THE JEREMIAH METZGER LECTURE

PATHOGENESIS OF ATHEROSCLEROSIS: REDOX AS A UNIFYING MECHANISM

R. WAYNE ALEXANDER

ATLANTA, GEORGIA

ABSTRACT

Excessive production of reactive oxygen species (ROS) occurs in many diseases and oxidation may be a common disease mechanism generally. The original "oxidation hypothesis" concerning the pathogenesis of atherosclerosis was posited in the context of the putative central role of oxidized LDL in the process. Atherosclerosis has three major characteristic features: inflammation with accumulation of T-cells and, in particular, monocytes, which become lipid rich foam cells; remodeling of the arterial wall; and the non-random localization of lesions to areas of disturbed flow or of low shear stress. The evidence is reviewed that each of these characteristics can be attributed to excessive ROS, which are derived from cellular oxidases, especially, the NAD(P)H oxidases. This expanded concept of the central role of oxidation in the pathogenesis of atherosclerosis has led to a renewed and intense interest in the potential role of antioxidants in therapy. The vascular protective effects of existing drugs such as statins and ACE inhibitors that are not related to serum lipid alterations are attributed to their indirect but effective roles as antioxidants. These data as well as evidence that newly developed antioxidant drugs show promise, not only in experimental animals but also clinically, are reviewed.

INTRODUCTION: OXIDATION IS A PATHOGENIC MECHANISM IN MULTIPLE DISEASES

The oxidative modification of proteins, lipids, and carbohydrates by reactive oxygen species (ROS) and the associated modification of func-

From the Department of Medicine, Emory University School of Medicine, Atlanta, GA.

Address for reprints: R. Wayne Alexander, M.D., Ph.D. ralexan@emory.edu voice 404-727-1749, Chair, Department of Medicine, EUH, H-153, fax 404-727-3099, 1364 Clifton Road NE, Mail Stop 16000021AA, Atlanta, GA 30322.

tion have been major foci in recent years of investigators interested in both normal physiologic processes and in the pathogenesis of multiple diseases. "Reactive oxygen species" is the generally accepted term for a broad category of oxygen containing molecules that includes "oxygen radicals" in which there are unpaired electrons resulting in their chemical interaction with other molecules. ROS also refers to substances such as hydrogen peroxide (H_2O_2) that can be very reactive and cause oxidative modification of other molecules.

ROS are generated normally by many cell types. The initial reaction frequently involves the one electron reduction of O_2 to generate superoxide (O_2^-), which is the initial product from which a large number of oxidants are derived (Figure 1). The generation of O_2^- by living cells was described decades ago in the case of neutrophils and was associated with an NADPH oxidase. In phagocytic cells NADPH oxidase is a multi-component, membrane associated enzyme that uses NADPH as an electron donor. Related enzymes, which may use NADH preferentially as the electron donor, have been found in a number of cell types including vascular endothelial and smooth muscle cells (reviewed in (1)). The assembly at the cell membrane of both cytosolic and plasma

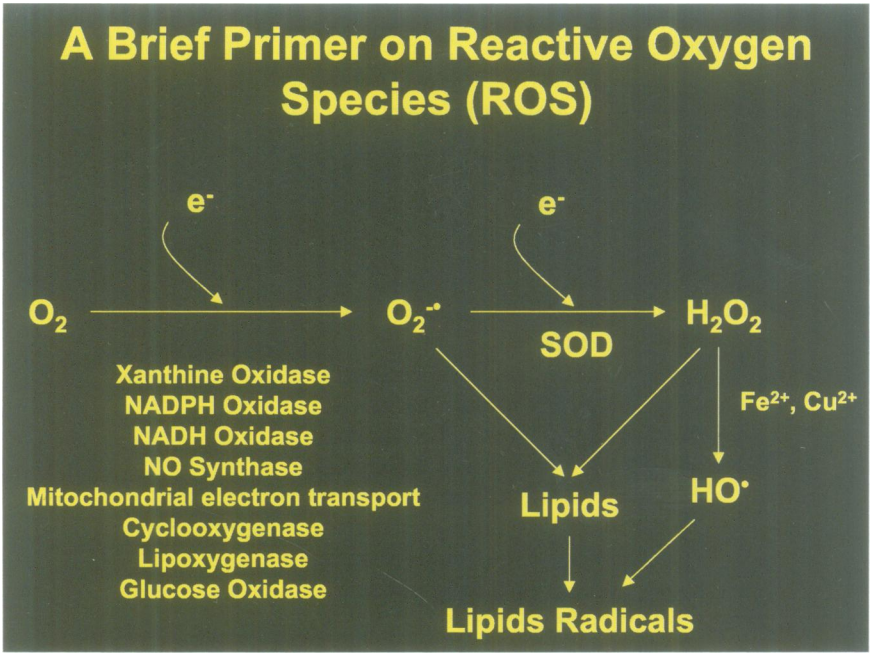
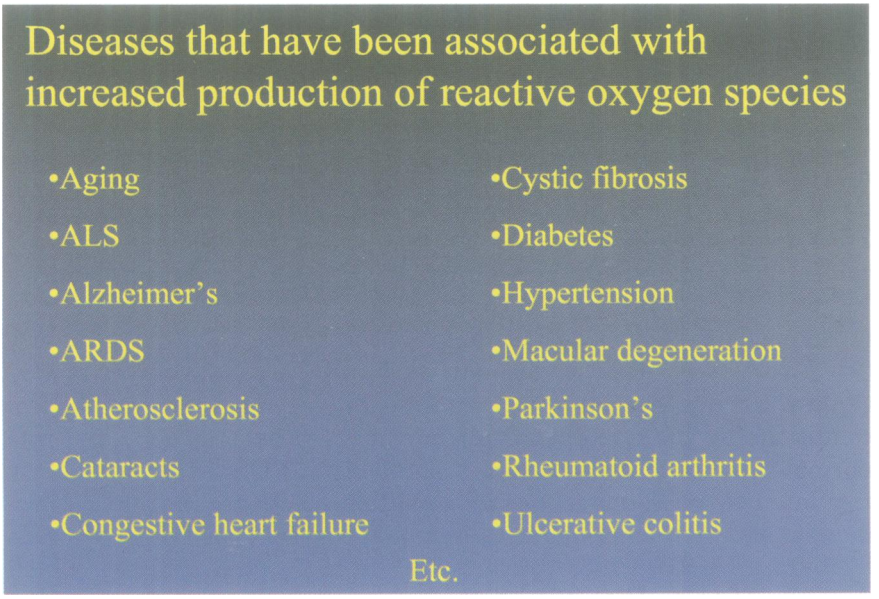


FIG. 1. Sources and basic chemistry of reactive oxygen species. (Courtesy of David G. Harrison, M.D.)

membrane bound components of the NAD(P)H oxidases to achieve an active, O_2^- producing enzyme characteristically requires the participation of the small GTPase rac-1. Attachment of rac-1 to the plasma membrane requires its modification or isoprenylation by the lipids farnesyl or geranylgeranyl. Both farnesyl pyrophosphate and geranylgeranyl pyrophosphate are products of the HMG CoA reductase pathway that is inhibited by statins, the cholesterol lowering drugs. Thus, inhibition of HMG CoA reductase, in addition to lowering blood cholesterol, could affect the metabolism of tissues including the arterial wall by decreasing the activity of the NAD(P)H oxidases and thus behaving effectively as antioxidants, as will be discussed subsequently (2). A variety of other oxidase enzymes also serve as sources of O_2^- (Figure 1). Furthermore, O_2^- may be produced under pathological conditions by the endothelial nitric oxide synthase (eNOS) and is a normal product of mitochondrial metabolism. ROS levels are tightly regulated by cells under normal conditions, both by controlling production and by promoting degradation by enzymes such as superoxide dismutase (SOD), which converts O_2^- to H_2O_2, and catalase, which degrades H_2O_2 to H_2O (Figure 1).

ROS are used by phagocytic cells to kill engulfed bacteria. They also are involved in generating signals that affect the internal workings of both phagocytic and non-phagocytic cells. For example, many G-protein coupled receptors, which traditionally have been viewed as acting primarily through the stimulation of cyclic nucleotide formation and/or calcium release or influx, have recently been shown to activate receptor tyrosine kinases such as the EGF receptor (3). This so-called transactivation is in several cases mediated by generation of ROS (3,4). Many cells also modulate their immediate external environment by releasing ROS. An example is the release of ROS by vascular smooth muscle cells to activate extracellular matrix metalloproteinases (5). Many of the effects of the reduction-oxidation (redox) state of the cell on signaling are mediated by changing the conformation and thus activity of proteins by regulating, for example, sulfhydryl groups that may cause bonding through disulfides leading to specific folding required for a particular function. Thus, ROS have come to be viewed as second messengers in much the same way as have cyclic nucleotides and calcium.

ROS, in general, are viewed as mediating stress responses, either physiologic or pathologic. Conversely, a reduced state is generally viewed as a quiescent one. Many diseases are associated with evidence of excessive oxidative stress (Table 1). In many cases the presence of oxidation protein or lipid end products has been documented in patho-



logic specimens (6,7). These oxidized products result from the formation of carbonyl groups from the action of ROS on proteins, carbohydrates, or lipids. Oxidation has been particularly well studied in atherosclerosis and in diabetes. Oxidized low density lipoprotein (oxLDL) and advanced glycation end-products (AGEs), the result of non-enzymatic glycation of proteins in the presence of high ambient glucose concentrations, have been associated prominently with the vasculopathies of these diseases (6,8,9). Both oxLDL and AGEs are biologically active and induce oxidative stress and pro-inflammatory responses. It is important to note here that alteration in the balance between reduction and oxidation (redox) has emerged as a general theme in the understanding of the pathogenesis of many, many diseases. Thus, common disease mechanisms including cell death, growth and migration, inflammation generally and aspects of malignant transformation and angiogenesis have been associated with alterations in redox state and excessive production of ROS.

PATHOGENESIS OF ATHEROSCLEROSIS: OVERVIEW

Atherosclerotic lesions have three general characteristics: inflammatory nature; non-random distribution with areas of disordered blood flow and low or oscillatory shear forces being sites of predilection; and

remodeling of the arterial wall (Figure 2). All of these features can be related to a significant extent to excessive production of ROS.

Inflammation

Atherosclerosis has been recognized as having a major inflammatory component since the early part of the twentieth century. In the 1930s and 1940s Leary described the role of the tissue macrophage in forming the foam cell that is a characteristic feature of the atherosclerotic lesion (10). The study of atherosclerosis in the early modern era of the 1960s through the 1970s emphasized primarily the role of lipoproteins and their metabolism. A shift in focus to the cell biology of the arterial wall and the impact of recruited inflammatory cells was in full force by the 1980s (11). The presence of activated T-cells as well as of macrophages in human atherosclerotic lesions (12) emphasized that the disease was predominantly inflammatory in nature. The accumulating evidence was summarized initially by Munro and Cotran (13).

The general features of the formation of an atherosclerotic lesion are shown diagrammatically in Figure 3. A key element is the

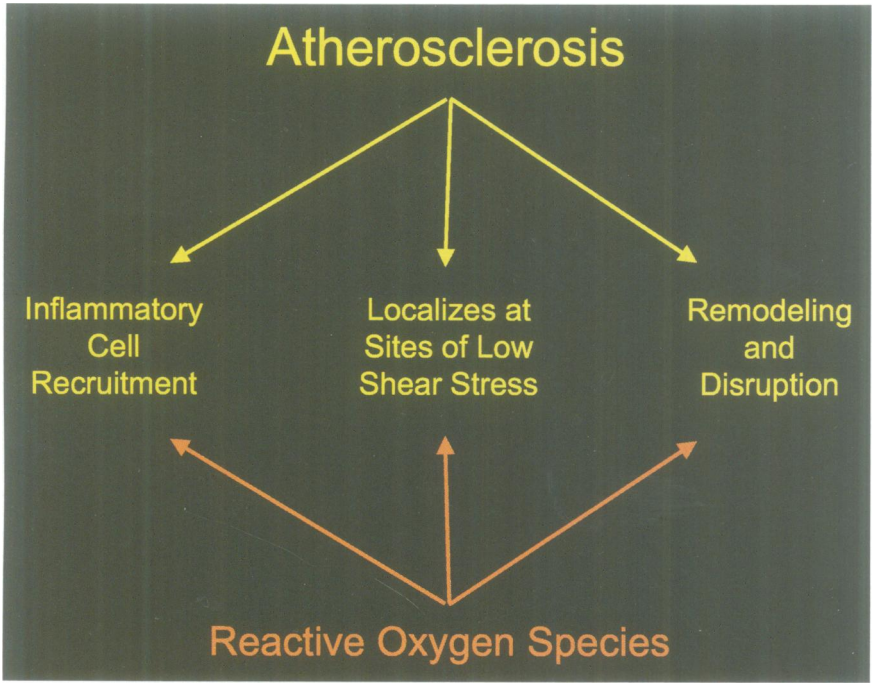


FIG. 2. Multiple roles of ROS in the pathogenesis of atherosclerosis.

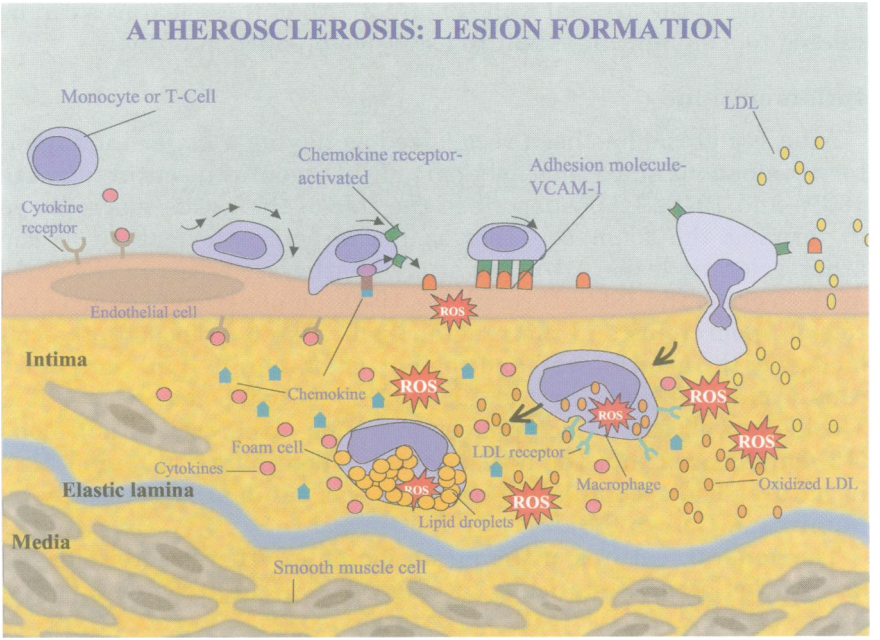


FIG. 3. Pathogenesis of Atherosclerosis—central role of reactive oxygen species in inflammatory cell recruitment, foam cell formation, and cell migration/remodeling.

activation of the endothelium to express adhesion molecules that recruit mononuclear or T-cells into the arterial wall. The mononuclear cells become tissue macrophages and can interact with T-cells (not shown in the intima in Figure 3) to result in the secretion of cytokines and chemokines that amplify the inflammatory response and attract additional inflammatory cells. LDL enters the arterial wall and becomes oxidized by ROS that are released in increased amounts by macrophages, by vascular smooth muscle cells that migrate into the intima from the media and become less differentiated or "modulated", as well as by the dysfunctional endothelial cells overlying the lesion. Only after the LDL is oxidatively modified in the ROS-enriched, inflammatory environment is it taken up by macrophages via scavenger receptors to form the intracellular lipid droplets that characterize the foam cell (9). Foam cells produce large amounts of tissue factor in addition to the ROS and the variety of pro-inflammatory molecules alluded to above. As foam cells die, the residual lipids and tissue factor become part of the lipid rich core of the advanced atherosclerotic plaque. This core is thus highly thrombogenic and, with plaque rupture into the artery lumen, can lead to thrombotic occlusion as discussed subsequently.

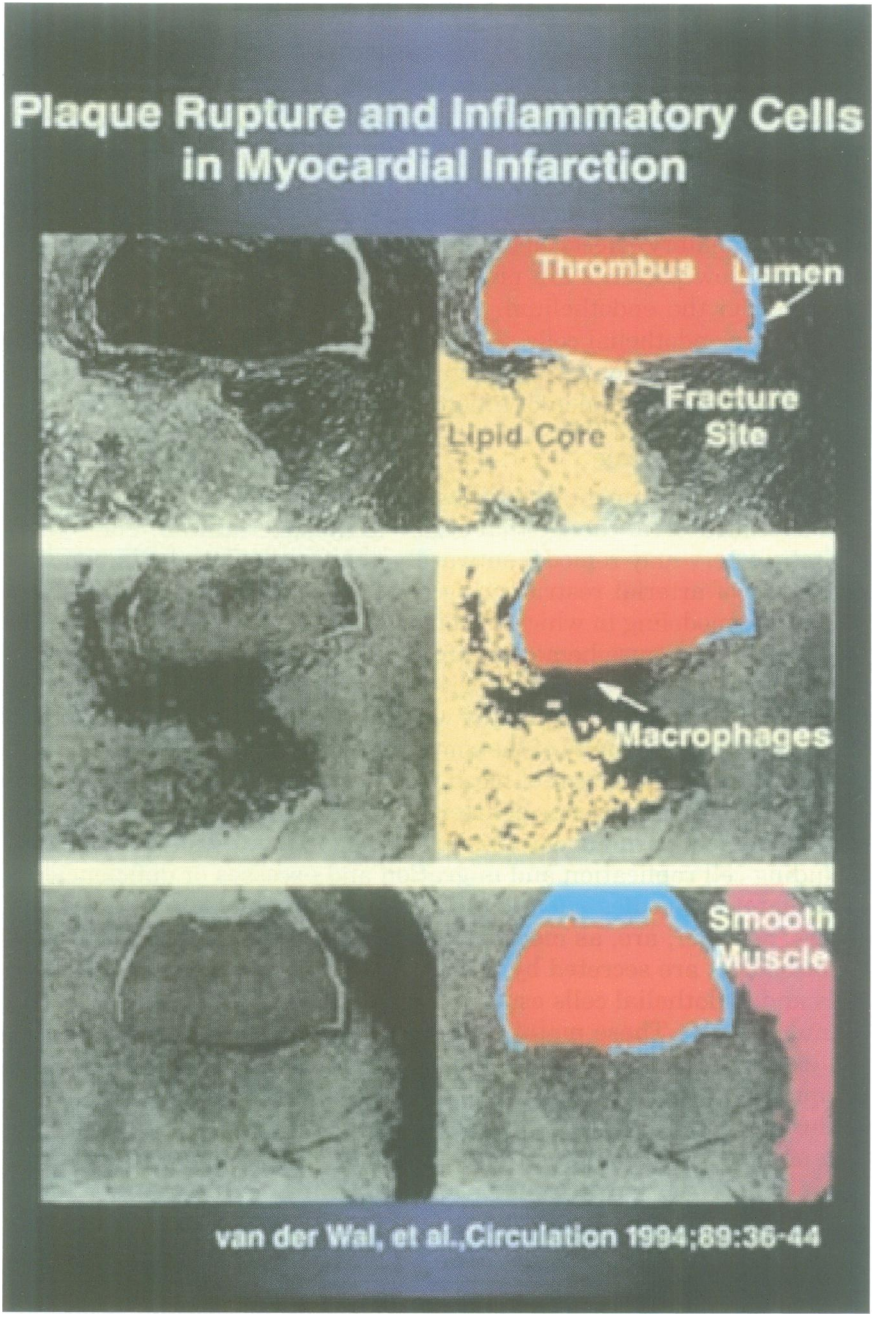
Hemodynamic Forces and Lesion Formation

As noted, the distribution of atherosclerotic plaques is not a random event as the predilection for lesion formation is at areas of low hemodynamic shear stress and of disturbed flow—i.e. oscillatory flow with a reversing component or non-laminar, turbulent flow (14,15). Such flow conditions commonly occur opposite to flow dividers or branch points. As will be discussed subsequently, laminar flow tends to have antioxidant, anti-inflammatory effects upon endothelial cells whereas non-laminar, disturbed flow patterns are pro-inflammatory and pro-oxidant in their influence on the endothelium. Arrest of leukocyte rolling by inducing adhesion to endothelial cells, which can be a result of non-laminar flow conditions, is a prerequisite for migration into the media and to initiation of the inflammatory lesion of atherosclerosis as will be discussed.

Plaque Remodeling

One of the fundamental features of atherosclerosis is the associated extensive structural change that occurs in the arterial wall. The atherosclerotic artery may remodel outwardly, thus protecting the lumen size. This form of arterial restructuring is adaptive as opposed to the maladaptive remodeling in which the enlarging plaque encroaches upon the lumen and may cause hemodynamically significant stenosis. Parenthetically, the outward remodeling with maintenance of relatively normal lumen diameter can be associated with considerable plaque burden that is not detected with many of the conventional diagnostic approaches that are based on the detection of blood flow obstruction.

The dramatic alterations of arterial structure that can be induced by atherosclerosis result from the interactions of multiple mechanisms including cell replication and migration and excesses or deficiencies of vasodilator molecules such as NO. Some of the most important elements, however, are, as mentioned previously, the matrix metalloproteinases that are secreted by macrophage/foam cells, vascular smooth cells and endothelial cells especially under conditions of inflammatory stimulation (5). These metal containing proteinases are released into the extracellular space in an inactive pro form that, when activated (frequently by ROS), degrade the connective tissue skeletal framework of the artery wall. This process not only facilitates the infiltration of inflammatory cells and migration of vascular smooth muscle cells from the intima to the media but also can compromise the structural integrity of the artery. The most dramatic manifestation of this outcome is rupture of an atherosclerotic plaque and the release of its thrombogenic contents into the lumen resulting in thrombosis, ischemia, and serious adverse clinical consequences such as myocardial infarction (16). Such a scenario is illustrated in Figure 4, which in the left hand


Plaque Rupture and Inflammatory Cells in Myocardial Infarction
Thrombus
Lumen
Fracture Site
Lipid Core
Macrophages
Smooth Muscle
van der Wal, et al.,Circulation 1994;89:36-44

panel shows three adjacent cross sections from a thrombosed coronary artery of a patient who died of an acute myocardial infarction. The first panel is stained for elastin and shows the breakdown of the connective tissue structure of the atherosclerotic plaque. The middle panel on the left is stained for macrophages and shows an intense localization of inflammatory cells at the site of rupture. The lower panel on the left demonstrates the absence of smooth muscle cells that would be expected to be stabilizing elements for plaque structure. The panels on the right of the figure are computer enhanced to illustrate better the two key points of the figure: *the inflammatory nature of atherosclerosis and the associated structural remodeling.*

INFLAMMATORY CELL RECRUITMENT INTO ATHEROSCLEROTIC LESIONS

Adherence of mononuclear cells to the arterial endothelium has been observed at all stages of atherosclerosis experimentally (Figure 3) (17,18) and is observed consistently in advanced lesions from human autopsy specimens. Efforts to define the mechanisms involved focused on the early stages of the disease in experimental animals. After initiating a high fat, high cholesterol diet in rabbits, attachment of mononuclear cells to the endothelium overlying lesion-susceptible areas (areas of low shear stress opposite flow dividers) is observed within several days (18). Using, as reagents, antibodies to adhesion molecules generated from in vitro studies with cytokine-stimulated endothelial cells, Gimbrone et al. (19) demonstrated the appearance of putative adhesion proteins at the endothelial surface of cholesterol-fed rabbits concurrently with the attachment of the monocytes (20). These presumptive leukocyte-docking proteins were initially called "athero-ELAM" for atherosclerosis-related, Endothelial cell Leukocyte Adhesion Molecule. It was demonstrated subsequently that "athero-ELAM" is the rabbit equivalent of the human adhesion molecule, vascular-cell

FIG. 4. Plaque rupture and inflammatory cells in acute myocardial infarction. The panels on the left show adjacent sections from a thrombosed coronary artery. The top panel is stained for elastin, which has been degraded at the fracture site. The middle panel has been stained to show the intense localization of macrophages at the fracture site. The bottom panel illustrates the virtual absence at the fracture site of vascular smooth muscle cells that would contribute to structural stability of the plaque. The panels on the right have been computer enhanced with color to illustrate more graphically the points made. Modified with permission from van der Wal AC, Becker AE, van der Loos CM, Das PK: Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. Circulation 1994;89:36–44 (16).

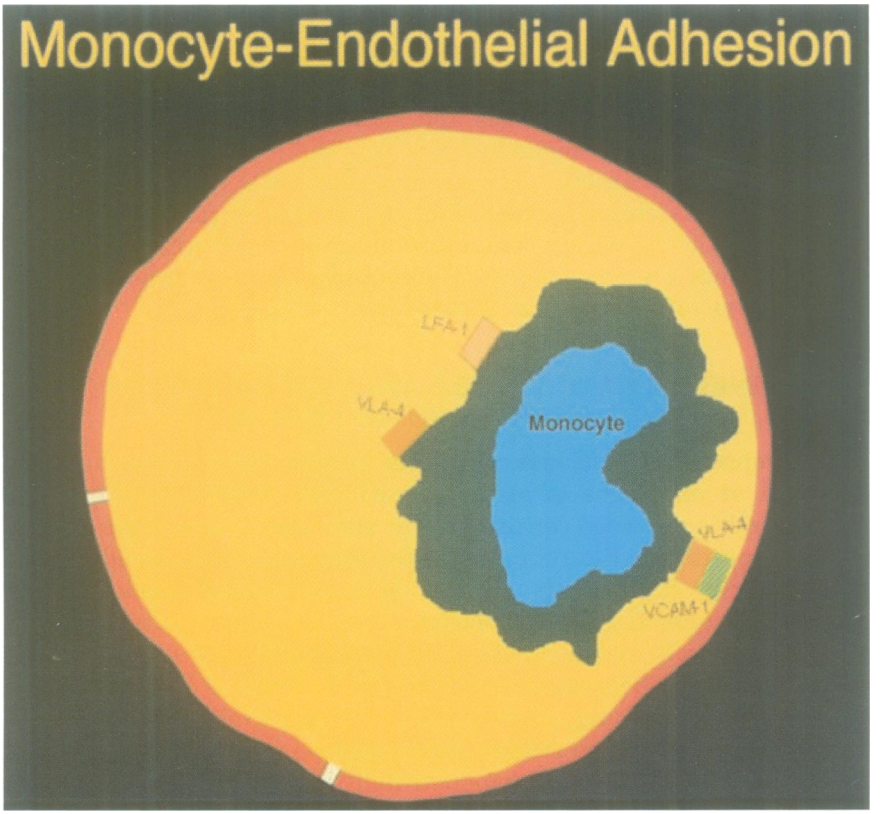


FIG. 5. Monocyte-Endothelial Adhesion. Monocyte attachment to the endothelial surface. Vascular cell adhesion molecule-1 (VCAM-1) is upregulated by cardiac risk factors such as hypercholesterolemia, diabetes, and hypertension and attaches to its counter ligand VLA-4 on the monocyte. This process of monocyte attachment which leads to migration into the arterial wall is an important aspect of the pathogenesis of atherosclerosis.

adhesion molecule-1 (VCAM-1) (20). VCAM-1 is not expressed constitutively in endothelial cells but is inducible. It interacts in a lock-and-key arrangement with its counter ligand, VLA-4, on monocytes and T-cells to contribute to localization and migration of the inflammatory cells into the vascular wall (21) (Figure 5).

In fact it was not known initially whether VCAM-1 expression was necessary and/or sufficient for mononuclear cell recruitment into atherosclerotic lesions or whether VCAM-1 was simply a surrogate for a class of adhesion molecules, the induction of which would be sufficient for disease development. The central pathogenic importance of

VCAM-1 was demonstrated subsequently, again by Cybulsky and colleagues (22). They partially rescued the embryonically lethal, null phenotype of the VCAM-$1^{-/-}$ mouse by disrupting the gene and creating a new allele, Vcam-$1^{D4D/D4D}$. The surviving mice expressing this allele had 2–8 percent of the mRNA and protein of the wild type VCAM-1 animals. The wild type and Vcam-$1^{D4D/D4D}$ mice were bred into a LDL receptor knockout background in which atherosclerosis develops because of hypercholesterolemia. Atherosclerosis was significantly reduced in the Vcam-$1^{D4D/D4D}$ but not in intercellular adhesion molecule-1 (ICAM-1) knockout mice in the LDL receptor$^{-/-}$ background. These data support the notion that VCAM-1 itself is critically important in the pathogenesis of atherosclerosis.

Redox State and Vascular Disease

It was hypothesized originally that lipid species might stimulate vascular pro-inflammatory signals in the *in vivo*, hyperlipidemic state and that this signal generation might in some way be a consequence of the metabolic stress induced by the hyperlipidemia (23). We posited, furthermore, that this stress might be manifested by enhanced production of reactive oxygen species. There were, at the time (early 1990's), examples of gene regulation in prokaryotes by oxygen radicals and we considered that such control mechanisms might be involved in VCAM-1 gene regulation.

At the time that the redox hypothesis for intracellular regulation of pro-inflammatory genes in endothelial cells was being developed, there was ancillary supportive evidence that atherosclerotic arteries were under oxidative stress. As alluded to, endothelial cells have been shown to oxidatively modify LDL (9) and the presence of oxidized LDL was demonstrated in both experimental atherosclerosis and in the human disease (24,25). During the same time period endothelial dysfunction in atherosclerosis as manifested by impairment of endothelial-dependent vasodilatation (26) was being demonstrated both clinically (27) and experimentally (28). Harrison and colleagues provided both indirect and direct evidence that the impairment of the endothelial vasodilator function was due, at least in part, to the excessive production of oxygen radical species that degraded nitric oxide (NO), which is produced by the endothelium and diffuses to the underlying vascular smooth muscle cells to cause relaxation (29–31) (Figure 6).

VCAM-1 Expression Is Redox-Sensitive

Because of the putative central role of VCAM-1 in the pathogenesis of the inflammatory component of atherosclerosis it was selected as a

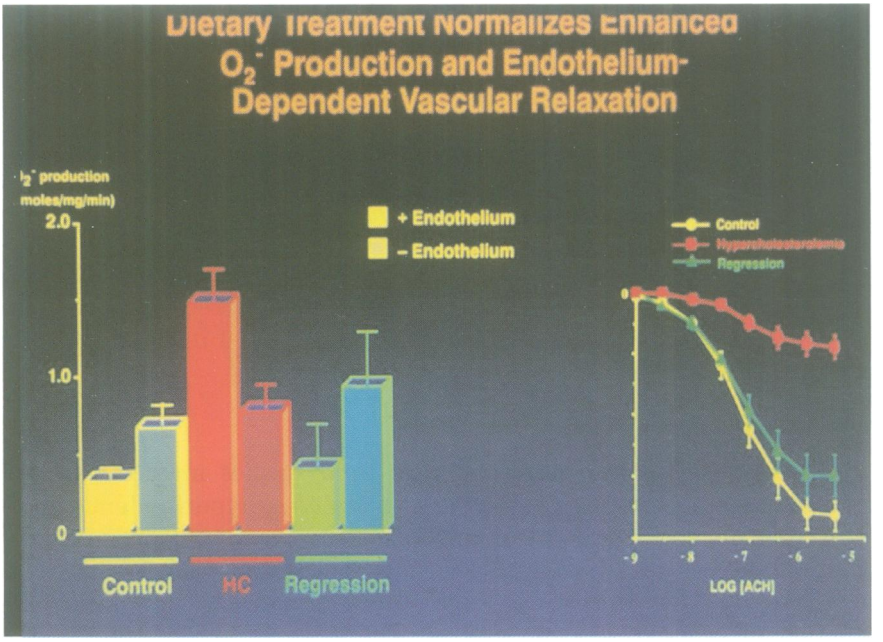


FIG. 6. Dietary Treatment Normalizes Enhanced O_2 Production and Endothelium-Dependent Vascular Relaxation. (Left) Bar graph shows vascular oxygen free radical (O_2^-) production by aortas taken from control rabbits, rabbits fed a 1% cholesterol diet (cholesterol-fed), or animals fed a 1% cholesterol diet for one month followed by a normal diet for one month (regression). Open bars are with intact endothelium and closed bars are with endothelium removed. Cholesterol feed was associated with increased O_2^- production and regression (treatment) resulted in normalization. (Right) Endothelial-dependent relaxation to acetylcholine of aortic rings from the three groups shown in left panel. Normalization of O_2^- production during regression was associated with normalization of endothelial-dependent relaxation. Modified with permission from Ohara Y, Peterson TE, Sayegh HS, et al: Dietary correction of hypercholesterolemia in the rabbit normalizes endothelial superoxide anion production. Circulation 1995;92:898–903 (31).

prototype of what was thought to be a set of vascular pro-inflammatory genes that was posited to be regulated by cellular redox state (23). As an initial approach to evaluating the redox sensitivity of VCAM-1, we performed studies in cultured human umbilical vein endothelial cells, which were stimulated with the cytokine interleukin-1 (IL-1) (23). We measured mRNA not only for VCAM-1 but also for E-selectin and for intercellular adhesion molecule-1 (ICAM-1). IL-1 resulted in a time dependent increase in mRNA for all three adhesion molecules but only that for VCAM-1 was inhibited by the intracellular antioxidant pyrolidine dithiocarbamate (PDTC) (Figure 7). Similar results were obtained if the endothelial cells were stimulated by tumor necrosis factor

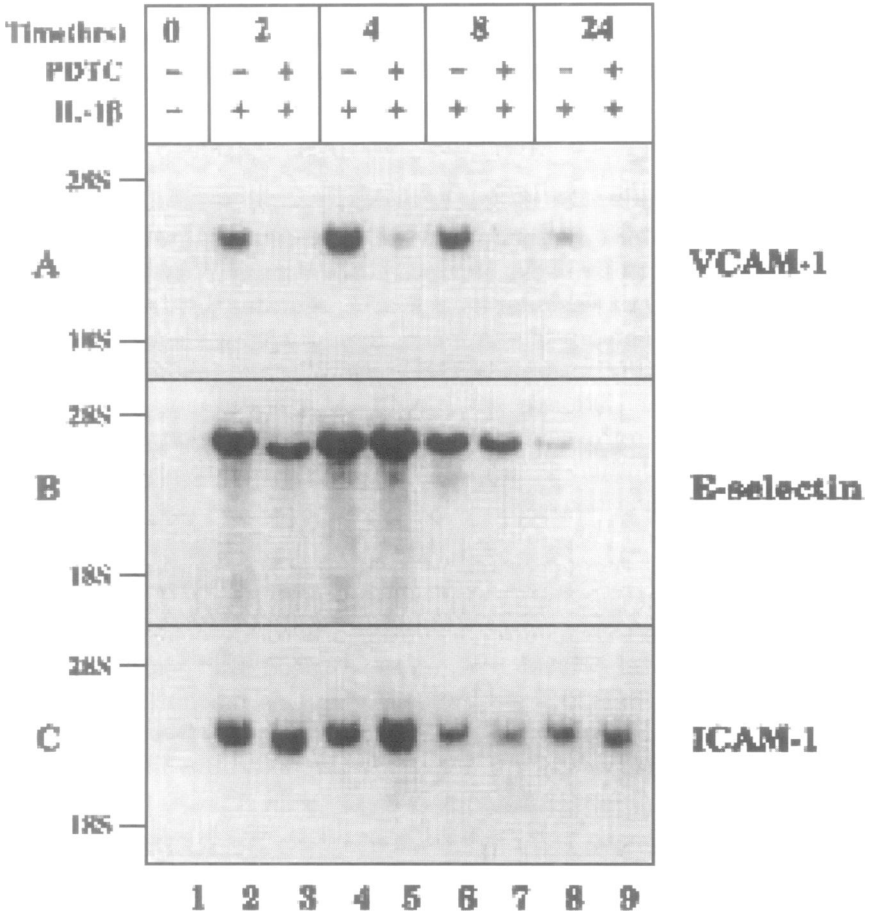


FIG. 7. Induction of HUVE VCAM-1 mRNA by IL-1β is selectively inhibited by the antioxidant PDTC. After pretreatment for 30 min with 50 μM PDTC, HUVE cells were exposed to IL-1β (10 U/ml) in the continuous presence of 50 μM PDTC. Total RNA was isolated and 20 μg size-fractionated by denaturing 1.0% agarose-formaldehyde gel electrophoresis, transferred to nitrocellulose, and hybridized to either ^{32}P-labeled human VCAM-1-specific (A), E-selectin-specific (B), or ICAM-1-specific (C) cDNA, and visualized by autoradiography. Lane 1, 0 h; lanes 2, 4, 6, 8, IL-1β alone for 2, 4, 8, and 24 h, respectively; lanes 3, 5, 7, 9, IL-1β with PDTC for 2, 4, 8, and 24 h, respectively. After washes, filters were exposed to X-ray film at −70°C with one intensifying screen for 18 h. Reproduced from The Journal of Clinical Investigation, 1993;92:1866–1874 (23) by copyright permission of The Rockefeller University Press.)

(TNF) or lipopolysaccharide (LPS) or if another, structurally unrelated antioxidant, n-acetylcysteine (NAC), were used as an inhibitor. PDTC inhibited not only the cell surface expression of VCAM-1 protein but

inhibited the binding of Molt-4 cells, which express the VLA-4 integrin counter ligand as discussed above. Thus, these data provided compelling evidence that VCAM-1 expression is mediated by redox sensitive factors. Furthermore, the level of control is at the transcriptional level and, in particular, involves nuclear factor-κB (NFκB).

Implications of redox regulation of VCAM-1

The regulation of endothelial VCAM-1 by redox state has several implications. First, it raises the more general possibility that subsets of pro-inflammatory vascular genes are regulated by the enhanced oxidative stress. This, in fact, appears to be the case since both monocyte-chemoattractant protein-1 (MCP-1) (32) and monocyte-colony-stimulating factor (32) are stimulated by oxidant-sensitive mechanisms. Secondly, in the context of the role of excessive oxygen radicals in promoting endothelial vasodilator dysfunction, it provides some potential mechanistic unification between fundamental features of atherosclerosis—namely, inflammation and vasomotor dysfunction. Thirdly, the redox regulation of pro-inflammatory signals in endothelial cells raises the possibility that antioxidants acting intracellularly might be effective therapeutic agents for atherosclerosis. Finally, the redox sensitivity of VCAM-1 regulation raised the possibility that alteration in redox state is a common underlying mechanism through which other cardiovascular risk factors, in addition to hyperlipidemia, exert their pro-atherogenic influences on the arterial wall.

PLAQUE REMODELING IS MODULATED BY REDOX STATE

As noted previously, arterial remodeling is a characteristic feature of the atherosclerotic lesion and is mediated to an important extent by matrix metalloproteinases (MMPS), which are a large family of endopeptidases with common structural features and mechanisms of action to degrade extracellular matrix components (5). As shown in Figure 8 an artery can undergo either constrictive remodeling with encroachment upon the lumen or expansive remodeling, the end-product of which can be either plaque rupture or aneurysm formation. As illustrated, these processes have been associated with variable expression of the multiple members of the MMP family. The inflammatory milieu of the atherosclerotic plaque and, in particular, the presence of the foam cell that secrets multiple cytokines which, in turn, stimulate pro-MMP secretion by the various constituent cell types provides the substrate for plaque remodeling (33–36). The foam cell is a major source of ROS, which can activate the pro-MMPs present in the arte-

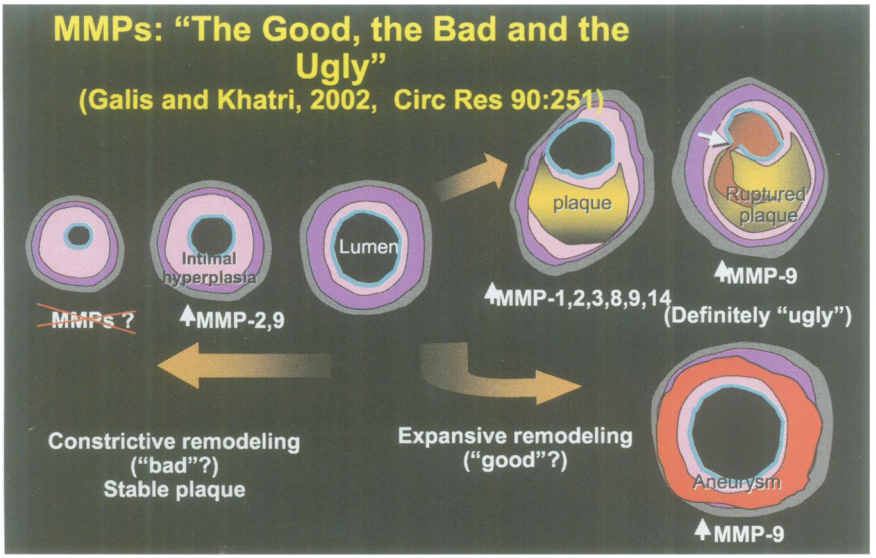


FIG. 8. Matrix metalloproteinases and atherosclerosis-induced arterial remodeling. Various MMPs are associated with the spectrum of remodeling that has been observed. Clinical outcomes obviously are dependent upon the type of remodeling that occurs. Reproduced with permission from Galis ZS, Khatri JJ. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. Circ Res 2002;90:251–262 (5).

rial wall (Figure 9) (5,37). MMP activity has been associated with the macrophages that localize in the shoulder of atherosclerotic plaques in human lesions (38). This area is known to be a "vulnerable" region of the plaque that is most subject to rupture and to be associated with acute events such as myocardial infarction. The localization of superoxide production by macrophages to this same area is illustrated in Figure 10. *In summary there is compelling evidence, but not yet definitive proof, that both adaptive and maladaptive remodeling of the arterial wall induced by atherosclerosis (including disruption and rupture as well as aneurysm formation) are related to the excessive production of ROS.*

HEMODYNAMIC FORCES, PLAQUE LOCALIZATION, AND OXIDATIVE STRESS

Atherosclerosis is, in its initial stages, not a generalized, randomly located disease but occurs somewhat predictably at branch points (15,39) (Figure 11). Hemodynamic modeling has shown that these areas, which are frequently opposite of flow dividers, experience *low*

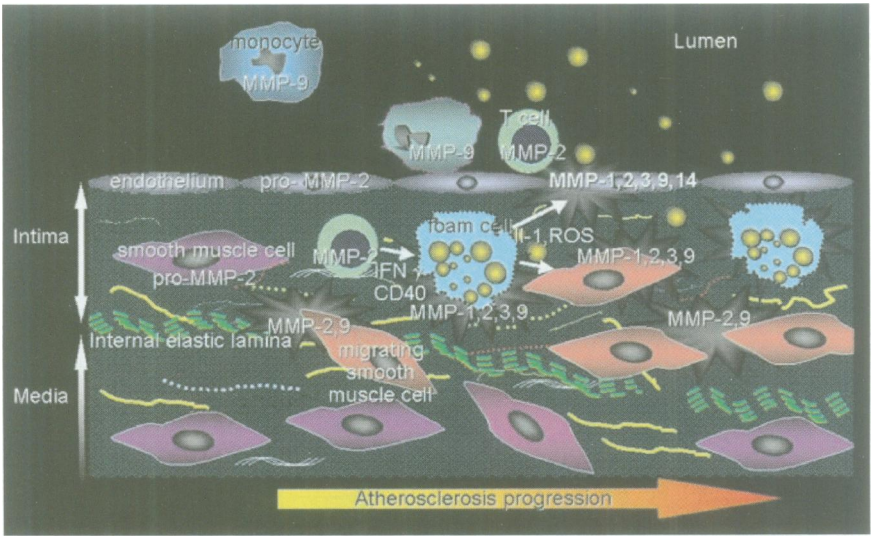


FIG. 9. Vascular cells and inflammatory cells produce and secrete MMPs in atherosclerosis. The spectrum of MMPs, which are secreted in a latent (pro-) form, is determined by the characteristic of the milieu in respect to the populations of cells present, cytokines secreted, and cell-cell as well as cell-matrix interactions. ROS can control not only the expression and secretion of MMPs but also the extracellular activation of the pro-forms. Degradation of matrix by MMPs facilitates cell migration and reorganization of the artery. This process, which is modulated significantly by ROS, can disrupt the structural integrity of the arterial wall and lead to plaque rupture. Reproduced with permission from Galis ZS, Khatri JJ. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. Circ Res 2002;90:251–262 (5).

shear stress and may also have non-laminar, disturbed patterns including reversing, oscillating flow (15,39). Laminar flow that occurs in linear areas of the arterial tree away from branch points generally induces resistance to the development of atherosclerosis. The molecular mechanisms, which have been defined in *in vitro* experiments with cultured endothelial cells, involve the inducement of resistance to oxidative stress and the consequent development of an anti-inflammatory state (40,41). For example, laminar flow induces eNOS expression in endothelial cells relative to static flow conditions (42). Endothelial NOS, by producing NO in a controlled fashion, acts as an effective antioxidant. Laminar flow conditions also stimulate the expression of SOD (43,44), and hemoxygenase-1 (43), as well as modulating the glutathione redox cycle (45), effects that would be associated with creating an antioxidant milieu. One would predict that the induction in endothelial cells of an antioxidant state would inhibit the expression of VCAM-1. In fact it was observed that prolonged laminar shear stress

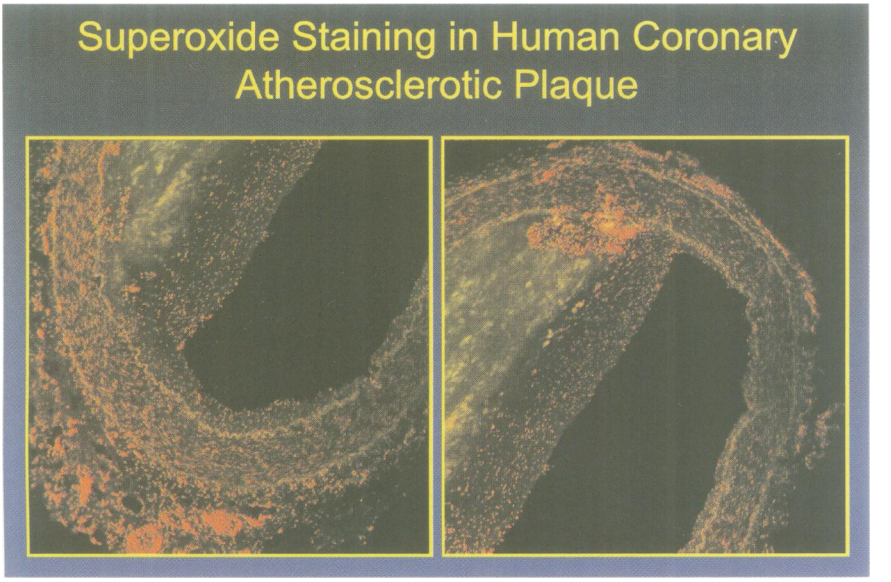


FIG. 10. ROS in Human Coronary Atherosclerotic Plaque. Staining of the section with a reagent that detects superoxide (O_2^-) shows intense localization in the vulnerable shoulder region of the plaque. This intense local oxidative stress would be expected to activate pro-MMPs and weaken the area potentially leading to rupture. (Courtesy of W. Robert Taylor, M.D.)

selectively inhibited the expression by endothelial cells of VCAM-1 but not ICAM-1 or E-selectin upon stimulation by the cytokine IL-1 (46). Detailed understanding is unavailable of the metabolic (*redox*) state of endothelial cells in localized areas of non-laminar flow in arteries *in vivo*. There are data, however, in normal animal models indicating that VCAM-1 is expressed in the endothelium in areas of the aorta that are predisposed to the development of atherosclerosis and that are known to be areas of low shear stress and disturbed flow—i.e., at branch points and opposite flow dividers (47). By inference from the *in vitro* data reviewed above these areas would be expected to exhibit enhanced oxidative stress. This extrapolation is also supported by observations that components of the NF-κB transcription factor system that is involved in VCAM-1 regulation and is redox sensitive, as discussed, are up-regulated in areas of the normal aorta predisposed to atherosclerosis development (48).

Summary: Redox State and Atherogenesis

The available data make a compelling case that excessive oxidation in the arterial wall plays a central role in the pathogenesis of athero-

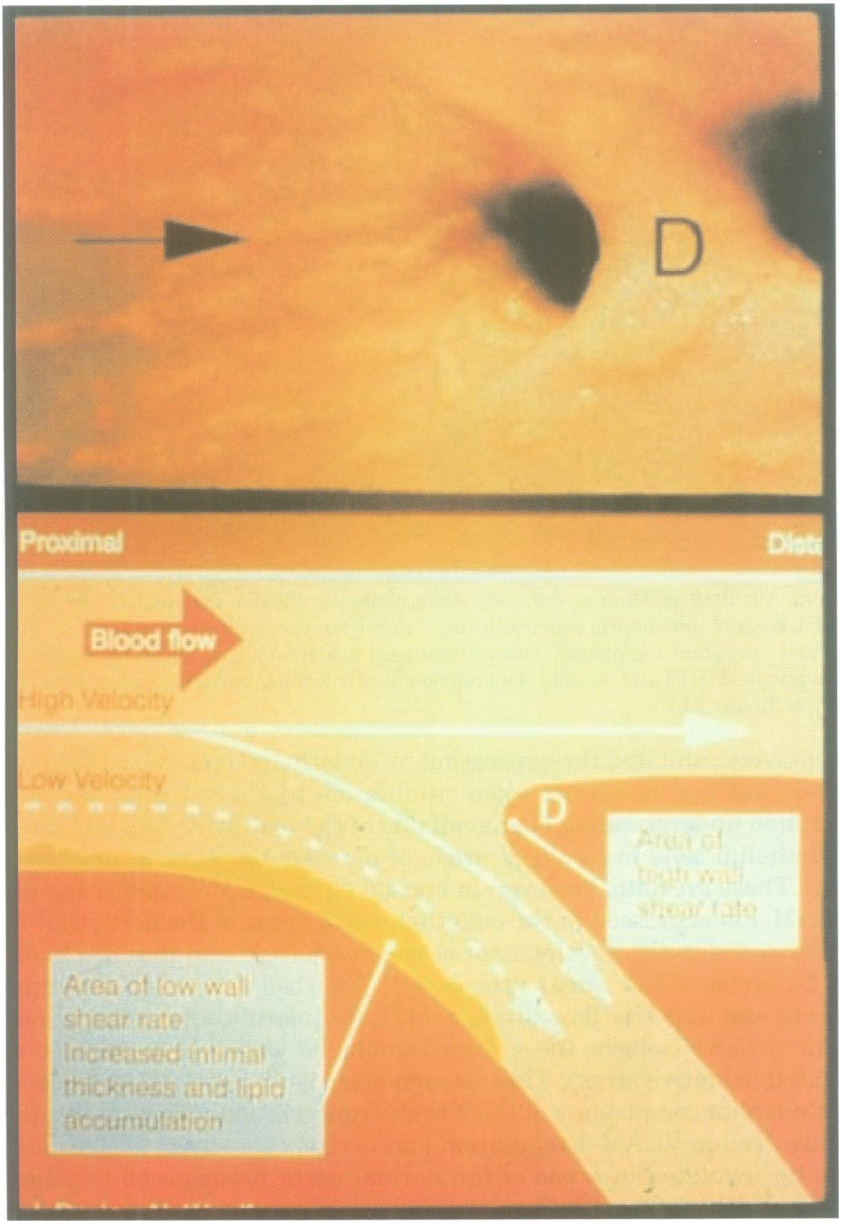


FIG. 11. Atherosclerosis develops initially at branch points and opposite to flow dividers. The upper panel illustrates fatty streak formation at the orifice of an arterial side branch and opposite the flow divider "D". Direction of blood flow is indicated by the arrow. As illustrated by the lower panel the susceptible area opposite to the flow divider is characterized by low flow velocity, thickening, and cell and lipid accumulation.

sclerosis at all stages of the disease. Whether this fact can be related to clinical observations and situations and, most importantly, whether new therapeutic approaches can be developed by attacking the disease with regimens that are effectively, directly or indirectly, antioxidants are major unresolved issues.

CLINICAL CORRELATIONS

There is a considerable body of evidence, though mostly indirect, supporting the notion that regimens, whether hygienic or pharmacological, that promote a "reduced" as opposed to an "oxidized" metabolic state are associated with decreased cardiovascular event rates (49). Since we currently have no generally accepted measures of the redox state of patients, the available data are inferential.

Renin-Angiotensin System and Atherosclerotic Cardiovascular Disease

The renin-angiotensin system (RAS) was originally characterized as one that was primarily involved in control of intravascular volume and blood pressure. Some of the earliest evidence that the RAS was involved in the pathogenesis of atherosclerotic vascular disease was published by Laragh and his group in the 1970's although the full implications were appreciated only 20–25 years later (50,51). These investigators, who were early advocates of "renin profiling" of patients with high blood pressure, originally reported that "high renin" hypertensives, who presumptively had elevated levels of angiotensin II (ANG II), had an increased incidence, relative to hypertensive patients with low renin, of cardiovascular events such as acute myocardial infarction. The findings were somewhat controversial at the time, in large part because there was little mechanistic context in which to place what were essentially phenomenological data. The fundamental story became more compelling when the same group reported similar findings in a much more incontrovertible manner in 1991 (52). Soon there after, studies evaluating the effects of angiotensin converting enzyme (ACE) inhibitors on ventricular enlargement and the development of congestive heart failure after acute myocardial infarction (AMI) were reported. Both the Survival and Ventricular Enlargement Trial (SAVE) (53) and SOLVD (54) studies reported a decrease in recurrence of AMI in patients treated with ACE inhibitors, which would be expected to decrease the ambient concentrations of ANG II. Although animal studies had shown previously that ANG II infusion dramatically exacerbated atherosclerosis in experimental animals

with hypercholesterolemia (55), insights into molecular mechanisms that potentially related the RAS to the pathogenesis of atherosclerosis began to emerge only in the mid 1990's. One of the key observations was that ANG II robustly stimulated oxidative stress in vascular cells *in vitro* by activating an NAD(P)H oxidase (56) (Figure 12). In *in vivo* animal experiments ANG II infusion induced endothelial dysfunction mediated by excessive oxidative stress (57). Predictably, based on the redox/VCAM-1-mediated vascular inflammatory mechanisms described previously (58), animals infused with ANG II also exhibited intense arterial inflammation with a mononuclear cell population (59). *Thus, a considerable body of experimental data* (reviewed in (55)) *supports the conclusion that the RAS generally and ANG II in particular mediate inflammatory responses in the vasculature that promote the development of atherosclerosis.* Chronic administration of ACE inhibitors also improves endothelial vasodilator dysfunction clinically (60), a fact that also is consistent with an effective antioxidant effect of these drugs (61). Finally, very recent clinical studies in hypertensive subjects and patients with known coronary artery disease have shown significant decreases in plasma markers of oxidative stress and inflammation in those treated with ANG II receptor blockers (62,63). *The*

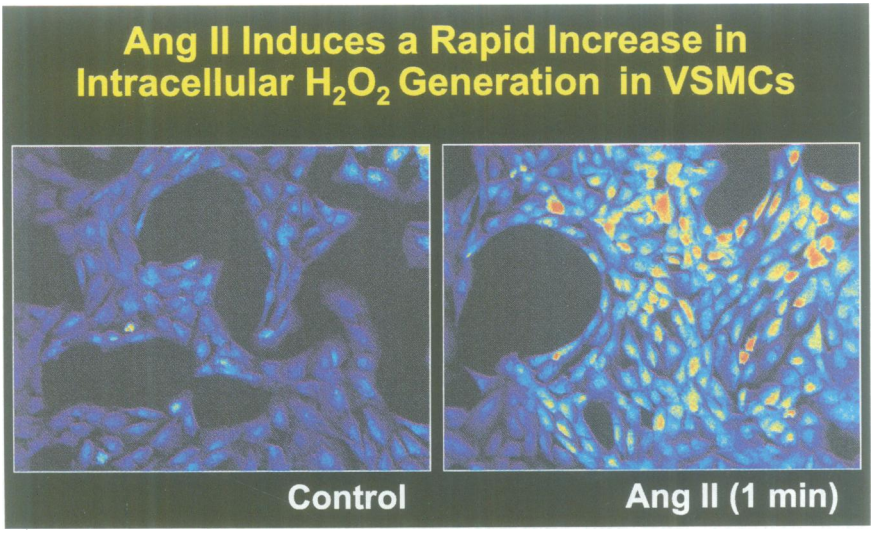


FIG. 12. Angiotensin II induces oxidative stress in vascular smooth muscle cells. Angiotensin II (10 nM) was applied to cultured rat vascular smooth muscle cells that had been treated with a detector for H_2O_2. Robust stimulation of H_2O_2 was detected after one minute. Angiotensin II induces oxidative stress in endothelial as well as vascular smooth muscle cells. NAD(P)H oxidases are major sources of ROS stimulated by Ang II.

apparent anti-atherosclerotic effects of ACE inhibitors in clinical trials can be understood in the context of this drug class acting, in part, indirectly as antioxidants by inhibiting ANG II stimulation of ROS production by vascular NAD(P)H oxidases. It is likely that similar conclusions will also be reached ultimately in the case of ANG II receptor blockers.

Anti-Atherosclerotic Effects of Statins and Oxidation

Statin therapy reduces cardiovascular event rates as has been demonstrated in multiple clinical trials involving both primary and secondary prevention (64). Inferentially, this very considerable body of data suggests that these drugs are modifying the biology of atherosclerotic lesions by diminishing the inflammatory response (65). Clinical imaging studies of the arterial wall in patients treated with statins are consistent with plaque stabilization and regression of the lipid core (66), but these changes are usually not readily apparent in conventional angiographic studies (67). As is frequently the case, we must extrapolate from experimental animal studies to gain insights into clinical observations. Lipid lowering by removing cholesterol from the diet decreases macrophage number and the MMP activity of lesions in hypercholesterolemic rabbits (68–70). Furthermore, dietary induction of hypercholesterolemia is associated with endothelial vasodilator dysfunction and increased production by the endothelium of O_2^- while restoration of normal cholesterol levels by reinitiating a normal diet returned both O_2^- generation and endothelial function to normal (71). A similar experimental approach recently confirmed that diet-induced hypercholesterolemia induces oxidant stress as well as endothelial activation with increased expression of VCAM-1 and both functions normalized with restoration of the usual chow diet (72). Statin therapy improves endothelial vasodilator function in patients with coronary artery disease (73). The most likely explanation, based on the available evidence, is that statins reduce oxidative stress. Part of the data upon which this assertion is based includes observations that acute administration to human subjects with vascular disease of the antioxidant vitamin C also improves endothelial vasodilator dysfunction, confirming that this functional abnormality is, at least in part, caused by excessive production of ROS (74). Statins may reduce oxidative stress by multiple mechanisms including: the reduction of the metabolic stress induced by elevated plasma lipid levels; decreased inflammatory cell recruitment that would result in diminished sources of ROS production; and increased production of NO by the endothelium because of the stimulation of eNOS expression (75). As alluded to previously,

statins also have the potential to block directly O_2^- formation by interfering with the assembly of the multiple components of NAD(P)H oxidase. Inhibition of HMG CoA reductase not only diminishes cholesterol synthesis but also interferes with the synthesis of isoprenyl lipid products such as geranylgeranyl phosphate and farnesyl phosphate. The isoprenylation of rac-1, the small GTPase that is an essential component of the active oxidase, is required for its translocation from the cytosol to the plasma membrane. Inhibition of prenylation of rac-1 by statins would interfere with the assembly at the plasma membrane of the various components of NAD(P) oxidase after stimulation by, for example, ANG II, as discussed previously (1). Such mechanisms have been postulated to provide an explanation for observations that the reduction of clinical events in prevention trials is frequently greater than can be accounted for by LDL lowering alone, the so-called "non-lipid lowering" effects of statins (76). Statins reduce inflammation in atherosclerotic lesions in non-human primates even if the dietary cholesterol is increased to maintain plasma cholesterol constant in animals receiving the drugs. *Thus, the anti-atherosclerotic effects of statins likely involve indirect antioxidant activity mediated by several mechanisms-both lipid lowering and non-lipid lowering.*

Clinical Trials With Antioxidants

Vitamins

A number of observational trials have suggested that increased intake of the antioxidant vitamin E is associated with decreased incidence of cardiovascular events in patients who either had established atherosclerotic cardiovascular disease or were at high risk for its development (reviewed in (77)). Subsequent prospective clinical trials have not confirmed that there is a beneficial effect of, in particular, vitamin E in reducing cardiovascular events. Although there has been some resulting skepticism about the potential efficacy of antioxidants as therapeutic agents in atherosclerosis (and the "oxidation hypothesis"), Parthasarathy and colleagues have critiqued the trials and developed a compelling case that they were flawed with respect to: patient selection; outcome expectations; choice of antioxidants, especially with respect potency and efficacy; lack of measurement of markers of oxidative stress markers; and lack of markers of vascular function (such as assessment of endothelial dependent vasodilator responses) (77). Very recently, however, an important study addressing some of these deficiencies was published. The Vitamin E Atherosclerosis Prevention Study (VEAPS) evaluated the effects of supple-

mental DL-α-tocopherol (400 IU daily) on progression of carotid intima-media thickness over the course of three years in a population of men and women 40 years old or older without evidence of cardiovascular disease (49). B-mode ultrasonograms were utilized. Compared with placebo, vitamin E supplementation did not reduce progression of carotid thickening although circulating vitamin E levels were raised ($P < 0.001$) and both circulating levels of oxidized LDL and the susceptibility of LDL to oxidation were decreased ($P = 0.03$ and $P < 0.01$, respectively). Thus, the weight of the evidence still is that vitamin E is ineffective in treating atherosclerosis. Taken at face value, either the general oxidation hypothesis is incorrect or the potency and/or tissue distribution of vitamin E limits its efficacy. The explanation is probably the former rather than the latter. Evidence supporting this assertion follows.

Antioxidant pharmaceuticals

Probucol is the only antioxidant pharmaceutical with which there is wide clinical experience (78). It was developed in the early 1960's in a program by the Dow Chemical Company to screen chemicals for antioxidant properties. Probucol was found to be a lipid lowering agent and was subsequently marketed. It was eventually withdrawn from the United States market for a variety of reasons (79) including: lack of efficacy as an adjunctive agent to cholestyramine in reducing femoral atherosclerosis in the Probucol Quantitative Regression Swedish Trial (PQRST) (80); modest lipid lowering effects (10–20 percent decrease in LDL in the context of a 20–30 percent lowering of the protective, high density lipoprotein (HDL); variable and limited oral bioavailability with high lipophilicity and poor cell permeability; and QT_C prolongation on the electrocardiogram (likely mediated by a metabolite) raising safety issues. On the other hand probucol, which is a very effective antioxidant chemically and which is still marketed in Canada and Japan, has been shown to be effective in reducing post-angioplasty restenosis in studies in which patients were started on the drug four weeks preceding the intervention (81,82). The biology of restenosis, which tends to be more proliferative than the predominantly inflammatory atherosclerotic lesion, none-the-less, has some similarities with the native disease, such as MMP activation, smooth muscle migration and modulation, and remodeling (83). The apparent efficacy of probucol in decreasing post-interventional restenosis, however, provided supporting evidence for the notion that drugs inhibiting oxidation mechanisms could have therapeutic benefits in human vascular

disease. Furthermore, this hypothesis has been bolstered further recently by the results from the Fukuoka Atherosclerosis Trial (FAST) in which the effects of probucol and pravastatin on carotid intima/media thickening (IMT) and on major cardiovascular events were compared with placebo over the course of two years (84). Probucol and pravastatin were equally efficacious in reducing IMT, which progressed in the placebo group. The effects of probucol were independent statistically of the reductions that it induced in both LDL and HDL. Probucol also reduced major cardiovascular events (2.4 percent) relative to the placebo group (13.6 percent; $P = 0.0136$).

The publication of the results of the success of probucol in the restenosis trials and in FAST appeared to validate efforts to develop antioxidants that were effective pharmaceuticals with acceptable absorption, bioavailability, and cell permeability characteristics. One such molecule was AGI-1067, which is a monosuccinate ester of probucol (79). AGI-1067 is a potent antioxidant and is more water soluble and cell permeable than is probucol and, unlike probucol, inhibited tumor necrosis factor alpha (TNF-α) stimulation of VCAM-1 expression in endothelial cells *in vitro* with a specificity in the presence of the antioxidant N-acetylcysteine similar to that shown in Figure 7. Moreover, AGI-1067 was shown to be a more efficacious anti-atherosclerotic agent than is probucol in several animal models. For example, hypercholesterolemic cynomolgus monkeys that were treated with AGI-1067 for one year had no histologic evidence of atherosclerosis and were indistinguishable from normal control animals (Figure 13a) and there was no QT_C prolongation on the electrocardiogram or other evidence of toxicity (79). In contrast cholesterol fed animals that were treated with probucol or vehicle for AGI-1067 had extensive coronary neointimal thickening and atherosclerotic lesion formation (Figure 13b). Thus, AGI-1067 appeared to have characteristics suitable for clinical testing for its effects in vascular disease.

The first clinical trial of AGI-1067 was the Canadian Antioxidant Restenosis Trial (CART-1) (85,86). This double blinded, placebo controlled, Phase 2 study compared the effects of three doses of AGI-1067 (70 mg, 140 mg or 280 mg once daily) with probucol (500 mg twice daily) and with placebo in 305 patients whom were undergoing percutaneous transluminal coronary angioplasty (PTCA) and were randomly assigned to treatment groups. Patients were treated for two weeks before and for four weeks after PTCA. Lesions were evaluated by intravascular ultrasound (IVUS) at baseline and at six months after the procedure. There was a dose-related reduction in late lumen loss at the PTCA site in the three AGI-1067 study arms ($p < 0.02$). Luminal

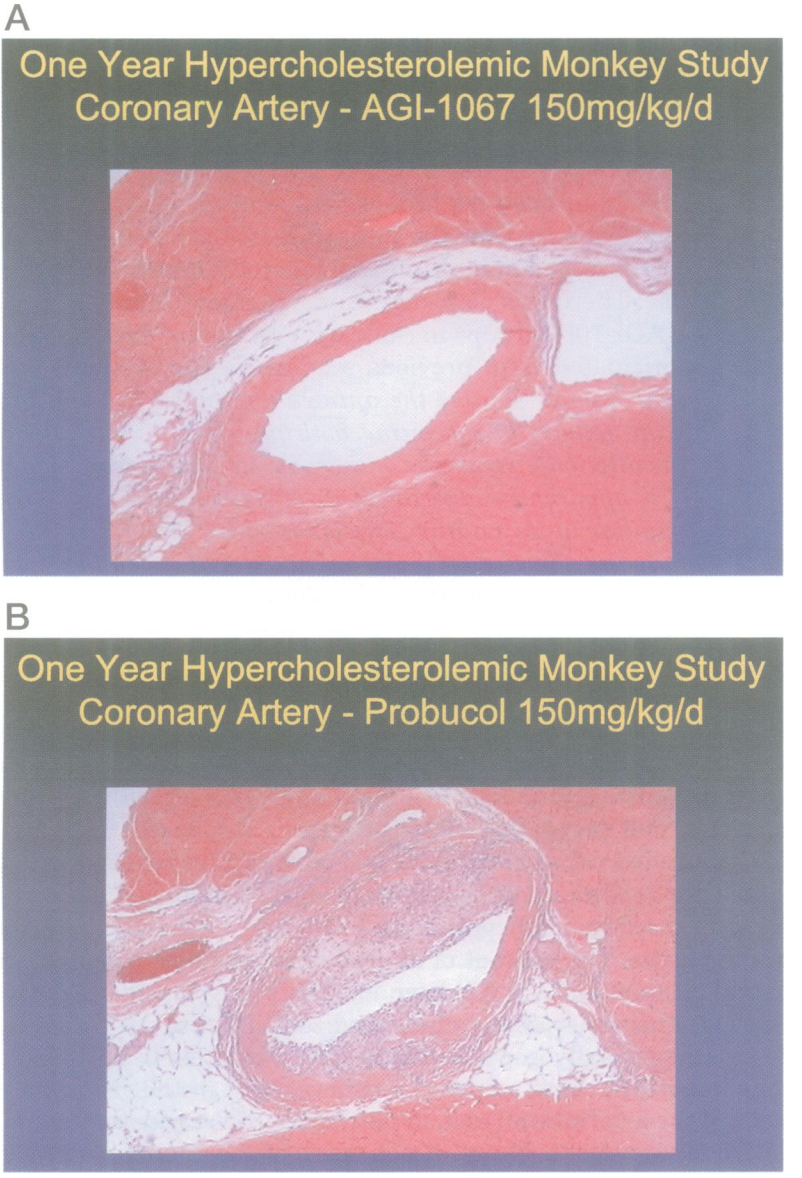


FIG. 13a Antioxidant therapy inhibits atherosclerosis in cynomolgus monkeys. Animals were fed a high cholesterol diet for one year and received 150 mg/kg/d of probucol monosuccinate (AGI-1067) in the chow. The coronary arteries were indistinguishable histologically from control animals fed normal chow. In contrast, untreated hypercholesterolemic animals and those receiving probucol (150 mg/kg/day) showed extensive coronary intimal thickening and lesion formation. Panel b. (Courtesy of Martin Wasserman, Ph.D., and Russell Medford, M.D., Ph.D., AtheroGenics, Inc.)

areas at six months were similar in the probucol group and in the group receiving 280 mg of AGI-1067 (3.69 +/− 2.69 mm^2 and 3.36 +/− 2.12 mm^2 respectively) as compared with 2.66 mm^2 for placebo ($P < 0.05$ for probucol and for 280 mg AGI-1067 versus placebo). Importantly, reference segment luminal volume decreased in the placebo group, was unchanged in the probucol group, and increased in the AGI-1067 140 mg and 280 mg groups. These latter observations provide the initial evidence that the anti-atherosclerosis effects of AGI-1067 that were observed in multiple animal studies may also obtain in human disease. There was no toxicity and, specifically, no QT_C prolongation in the AGI-1067 groups in contrast with the predictable prolongation, as anticipated from previous studies, in the probucol group. *Thus, in initial human studies the antioxidant AGI-1067 appears to have significant activity in inhibiting both post-PTCA restenosis and atherosclerosis after only six weeks of therapy. In comparison to probucol AGI-1067 is apparently superior in its effects to inhibit atherosclerosis and is similar in its salutary effects on post-PTCA restenosis but without the adverse effect of QT_C prolongation or the strikingly unfavorable pharmacokinetic properties of probucol.*

SUMMARY

Thus, there is now a large and compelling body of data suggesting that ROS play critical and specific roles in cell signaling and are involved in multiple functions including migration, growth, apoptosis, metabolism, and generation of inflammatory responses. ROS are involved generally in stress responses and maladaptive responses involving excessive generation of oxygen radicals or diminished antioxidant reserves in diseases as disparate as Alzheimer's dementia and rheumatoid arthritis. The concept of altered redox state as a central, fundamental cause of disease, has been most fully developed, arguably, in the case of atherosclerosis in which oxidized LDL was identified early on as a pathogenically important molecule. Oxidation of LDL is likely but one important consequence of a general shift in the redox state of the cells of the arterial wall and, in particular, of the endothelium to an oxidative environment. Many, if not most, of the common cardiovascular risk factors such as diabetes mellitus, hypertension, and hyperlipidemia appear to evoke pathological responses in the vasculature by stimulating the expression or activity of a number of oxidases and, particularly, the family of enzymes that utilize NAD(P)H as electron donors. At least three fundamental characteristics of atherosclerosis-distribution in the arterial tree, inflammatory cell recruitment and

foam cell formation, and vascular remodeling and plaque rupture can be understood as being modulated by oxidation. Oxidation is thus proposed as an organizing principle in the pathogenesis of atherosclerosis. These developments have led in turn to interest in antioxidants as potential therapeutic agents for the disease. In fact, the anti-atherosclerotic effects of standard drugs such as ACE inhibitors and statins are increasingly being viewed as acting, at least in part, indirectly as antioxidants. Furthermore, the vascular protective effects of hygienic measures such as exercise are known to be protective in part because they induce a reduced/antioxidant state in arteries. The general concept has led to reassessment of the role as anti-atherosclerotic agents of existing drugs such as probucol, which is a strong antioxidant, and to the development of new antioxidants as therapeutic agents that have pharmacologic properties more favorable than those of probucol. One of these, AGI-1067, is in active clinical testing and the initial data suggest therapeutic efficacy in both post-PTCA restenosis and atherosclerosis.

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