure 1 legend. Values expressed are in mg/dL.

Results

Serum Lipid Values
Serum lipid values at the start of the experiment, after 24 weeks of cholesterol supplementation, and after the different cholesterol withdrawal periods are shown in the Table. The hypercholesterolemic diet induced a pronounced hypercholesterolemia at week 24, which was completely reversed after 24 weeks of dietary lipid lowering.

Atherosclerotic Plaques of Cholesterol-Fed Rabbits
The induced atherosclerotic plaques showed a clear distinction between a luminal layer composed of numerous lipid-rich foam cells of macrophage origin and a deep layer that was fibrous, containing extracellular lipid deposits and few smooth muscle cells. The plaques showed signs of cell replication (Ki-67) and apoptotic cell death (1% to 2% TUNEL-positive nuclei). After 24 weeks of dietary lipid lowering, the plaque size was unchanged. However, the cell population was profoundly altered. Macrophages disappeared from the plaques, whereas residing smooth muscle cells lost their lipid content. Apoptosis was nearly absent (<0.1% TUNEL-positive nuclei). The changes were associated with a reduction of cell replication and increased deposition of fibrillar collagen fibers in the plaques, which points to plaque stabilization.

Oxidative DNA Damage in Atherosclerotic Plaques of Cholesterol-Fed Rabbits
To investigate the extent and distribution of oxidative DNA modifications in plaques, the DNA damage marker 8-oxoG was demonstrated by immunohistochemistry. Arteries of normocholesterolemic animals showed no immunoreactivity for 8-oxoG (Figure 3A), except for a small number of arteries in which the endothelial cells of the adventitia and at the luminal border were stained. In contrast, strong nuclear 8-oxoG immunoreactivity was found in the atherosclerotic plaques of cholesterol-fed rabbits (Figures 3B and 3C) but not in the underlying media. 8-oxoG immunoreactivity was present predominantly in the superficial layer of the plaque. Although both the plaque macrophages and smooth muscle cells were labeled, strong 8-oxoG immunoreactivity was mainly localized in the macrophage population. Furthermore, the endothelial cell layer and a small group of cells present in the adventitia, mainly endothelial cells, were positive for 8-oxoG. The majority of 8-oxoG immunoreactive cells were not labeled by the TUNEL technique.

DNA strand breaks in cells derived from freshly isolated segments of the thoracic aorta were determined by alkaline single-cell gel electrophoresis. Comet slides contained both macrophages and smooth muscle cells in a ratio similar to the one found in tissue sections. The number of DNA strand breaks was significantly higher in the plaque as compared with the samples of an age-matched control group (Figure 4). Each cell had the appearance of a comet, with a brightly fluorescent head and a tail with an intensity that was related to the amount of damage sustained by the cell. There was a significant difference in tail length between cells isolated from segments proximal to the aortic arch and cells derived from the most distal part of the thoracic aorta (Figure 4). The tail moment (ie, tail length×percentage DNA in tail) in cells from proximal aorta segments was about twice as high as compared with the distal tail moment values. The majority of...
cells examined by the alkaline comet assay had a normal morphology and were not apoptotic as demonstrated by a negative TUNEL staining. Histological examination of the aorta segments revealed a much more pronounced lesion formation in the upper (proximal) region of the thoracic aorta (Figures 5A, 5C, and 5D). The RAM-11 immunoreactive area of the proximal lesions was not significantly different from the most distal lesions of the thoracic aorta (Figure 5B). The plaque area correlated with the number of DNA strand breaks ($R=0.593$, $P=0.010$) (Figures 4 and 5).

DNA Repair in Atherosclerotic Plaques of Cholesterol-Fed Rabbits

Several proteins involved in DNA repair were upregulated in the plaque as compared with arteries of normocholesterolemic rabbits (control animals euthanized at 48 weeks). Western blots revealed an increased expression of p53, p53 phosphorylated at Ser392, PARP-1, and XRCC1 (x-ray repair cross-complementing 1), but a constitutive expression of DNA polymerase $\beta$ (Figure 6).

Upregulation of DNA repair enzymes was found predominantly in the macrophage-derived foam cells, present in the superficial layer of the plaque (Figure 7). However, increased immunoreactivity for PARP-1 and p53 was also found in some smooth muscle cells surrounding the macrophage-rich area of the plaque. The smooth muscle cells in the adjacent media did not overexpress PARP-1 or p53.

DNA Damage and Repair in Atherosclerotic Plaques of Cholesterol-Fed Rabbits After Dietary Lipid Lowering

Dietary lipid lowering was associated with a strong reduction of oxidative DNA damage. After a lipid-lowering period of 12 to 24 weeks, 8-oxoG immunoreactivity was significantly reduced but not entirely abrogated (Figures 3D through 3F). Single-cell gel electrophoresis showed a fast decrease in DNA strand breaks (Figure 4). Both tail length and tail moment values normalized after 4 weeks of cholesterol withdrawal, whereas the differences in DNA strand breaks between the proximal and distal parts of the thoracic aorta were no longer significant. This was in parallel with a decline of the total plasma cholesterol and LDL cholesterol levels (Table). It should be noted, however, that the plaque cell composition was unchanged at this time point.

The expression of DNA repair enzymes, which were upregulated during the cholesterol load, decreased progressively and normalized after 24 weeks of cholesterol withdrawal (Figures 6 and 7).

Discussion

Prolonged diet-induced hypercholesterolemia (0.3% cholesterol for 24 weeks) in rabbits causes intimal thickening and rapid cellular turnover, eventually culminating in the formation of an atherosclerotic lesion. We previously reported that these lesions fulfill several criteria of human atherosclerotic plaques. In the present study, we provide evidence that cholesterol-induced plaque formation is associated with DNA repair mechanisms.
damage most likely resulting from a high degree of oxidative stress.

Mammalian cells undergo \( \approx 10,000 \) measurable DNA modification events per cell per hour. DNA adduct formation, however, may increase substantially under conditions of oxidative stress. In general, DNA damage resulting from attack by oxygen free radicals includes base modifications, sugar damage, strand breaks, abasic sites, and DNA protein cross-links. \(^{13} \) 8-oxoG is one of the most abundant lesions in DNA generated by oxidative stress. \(^{26} \) Using monoclonal antibody N45.1, which is highly specific for 8-oxoG, \(^{27–29} \) elevated levels of 8-oxoG were found in the superficial layer of the plaque containing numerous macrophage-derived foam cells but not in the underlying media. Moreover, alkaline single-cell gel assays demonstrated that the number of DNA strand breaks raised significantly in the plaque as compared with samples of normolipemic animals. Cells containing a large number of DNA strand breaks were found predominantly in segments close to the aortic arch that showed pronounced atherosclerotic lesions. This suggests a relationship between the number of DNA strand breaks and plaque size. Theoretically, the alkaline single-cell gel assay allows detection of both single-strand and double-strand DNA breaks. Double-strand breaks are generally considered to be of greater biological consequence than single-strand DNA breaks, because they can lead directly to chromosome aberrations and loss of genetic material. However, it has been reported that ROS induce solely nicks into DNA, \(^{3–5} \) so we may assume that the DNA breaks detected in the plaque are mainly single-stranded.

Although cells possess many defense systems that prevent formation of DNA damage, the BER pathway is believed to be the primary defense against oxidative damage. \(^{14–16} \) BER

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**Figure 6.** Western blot analysis of DNA repair enzymes in distal thoracic aorta segments (n=4) of rabbits before and after cholesterol withdrawal. Total protein samples were prepared from animals that received a cholesterol-rich diet (0.3%) for 24 weeks (chol 24 wk) or 48 weeks (chol 48 wk), as well as from cholesterol-fed animals that were killed after a cholesterol withdrawal period of 4 weeks (chol wd 4 wk), 12 weeks (chol wd 12 wk), or 24 weeks (chol wd 24 wk). Animals that received a cholesterol-free diet for 48 weeks (control 48 wk) were used to evaluate protein levels under normal physiological conditions. Experiments were done in duplicate. A representative immunoblot for each DNA repair enzyme is shown.

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**Figure 7.** Expression of DNA repair enzymes in atherosclerotic plaques of rabbit thoracic aorta. Tissue sections of atherosclerotic plaques at 24 weeks were immunostained for PARP-1 (A) or p53 (B). DNA repair was confined mostly to macrophages. Bar graphs showing quantification of PARP-1 (C) or p53 (D) immunoreactive cells before and after the different cholesterol withdrawal periods illustrate the progressive decline of expression levels. **P<0.01, ***P<0.001 vs animals fed a diet supplemented with low-dose cholesterol for 24 weeks (1-way ANOVA followed by the Bonferroni test). Scale bar=20 \( \mu \)m. C and D, Abbreviations as in Figure 1.
involves several proteins that act to excise a damaged nucleobase from DNA and replace it with the correct undamaged nucleotide. 8-oxoG lesions are typically removed by BER. In contrast, DNA strand breaks are sensed by several kinases such as the DNA-dependent protein kinase\(^{26}\) or the ataxia-telangiectasia mutated gene product ATM\(^{31}\) leading to phosphorylation and activation of p53. Recently, it has been postulated that this phenomenon plays a principal part in the progression of atherosclerosis, because absence of p53 accelerates atherosclerosis by increasing cell proliferation in vivo.\(^{32}\) After 24 weeks of cholesterol supplement, plaques showed a strong upregulation of p53. The protein was detectable predominantly in the macrophage population of the plaque and was absent in the adjacent media as well as in the aorta of age-matched normcholesterolemic rabbits. At present, multiple p53 posttranslational modifications are known that may stabilize the protein.\(^{18}\) We found that p53 became phosphorylated in the plaque on Ser392, but we cannot exclude other modifications taking place as well.

It is well established that oxidative DNA damage can induce apoptosis through activation of p53. Active p53 transmits the apoptotic signal by a complicated mechanism that involves, at least in part, its ability to transactivate target genes such as BAX and a series of p53-inducible genes.\(^{33}\) Although many smooth muscle cells in the plaque express Bax,\(^{21}\) apoptotic nuclei as visualized by the TUNEL technique were scarce and did not correlate with the number of 8-oxoG immunoreactive cells. Indeed, the TUNEL technique detects oligonucleosomal DNA breaks but is probably not sensitive enough to identify low numbers of DNA breaks. This indicates that cells in the plaque may contain 8-oxoG modifications and a small number of DNA breaks (detectable by the comet assay) without undergoing the execution phase of apoptosis.

In addition to p53, PARP-1 was stimulated to participate in DNA repair. PARP-1 binds both double- and single-stranded DNA breaks in a non–sequence-dependent manner and catalyzes the addition of long branched chains of poly(ADP-ribose) to a variety of nuclear proteins, most notably PARP-1 itself.\(^{17}\) According to recent studies, PARP-1 interacts directly with the BER protein XRCC1\(^{34}\) and appears to play an important role in p53 activation and regulation after DNA damage.\(^{17}\) XRCC1 in turn interacts with the BER proteins DNA ligase III\(^{35}\) and DNA polymerase \(\beta.\)\(^{36}\) Consequently, it was concluded that PARP-1 could detect nicks in DNA and then recruits other members of the BER pathway to the site of DNA damage. Because atherosclerotic plaques showed elevated levels of both PARP-1 and XRCC1, these processes may play a relevant role in atherosclerosis to preserve the genome. However, in case of massive DNA damage, PARP-1 activation leads to cellular energy depletion and necrosis.\(^{19}\) In this regard, DNA repair may also contribute to necrotic core formation, the hallmark of an advanced unstable lesion.

Recent studies have shown that the discontinuation of a cholesterol-rich diet in rabbits has beneficial effects on the architecture of the lesions.\(^{21,37}\) After a prolonged cholesterol withdrawal period, morphological changes include increases in smooth muscle cells and collagen, as well as reductions in macrophage number, extracellular lipid, and calcifications. Moreover, smooth muscle cells lose their lipid accumulation and show reduced expression of the proapoptotic factor Bax.\(^{21,38}\) Together with a less frequently observed rate of apoptosis and a drastic increase of cross-banded collagen fibers, these changes point to an increased tensile strength of the plaque. In addition to these morphological benefits, we demonstrate here that the lesions are cleared of oxidative DNA damage, indicating presence of an efficient DNA repair mechanism. Because oxidative damage to DNA can exert a number of deleterious effects, including mutations and induction of apoptosis,\(^{13}\) efficient removal of DNA adducts may contribute to stabilization of the plaque. Interestingly, repair of DNA strand breaks as detected by the alkaline comet assay proceeded much faster than repair of 8-oxoG lesions, so we can assume that decreased levels of DNA strand breaks reflect an early event in plaque stabilization. This is consistent with earlier reports showing that repair of endogenous damage highly depends on the type of DNA lesion. Apurinic/apyrimidinic sites, for example, are removed over relatively short times, whereas repair of oxidized bases (especially purine lesions such as 8-oxoG) is a rather slow process.\(^{16}\)

Taken together, our data fit into the following model. During hypercholesterolemia-induced atherogenesis, ROS are formed resulting in oxidative DNA damage. DNA damage is followed by increased DNA repair activity so that initial damage is efficiently repaired and, more importantly, remains sublethal. However, some cells may reach a point of no return, after which the DNA repair systems can no longer cope with the extensive damage and apoptotic cell death becomes imminent. During cholesterol withdrawal, most macrophages disappear from the plaque. A reduction of both ROS formation and oxidative tissue injury can be observed as well. Subsequently, DNA repair systems normalize and the vessel wall starts to recover. More work is certainly required to understand the potential role of oxidatively damaged DNA in the pathogenesis of atherosclerosis. Nevertheless, we believe that this model may help to explain the benefits of lipid-lowering therapy on plaque stabilization.

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References


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