Elevated Levels of Oxidative DNA Damage and DNA Repair Enzymes in Human Atherosclerotic Plaques

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Background—The formation of reactive oxygen species is a critical event in atherosclerosis because it promotes cell proliferation, hypertrophy, growth arrest, and/or apoptosis and oxidation of LDL. In the present study, we investigated whether reactive oxygen species–induced oxidative damage to DNA occurs in human atherosclerotic plaques and whether this is accompanied by the upregulation of DNA repair mechanisms.

Methods and Results—We observed increased immunoreactivity against the oxidative DNA damage marker 7,8-dihydro-8-oxo-2'-deoxyguanosine (8-oxo-dG) in plaques of the carotid artery compared with the adjacent inner media and nonatherosclerotic mammary arteries. Strong 8-oxo-dG immunoreactivity was found in all cell types of the plaque including macrophages, smooth muscle cells, and endothelial cells. As shown by competitive ELISA, carotid plaques contained 160±29 8-oxo-dG residues/10^5 dG versus 3±1 8-oxo-dG residues/10^5 dG in mammary arteries. Single-cell gel electrophoresis showed elevated levels of DNA strand breaks in the plaque. The overall number of apoptotic nuclei was low (1% to 2%) and did not correlate with the amount of 8-oxo-dG immunoreactive cells (>90%). This suggests that initial damage to DNA occurs at a sublethal level. Several DNA repair systems that are involved in base excision repair (redox factor/AP endonuclease [Ref 1] and poly(ADP-ribose) polymerase 1 [PARP-1]) or nonspecific repair pathways (p53, DNA-dependent protein kinase) were upregulated, as shown by Western blotting and immunohistochemistry. Overexpression of DNA repair enzymes was associated with elevated levels of proliferating cell nuclear antigen.

Conclusions—Our findings provide evidence that oxidative DNA damage and repair increase significantly in human atherosclerotic plaques. (Circulation. 2002;106:927-932.)

Key Words: atherosclerosis ■ oxidative stress ■ apoptosis

Numerous studies have linked excess generation of reactive oxygen species (ROS) with cellular damage and atherogenesis.1,2 Although this notion is widely held, thorough factual evidence is lacking. ROS have been implicated in a variety of distinct cellular processes, including initiation of gene expression and promotion of cell proliferation, hypertrophy, growth arrest, and/or apoptosis.1,2 On the other hand, ROS are involved in oxidation of LDL, which is considered a fundamental step in the initiation and progression of atherosclerosis. It is also tempting to speculate that ROS may have some deleterious effects on DNA. Indeed, ROS can provoke extensive oxidative DNA damage, DNA strand breaks, and chromosomal aberrations.3 Significant damage to DNA resulting from endogenous free radical attack has already been suggested to contribute to the pathology of cancer4 and several neurodegenerative diseases.5,6 A growing body of evidence indicates that oxidative DNA damage is also a prominent feature of atherosclerotic plaques.7-9 In light of this, we have recently described elevated levels of oxidative DNA damage and repair in the thoracic aorta of cholesterol-fed rabbits.10 The aim of the present study was to investigate whether oxidative DNA damage occurs in human atherosclerotic plaques and whether DNA repair mechanisms are upregulated in response to DNA damage.

Methods

Carotid Endarterectomy Specimens

Human carotid endarterectomy specimens (n=13) were obtained from patients with a carotid stenosis >70%, as demonstrated by digital subtraction angiography and duplex ultrasonography. The specimens were opened along their longitudinal axis. Half of the specimen was fixed in 4% formalin within 2 minutes after surgical removal. The other half was gently pressed against an agarose-coated slide to examine DNA strand breaks by the alkaline single-cell gel electrophoresis assay (see below). Tissue was frozen in liquid nitrogen to be used as cryosections for RNA/DNA extraction and for Western blotting. Complete longitudinal sections of formalin-fixed, paraffin-embedded specimens contained the inner wall of the distal...
common carotid artery, the proximal part of the external carotid artery, and the carotid sinus.11 Nonatherosclerotic mammary arteries (n=8) obtained during bypass surgery were used as negative controls and were manipulated similarly. Characteristics of the study and of the control group are summarized in the Table. Names of patients and specimens were irreversibly encrypted. The local ethics committee approved this protocol.

Antibodies

The following mouse monoclonal antibodies were used: anti-PARP-1 (clone C2-10; PharMingen), anti-7,8-dihydro-8-oxo-2'-deoxyguanosine (clone N45.1; Japan Institute for the Control of Aging), anti-p53 (clone DO-7), anti-CD68 (clone PG-M1), anti-hOgg1 from Novus Biologicals; anti-phospho-p53 (S15; BIOMOL); anti-nitrotyrosine (Upstate Biotechnology); and anti-Ref-1 (C-20; Santa Cruz Biotechnology). Goat anti-mouse and sheep anti-rabbit peroxidase-conjugated secondary antibodies were purchased from Jackson and DAKO, respectively.

Immunohistochemistry and DNA In Situ End Labeling

The immunohistochemical reactions were performed by an indirect peroxidase antibody conjugate method.12 For the detection of oligonucleosomal DNA cleavage, a stringent terminal deoxynucleotidyl transferase end-labeling (TUNEL) technique was used.13 TUNEL staining was combined with an immunohistochemical stain for SC-35 to avoid aspecific labeling.13

Protein Isolation and Immunoblot Assays

Four human carotid endarterectomy specimens and 4 control samples were pooled to reduce specimen-to-specimen variation and were subsequently homogenized.10 Immunoblot assays were performed as previously described.10

Results

Plaque Composition

The carotid endarterectomy specimens were in the form of complete casts of carotid bifurcations. Thin fibrous cap atheromata alternated with other stages of atherosclerosis (fibrous cap atheromata and intimal xanthomata) in the same specimen, in accordance with the adapted American Heart Association classification scheme, as modified by Virmani et al.11

Figure 1. Immunohistochemical markers for oxidative stress and apoptotic cell death in advanced plaques of carotid endarterectomy specimens. The regions shown are macrophage-rich and located around the necrotic core (nc). A, Inducible nitric oxide synthase; B, nitrotyrosine; C, cleaved caspase-3 (arrows); and D, splicing factor SC-35 (blue) combined with TUNEL labeling (brown). Arrowheads indicate one apoptotic body in the middle of SC-35 positive cells. Scale bar=50 μm.
There were numerous foam cells of macrophage origin (CD68-positive cells) present around the necrotic core of thin fibrous cap atheromata. Smooth muscle cell actin immunoreactive cells were nearly absent in these regions (<0.2% of the total area). Some foam cells surrounding the necrotic core were strongly immunoreactive against inducible nitric oxide synthase (Figure 1A). Nitrotyrosine was also detected in this region (Figure 1B). Insoluble multilaminated material composed of oxidized lipids and proteins was present in the cytoplasm of many macrophages. We found some larger extracellular lipid deposits in the necrotic core.

Oxidative DNA Modifications

Smooth muscle cells (SMCs) of mammary arteries showed weak immunoreactivity for the oxidative DNA damage marker 8-oxo-dG. In contrast, strong 8-oxo-dG immunoreactivity was found in all cell types of the plaque (>90% of the total area), including macrophages, SMCs, and endothelial cells (Figures 2A and 2D). 8-oxo-dG immunoreactivity was localized predominantly in the nucleus of the labeled cells. Adjacent fragments of the inner media stained minimally or did not stain at all. Plaque cells showed an ≈55-fold higher level of 8-oxo-dG in comparison with mammary arteries (160 ± 29 versus 3 ± 1 8-oxo-dG residues/10^5 dG; Figure 3). The majority of 8-oxo-dG immunoreactive cells in the plaque (>95%) showed immunoreactivity for splicing factor SC-35 but were negative for active caspase-3 (Figure 1C) and were not labeled by the TUNEL technique (Figure 1D). Immunoreactivity for 8-oxo-dG did not differ in intimal xanthomata from thin fibrous cap atheromata (data not shown).

DNA Strand Breaks

Alkaline single cell gel electrophoresis revealed that the number of DNA strand breaks was significantly higher in cells of the carotid endarterectomy specimens compared with cells derived from mammary arteries (Figures 4A and 4B). Most cells in the plaque (>90%) contained DNA strand breaks with varying outcomes (Figures 4A and 4B). However, only a minority of cells (1% to 2%) showed TUNEL reactivity. Cells containing a massive amount of DNA strand breaks were localized predominantly in regions of the plaque that contained large amounts of oxidized lipid deposits (Figure 4E). In mammary arteries (Figures 4A and 4C) and peripheral blood monocytes (Figure 4D), DNA strand breaks were scarce or completely absent.

Expression of DNA Repair Enzymes

Several proteins involved in DNA repair were upregulated in plaques of carotid endarterectomy specimens when compared with nonatherosclerotic vessels. Western blots revealed an increased expression of p53, p53 phosphorylated at Ser15 and Ser392, DNA-PK, redox factor/AP endonuclease Ref-1, and PARP-1, but a constitutive expression of DNA polymerase β, the N-glycosylases hOgg1 and hNTH1, and 8-oxoGTPase (MTH1; Figure 5). Western blots were confirmed by immunohistochemistry. Strong nuclear immunoreactivity for Ref-1 and DNA-PK was present in the entire plaque (>90% of the total area) in both macrophages and SMCs (Figures 2B, 2C, 2E, and 2F). The carotid artery endothelium and endothelial cells from microvessels were negative for Ref-1 and DNA-PK or showed occasional staining. SMCs from the media adjacent to the plaque and from mammary arteries stained minimally. In contrast with Ref-1 and DNA-PK
immunolabeling, only a subpopulation of cells in the plaque stained for p53, phospho-p53, or PARP-1 (Figures 6A and 6B). Upregulation of p53 and PARP-1 occurred predominantly in the macrophage population of the plaque but was undetectable in the adjacent media and mammary arteries.

Overexpression of DNA repair enzymes was associated with elevated levels of PCNA (Figure 6C).

Immunoreactivity for Ref-1 and DNA-PK did not differ in intimal xanthomata from thin fibrous cap atheromata. However, overexpression of PARP-1, p53, and PCNA was found predominantly in macrophages around the necrotic core of thin fibrous cap atheromata (Figure 6).

Discussion

In the present study, elevated levels of oxidative DNA damage were found in human atherosclerotic plaques. This finding supports the current assumption that oxidative tissue injury is aggravated during plaque formation, most likely as a result of oxidative stress. 8-oxo-dG is one of the most abundant oxidative lesions in mammalian DNA. We observed an increased amount of 8-oxo-dG in human plaques compared with the underlying media or nonatherosclerotic mammary arteries. Strong immunoreactivity for 8-oxo-dG was found in all cell types of the plaque, including macrophages, SMCs, and endothelial cells. Interestingly, 8-oxo-dG is strongly mutagenic and leads to an increased frequency of spontaneous G:C → A:T transversion mutations in repair-deficient cells.

Previous reports showed an increased mutation rate and widespread microsatellite instability in human atherosclerotic lesions. These may be related to the accumulation of oxidized base residues. In addition, ROS may also generate DNA strand interruptions. Single-cell gel electrophoresis revealed that the number of DNA strand breaks was raised significantly in the plaque compared with nonatherosclerotic vessels. Because DNA strand breaks are fragile sites, they can attract undesirable recombination events. Elevated levels of
DNA strand breaks in the plaque may therefore account for the chromosomal rearrangements described previously. It is important to note that extensive chromosome abnormalities in SMCs can be associated with transformation events (eg, intimal uterine leiomyosarcomas). Atherosclerotic plaques contain a large monoclonal population of SMCs and thus may be regarded as monoclonal neoplasms of the arterial wall. This is consistent with the findings that DNA extracted from atherosclerotic plaques had a transforming ability when transfected into NIH3T3 cells and that SMCs cultured from plaque tissue retained transforming potential throughout many cell passages. Monoclonality of plaque SMCs could be caused by the expansion of a large, preexisting patch of SMCs. An alternative mechanism for monoclonality that corresponds with increased levels of oxidative DNA damage is the creation of a new lineage of SMCs. This may occur either through genetic mutation or through epigenetic changes. If true, somatic mutations of SMCs may play an important role in the pathogenesis of atherosclerotic plaques.

Severe oxidative DNA damage can induce apoptosis by activating a variety of proapoptotic proteins. In the present study, atherosclerotic plaques were characterized by the appearance of apoptotic cell death, but the overall number of TUNEL-positive nuclei was low (1% to 2%) and did not correlate with the amount of 8-oxo-dG immunoreactive cells (>90%). Therefore, we assume that cells in the plaque contain a sublethal level of oxidative DNA modifications and/or strand breaks and thus do not undergo the execution phase of apoptosis. On the contrary, most cells surrounding the necrotic core are labeled for splicing factor SC-35, suggesting that these cells are viable and metabolically active. However, we cannot rule out the possibility that DNA lesions accumulate with time and eventually induce plaque cells to undergo apoptotic cell death.

DNA damage cannot be tolerated in mammals if left un repaired. Therefore, cells have developed many defense systems to remove DNA damage. A major repair mechanism for oxidative DNA damage, including 8-oxo-dG and DNA strand breaks, is the base excision repair pathway. In the present study, Western blot analyses and immunohistochemical stains indicated that there are several DNA repair enzymes that are upregulated in the plaque, either those involved in base excision repair (Ref-1, PARP-1) or in nonspecific repair pathways (p53, DNA-PK). Furthermore, we demonstrated that plaque p53 contains at least 2 phosphorylated residues, Ser15 and Ser392, which stabilize and activate the protein in the plaque. Activation of p53 by phosphorylation is an important regulatory event in the arterial vessel wall because p53 deficiency, specifically in macrophages, leads to a significant doubling of atherosclerotic lesion size. Overexpression of some base excision repair enzymes does not always lead to increased protection against DNA-damaging agents. For this reason, it remains to be determined whether the capacity of the vascular cells to repair endogenous lesions after upregulation of specific repair enzymatic activity is increased. A nonphysiological level of certain repair proteins may even prove detrimental to mammalian cells. This is particularly clear for PARP-1, whose imbalanced production leads to necrotic cell death as a result of NAD⁺ and ATP depletion. In this case, PARP-1 overexpression may also contribute to the formation of a necrotic core, which is the hallmark of an atherosclerotic lesion.

Immunohistochemical analysis revealed the abundant expression of both DNA-PK and Ref-1 in the entire plaque. DNA-PK is a serine/threonine kinase that serves as an essential upstream activator of p53. Nevertheless, the significance of the upregulation of Ref-1 in atherosclerotic plaques is unclear and remains to be elucidated. Herring et al could not demonstrate increased resistance to DNA damage in cells overexpressing Ref-1, indicating that increased levels of Ref-1 cannot improve the repair efficiency. It is important to note that Ref-1 is also a reductase factor and, thus, a widespread regulator of transcription factors that control multiple events in the life cycle of various cell types. Downregulation of Ref-1 expression after induction of hypoxia into endothelial cells precedes DNA fragmentation and apoptosis.
apoptotic cell death. This suggests that Ref-1 is a crucial enzyme that determines cell fate.31

Overexpression of DNA repair enzymes was associated with elevated levels of PCNA. Because this protein is essential for DNA replication, many groups have used it as an important marker for cell proliferation. However, recent evidence suggests additional roles for PCNA in chromatin assembly, RNA transcription, and DNA repair.32 We assumed that high levels of oxidative stress in the plaque could trigger a unconventional repair pathway that modulates PCNA expression, given that mammalian cell extracts can repair oxidative DNA modifications by an alternative PCNA-dependent base excision repair pathway.33 Similar conclusions were reported for macrophages treated with oxidized LDL,34 as well as for cardiac myocytes in dilated cardiomyopathy.35

In summary, our findings show that oxidative DNA damage and repair increase significantly in human atherosclerotic plaques. There is currently a lack of essential information regarding the functional consequences of damaged DNA in human plaques, besides the potential role in carcinogenesis and cell death. Of particular interest, however, is the finding that several factors associated with the presence of DNA adducts in aorta SMCs (ie, age, smoking habits, blood lipids, and blood pressure) are also known to play a major role in atherogenesis.7 In this context, detection of DNA damage may provide a useful biomarker not only in carcinogenesis but also in atherogenesis.

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