Title

Lipoprotein oxidation: mechanisms and implications for atherogenesis

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Author

Palinski, W

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13 Oxidative stress and diabetes mellitus

W Palinski and J L Witztum

Background

Epidemiological studies provide convincing evidence for an increased incidence of cardiovascular disease in diabetes mellitus [1]. However, the pathogenesis of atherosclerosis in general, and the mechanism(s) increasing plaque formation in diabetic subjects in particular, remain unknown. Research on the causes of atherosclerosis is impaired by the slow progression of the disease, the complexity of the lesions and the lack of techniques suitable to follow the cellular development of lesions. During the last two decades, important new pathogenic mechanisms have been discovered, and well-known ones previously viewed as distinct entities are now recognized to interact. Several hypotheses have been proposed, which link hypercholesterolemia, oxidative modification of lipoproteins and vascular injury [2,3]. Recent advances in our understanding of another form of protein (and lipoprotein) modification, induced by non-enzymatic glycation (NEG), and of the interaction between oxidation and glycation, provide a potential explanation for the increased arteriosclerosis so frequently associated with diabetes mellitus. In this chapter we will review the role of the oxidative modification of lipoproteins in the pathogenesis of atherosclerosis, and discuss the manifold analogies and interactions between oxidation and glycation.

Hyperlipidemia, modified lipoproteins, and foam-cell formation

It is generally accepted that hypercholesterolemia is a major contributor to atherosclerosis [4]. The correlation between lipid lowering and coronary heart disease has been clearly established by numerous clinical intervention trials [5]. Angiographic studies have not only shown that lipid lowering reduces the progression of atherosclerosis, but suggest that such intervention may even lead to regression of preexisting lesions [6]. One of the earliest manifestations of atherosclerosis, if not the initiating event itself, is the accumulation of lipoproteins in the arterial intima. Plasma low-density lipoproteins (LDL) are the principal source of lipoproteins in the atherosclerotic lesions, at least in humans. However, the mechanisms leading to the intimal accumulation of LDL are not fully understood.

Predilection sites for atherogenesis frequently coincide with areas of non-laminar flow, resulting in localized differences in tensile and shear stress. The intimal lipoprotein accumulation has therefore been thought to be caused by increased influx of LDL resulting from endothelial damage. Experimental evidence for such damage includes more frequent endothelial mitosis at such sites [7]. Studies of lipoprotein flux through the vascular wall seem to indicate that increased residence time, rather than increased influx, may be responsible for the intimal accumulation [8]. Furthermore, plasma LDL may not account for the second characteristic element of early atherosclerotic lesions (i.e. the accumulation of large numbers of monocyte-derived macrophages and foam cells). LDL receptors (also known as B/E receptors) mediate most of the cellular uptake of LDL, and account for the clearance of more than two-thirds of the plasma LDL, predominantly by liver cells [9]. However, LDL receptors are unlikely to account for the rapid uptake of cholesterol-rich lipoproteins by intimal macrophages, because the expression of these receptors is down-regulated by the intracellular LDL content. The need for a mechanism capable of inducing foam cell formation is particularly apparent in patients with familial hypercholesterolemia, characterized by an inherited lack of functional LDL receptors, hypercholesterolemia and extensive premature atherosclerosis.

The description of a separate 'scavenger' or 'acetyl-LDL' receptor provides a possible explanation [10]. This receptor is present on macrophages and sinusoidal Kupffer cells, and recognizes several forms of chemically modified lipoproteins. Uptake of modified LDL by macrophages via this receptor is many times higher than the uptake of native (unmodified) LDL by the LDL receptor, and induces foam cell formation *in vitro*. Furthermore, expression of the scavenger receptor is not down-regulated by high intracellular LDL concentrations. The scavenger receptor recognizes epitopes on modified LDL that appear to be formed at least in part by derivatization of free amino groups of amino acid residues (prevalently lysines) of apoprotein B (apoB). Derivatization of lysines not only has profound consequences for the recognition of lipoproteins by specific receptors and for lipoprotein metabolism [11], but may also affect numerous properties of proteins in general. The presence on macrophages of receptors recognizing a broad spectrum of chemical modifications suggests that the removal of modified lipoproteins may be an important biological function of macrophages.

The search for naturally occurring forms of modified lipoproteins was greatly enhanced by the discovery that LDL incubated with endothelial cells undergoes extensive structural modification, and is recognized by the scavenger receptor [12]. Other cell lines of the arterial wall — smooth muscle cells, macrophages and lymphocytes — are also capable of modifying LDL in a similar way [13–16]. Subsequent studies have determined that the cell-mediated modification of LDL is an oxidative process, associated with extensive peroxidation of its polyunsaturated fatty acids, formation of adducts between lipid peroxidation products and lysine residues of apo B, and concomitant breakdown of the apoprotein [2,13,17]. Immunocytochemical studies of atherosclerotic lesions of several species have since demonstrated that such oxidative modification occurs *in vivo* [18–22]. In addition, LDL extracted

from lesions has all of the properties of oxidized LDL (OxLDL) prepared *in vitro* [23]. Furthermore, it is now apparent that more than one receptor for OxLDL exists [24] (for detailed reviews of OxLDL, see [2,25,26]).

Oxidation is not the only mechanism resulting in rapid uptake of cholesterol-rich lipoprotein particles. NEG of proteins by reducing sugars leads to an analogous modification of lysine residues of proteins [27–29], and receptors for advanced glycation end products also occur on macrophages [30–34]. The consequences of an increased uptake of modified lipoproteins for atherogenesis and the pathogenic mechanisms of both forms of modifications will be discussed later.

The oxidative modification of LDL

Although physical and chemical properties of LDL affected by oxidative modification have been rapidly identified, the definition of 'oxidized LDL' remains elusive. Two main events occur during the oxidation of LDL. Lipids and phospholipids in the core and surface of the particle undergo peroxidation, and products of lipid peroxidation can react with the apoprotein moiety. Both lipid peroxidation and apoprotein modification yield a large number of products, many of which have atherogenic properties. However, the chemical structure and quantitative occurrence of individual reaction products is not only determined by the experimental conditions used to generate OxLDL *in vitro*, but may also depend on the composition of the individual LDL preparation used, which is far from homogeneous. The assessment of the biological properties of byproducts of OxLDL is equally difficult. The results of *in vitro* studies have to be viewed with great caution [35], and few reliable data are yet available about the extent of lipoprotein oxidation occurring in atherosclerotic lesions, or about the nature and prevalence of individual products formed *in vivo*.

The oxidative modification of LDL has been intensely studied, and it is now evident that the chemical processes involved are extraordinarily complex [25]. It is generally assumed that oxidation is initiated by abstraction of hydrogen atoms from unsaturated fatty acids of the LDL particle. *In vitro*, oxidation may be induced by metal ions. Incubation of LDL with copper ions in the absence of cells generates a form of OxLDL which closely resembles cell-induced OxLDL, and both copper- and cell-induced oxidation of LDL are effectively prevented by ethylenediamine-tetraacetic acid (EDTA) and other metal chelators [2]. However, it is uncertain whether free metal ions are present in the extracellular space in sufficient quantities to initiate lipid peroxidation, or whether complexes of metal ions such as ceruleoplasmin may also act as catalysts [36]. Although metal ions clearly promote lipid peroxidation, oxidation *in vivo* may also be initiated by vascular cells. This could occur for example by a gradual enrichment of the particle with lipid hydroperoxides derived from cells. Vascular cells, particularly activated macrophages, could achieve this via 15-lipoxygenase activity [37], which could then transfer the lipid hydroperoxides to extracellular LDL. In addition, macrophages could induce oxidation of LDL by secretion of superoxide and other reactive oxygen species [25]. Cells may also use other mechanisms such as the release of thiols (in the case of macrophages) or myeloperoxidase [38].

Once formed, lipid hydroperoxides decompose to more reactive peroxy radicals, which in turn may generate more lipid hydroxides. This process is greatly accelerated by the presence of metal ions. The susceptibility of the LDL particle to oxidation depends on a number of properties, in particular its antioxidant content. Only when the antioxidative defenses have been overcome does the oxidative process enter a rapid propagation phase. This phase is characterized by formation of lipoperoxides and rearrangement of double bonds, which can be monitored by increased absorbance at 234 nm. The propagation phase is greatly promoted by the phospholipase A_2 activity intrinsic to the LDL particle, and results in extensive breakdown of lecithin to lysolecithin. *In vitro*, OxLDL can be generated by incubation of LDL with phospholipase A_2 and lipoxygenase in the absence of cells, whereas addition of phospholipase A_2 inhibitors prevents the oxidative modification [37]. Decomposition of oxidized fatty acids then leads to the formation of highly reactive aldehydes and ketones, such as malondialdehyde and 4-hydroxynonenal. The chemical and biological properties of these lipid peroxidation products, the mechanisms of their formation and their metabolism have been excellently reviewed [39,40].

During the progression of the oxidative modification, these reactive aldehydes covalently bind to free amino groups of amino acids of the apoprotein, predominantly lysines [41]. The increase in aldehydes can be detected by an increase in fluorescence and thiobarbituric acid reactive substances (TBARS). The Schiff base mechanism that occurs when lysines react with aldehydes such as malondialdehyde or 4-hydroxynonenal is not the only pathway leading to protein modification. Lipid hydroperoxides may also directly interact with the apoprotein, without prior fragmentation to reactive aldehydes [42]. Progressive modification of the approximately 360 lysine residues of apo B-100 initially results in decreased recognition by the LDL receptor and, once a threshold number of lysines has been modified, in recognition by scavenger receptors [41]. It should be noted that the products of lipid peroxidation originating from LDL or other lipoprotein particles may not only react with apo B, but also with collagen and other matrix proteins.

Factors determining the susceptibility of lipoproteins to oxidation

In vivo, the extent to which LDL is oxidized is determined by a number of factors intrinsic to the LDL particle, as well as by extrinsic factors that determine the pro-oxidant stress [25]. The fatty acid composition of LDL, in particular its polyunsaturated fatty acids content, is a major determinant of its susceptibility to oxidation [43–47]. Polyunsaturated fatty acids of LDL are the primary substrate of lipid peroxidation in lipoproteins, and particles rich in monounsaturated fatty acids, (e.g. oleic acid, (18:1)) are therefore more resistant to oxidation than particles rich in polyunsaturated fatty acids (e.g. linoleic acid, (18:2) or arachidonic acid, (20:4)) [43–47]. A second major factor is the concentration of endogenous antioxidants, in particular vitamin E [48]. When the formation of conjugated dienes is used as the measure of lipid peroxidation, in an in-vitro system using copper as catalyst, the rapid propagation phase of oxidation occurs only after the endogenous antioxidant content of the LDL

particle has been depleted [48]. The length of the 'lag time' (the time between the addition of copper and the rapid rise in conjugated dienes), is proportional to the concentration of vitamin E, and can be prolonged several fold by enrichment of the particle with natural or synthetic antioxidants [48–51]. It is also well documented that the size of the LDL particle is of importance, as small dense LDL particles are more susceptible than larger, more buoyant ones, though the mechanisms responsible are not yet clear [52]. Finally, it is also conceivable that the phospholipase A_2 activity of the LDL particle may contribute to its susceptibility to oxidation [2]. Extrinsic factors include the ability of intimal cells to contribute to pro-oxidant conditions and the concentrations in the extracellular fluid of hydrophilic antioxidants such as vitamin C. (When vitamin E acts as an antioxidant, it is converted to a vitamin E radical. Vitamin C regenerates reduced vitamin E, which once again acts as an antioxidant.) Finally, high-density lipoprotein (HDL) is capable of inhibiting oxidation of LDL *in vitro* [53], and it is possible that it plays a similar role *in vivo*.

Potential atherogenic mechanisms of oxidized LDL

Extensive evidence suggests that many products formed during the oxidation of LDL contribute to atherogenesis by mechanisms distinct from the rapid uptake of OxLDL by macrophages [2,25,26]. Oxidized lipid and modified apoprotein fragments may trigger numerous events that may increase lesion formation. For example, OxLDL or its byproducts may contribute to the accumulation of monocytes/macrophages in the intima, modulate gene expression in arterial cells, affect vascular cells by their cytotoxic properties, provoke immune reactions, modulate the regulation of the vascular tone and exert a pro-coagulatory influence. In the following, we will try to organize these multiple effects to establish a potential sequence of events. However, the reader should keep in mind that the presumed contribution of these pathways is to a large extent based on results obtained *in vitro*, and that a single chain of events is most unlikely to exist, in particular in the advanced stages of lesions. The experimental determination of the contribution of individual mechanisms to lesion formation *in vivo* has been severely limited by the lack of appropriate techniques and animal models until recently.

Figure 1 summarizes hypothetical pathways which may lead to the oxidation of LDL in the intima, and the mechanisms by which OxLDL and its byproducts could contribute to lesion formation. Fatty streaks, the earliest stage of atherosclerotic lesions, are characterized by the accumulation of lipids and monocyte-derived macrophages in the intima. In cholesterol-fed rabbits, the focal accumulation of LDL precedes that of monocytes [8]. Some of the LDL penetrating the intima would be oxidized. Although all major cell lines found in the vascular wall are capable of oxidizing LDL, it is reasonable to assume that the initial oxidation of LDL present in the intima would be mediated by endothelial cells, rather than by macrophages or smooth muscle cells. Furthermore, some LDL particles entering the arterial wall may already be partially seeded with lipid hydroperoxides. Previously, we thought it unlikely that significant amounts of OxLDL would be present in the circulation. In view of the excellent antioxidant protection of plasma, it was presumed that the oxidation of LDL occured predominantly in the vascular wall, where pro-oxidant factors may predominate in microdomains adjacent to cells [2]. However, subtle modifications insufficient to cause rapid removal by scavenger receptors in the hepatic sinusoidal system may be present in some circulating LDL particles [54]. Furthermore, there is a constant flux of LDL into and from the aorta, and less than 15% of LDL particles entering the arterial wall are trapped or degraded there [8]. It is therefore conceivable that LDL particles that have been subjected to minimal oxidation during their passage through the vascular wall might return to the circulation. LDL may also be modified in other sites, and marked hypercholesterolemia may contribute to induce increased oxidative stress [55,56]. Once it has entered the vascular wall, such 'seeded' LDL would be much more susceptible to further cell-mediated oxidation, and would then more rapidly reach the degree of oxidation required for recognition by scavenger receptors.

OxLDL may also alter gene expression of neighboring cells. This may not require the same extent of modification that is necessary for scavenger receptor recognition. In-vitro, minimally modified LDL (i.e. oxidatively modified LDL that is not yet recognized by the scavenger receptor) induces endothelial cells to express adhesion molecules, chemotactic factors (e.g. monocyte chemoattractant protein-1 (MCP-1)) and colony-stimulating factors (e.g. macrophage colony-stimulating factor (MCSF)) [57]. The activation of inflammatory mediator genes and the activation of NF-kB-like transcription factors by lipid peroxidation products may indicate common pathways for many of the atherogenic consequences of moderately oxidized LDL [58].

Products of OxLDL such as lysophosphatidylcholine also exert a direct chemotactic effect on circulating monocytes, whereas they inhibit the motility of macrophages [59]. Thus, OxLDL could promote the intimal accumulation of macrophages by attracting monocytes via the mechanisms mentioned, as well as by trapping them in the lesion once they undergo phenotypic differentiation.

Other mechanisms by which OxLDL may be atherogenic include the cytotoxicity of oxidized lipid components [60], which could affect endothelial function or even result in endothelial denudation. Although endothelial coverage of earlier stages of atherosclerotic lesions is usually intact, damage to endothelial cells at later stages, or at shoulder regions of lesions overlaying foam cells could lead to procoagulant conditions. OxLDL may also directly influence the coagulation system by activating platelets or interfering with anticoagulant functions of the endothelium [61], or by a direct stimulation of tissue factor release [62]. Finally, OxLDL has been reported to inhibit vascular relaxation in response to endothelium-derived relaxing factor (EDRF) [63], and antioxidant therapy has been reported to protect against this. For a review of the regulation of the vascular tonus by EDRF (nitric oxide), see reference [64].

Activated macrophages could then increase lesion formation by inducing the expression or secretion of potentially atherogenic factors. Macrophages and foam cells are capable of releasing MCP-1 and MCSF which could lead to further recruitment of monocytes from the circulation or promote macrophage differentiation in the intima. Macrophages could enhance the oxidation of

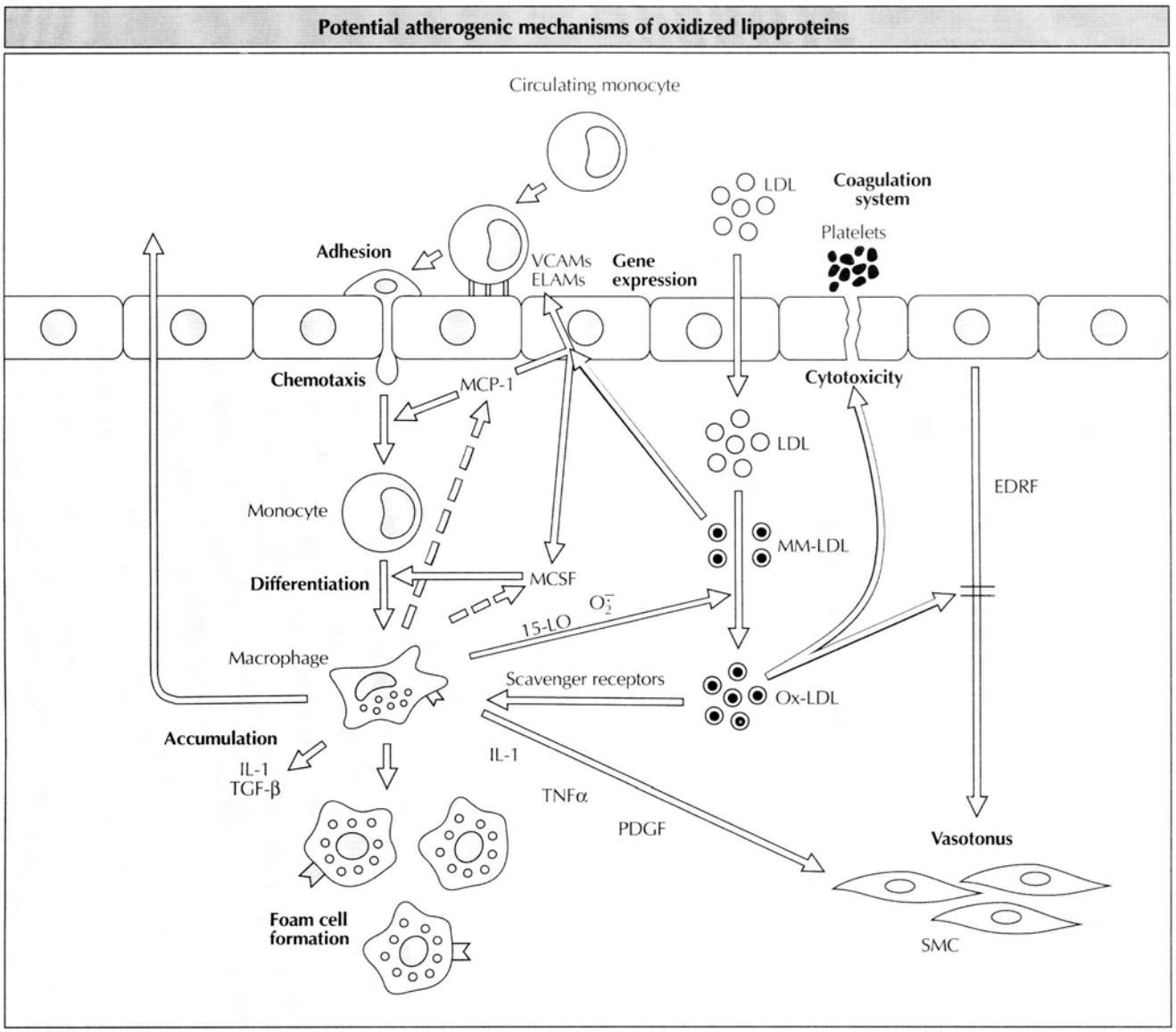

Fig. 1. Potential atherogenic mechanisms of oxidized lipoproteins. EDRF, endothelium-derived relaxing factor; ELAM, endothelial leukocyte adhesion molecule; IL-1, interleukin-1; LDL, low-density lipoprotein; 15-LO, 15-lipoxygenase; MCP-1, monocyte chemoattractant protein-1; MCSF, macrophage colony-stimulating factor; MM-LDL, minimally modified-LDL; OxLDL, oxidized low-density lipoprotein; PDGF, platelet-derived growth factor; SMC, smooth muscle cells; TGF-β, transforming growth factor-beta; TNFα, tumor necrosis factor-alpha; VCAM, vascular cell adhesion molecule.

LDL, either by an increased expression of 15-lipoxygenase (15-LO) activity, or by the release of superoxide and other oxidizing agents. Finally, macrophages may release a large number of other cytokines and growth factors which could promote smooth muscle proliferation and affect multiple regulatory mechanisms [3].

Evidence for the occurrence of oxidized LDL *in vivo*

In the past few years, substantial evidence has accumulated for the occurrence of oxidative modification of lipoproteins *in vivo*. The first line of evidence was provided by the immunocytochemical demonstration of several epitopes typical of OxLDL in atherosclerotic lesions of several species, including humans [18–21]. Oxidation of LDL is accompanied by the formation of a large spectrum of lipid peroxidation products that may react with lysine residues [39,40]. Thus, many different adducts can be postulated to occur in OxLDL, and the co-localization of several such adducts in tissue sections and arterial LDL extracts would be indicative of the occurrence of lipoprotein oxidation. Even minor modifications of LDL render it highly immunogenic [65]. This was used to generate antisera and monoclonal antibodies against

'model' epitopes of OxLDL by immunizing guinea-pigs and mice with homologous LDL, modified *in vitro* with aldehydes such as malondialdehyde and 4-hydroxynonenal [19,66]. The resulting antibodies were specific for malondialdehyde–lysine and 4-hydroxynonenal–lysine, respectively. In addition, monoclonal antibodies were generated against other, as yet unidentified, lipid–protein adducts [21,66]. Immunocytochemistry with a panel of these antibodies demonstrated that 'oxidation-specific' epitopes occur in atherosclerotic lesions ranging from early fatty streaks to very advanced fibrous plaques [18–20]. In early lesions and fibrous plaques staining was predominantly found in macrophage-rich areas. In addition to the prevalent cellular staining of macrophages and foam cells, transitional and advanced lesions displayed areas of diffuse extracellular staining in the intima. In some lesions an apparent inverse correlation was observed between the presence of oxidation-specific epitopes and native unmodified LDL [20]. The prevalence of oxidation-specific epitopes within, or in the immediate vicinity of, macrophages is consistent with the postulated rapid uptake of OxLDL via the scavenger receptor and the reduced intracellular metabolism of OxLDL. However, the staining would also be compatible with intracellular oxidation of LDL. Staining in the core of fibrous plaques was predominantly diffuse and extracellular, probably reflecting epitopes of OxLDL released from necrotic foam cells.

LDL and other lipoproteins provide the principal source of lipids in atherosclerotic lesions, and probably constitute the major source of lipid peroxidation products. However, adduct formation is not necessarily limited to the apoprotein and may involve other proteins as well. The immunostaining in lesions therefore may in part reflect modified lysine residues on vascular matrix proteins. The conclusion that the immunostaining represents, at least in part, OxLDL is derived from the demonstration of oxidation-specific epitopes in LDL gently extracted from atherosclerotic lesions. In contrast to LDL extracted from non-lesioned arteries or plasma LDL, such 'lesion-LDL' had the physical, chemical, and immunological properties of LDL oxidized *in vitro*, and was recognized by the scavenger receptor of macrophages [23].

Given the immunogenic potency of modified LDL, it is likely that OxLDL present in lesions may induce the formation of antibodies *in vivo*. The prevalence in several species of auto-antibodies that recognize epitopes of OxLDL therefore provides additional evidence for the occurrence of lipoprotein oxidation [19,22,67]. Such circulating autoantibodies are capable of binding to OxLDL in lesions [19,20, 22]. Furthermore, we have recently demonstrated that immunoglobulins isolated from atherosclerotic lesions of LDL receptor-deficient rabbits and humans recognize OxLDL, and that immune complexes of these autoantibodies with OxLDL are present in lesions [68]. The role of these antibodies, and that of the immune system in general, in atherogenesis will be discussed in greater detail below.

As indicated in Figure 1, OxLDL could cause or mediate a multitude of other atherogenic processes. Some of the postulated mediators of atherogenicity have indeed been demonstrated in lesions. For example, studies using *in situ* hybridization and immunocytochemistry have shown that gene expression for MCP-1, MCSF, 15-LO, interleukin (IL)-1 and the respective proteins occur in macrophage-rich areas of lesions, consistent with their hypothesized role in atherogenesis [69–72]. However, the role that each of these individual factors plays in atherogenesis remains to be established.

Antioxidant intervention studies

The oxidation hypothesis was considerably strengthened by the observation that antioxidants were capable of reducing atherogenesis in animal models. Probucol, a lipid-lowering drug with strong antioxidant properties, inhibits atherosclerosis in LDL receptor-deficient rabbits [73,74] by a mechanism independent of its hypolipidemic effect [73]. The results obtained with probucol were later confirmed in cholesterol-fed rabbits [75], and in non-human primates [76]. Similar data were also obtained with other potent antioxidant compounds which lack the plasma-lowering effect of probucol, such as butylated hydroxytoluene (BHT) [77], N,N'-diphenyl-phenylenediamine (DPPD) [78] and a probucol analogue [79]. Although other cellular effects of probucol have since been described, the chemical diversity of these antioxidants supports the view that their antiatherogenic potency is indeed due to their antioxidant effect.

The results with these synthetic antioxidants raise the question of whether natural lipophilic antioxidants may be equally effective in reducing lesion formation [80]. Some epidemiological data in humans indicate that large supplements of vitamin E may reduce the risk of coronary heart disease ([81–83], reviewed in [84]). However, a recent clinical intervention trial in heavy smokers showed no protection by very small doses of vitamin E [85]. Vitamin E also failed to reduce lesions in LDL-receptor-deficient rabbits [86]. The observation that a probucol analogue also did not inhibit atherosclerosis prompted the proposal of a 'threshold hypothesis' [51]. This hypothesis proposes that for any given degree of oxidant stress, an antioxidant must convey a threshold level of protection to lipoprotein particles in order to prevent them from oxidation during their passage through the intima. The prolongation of the lag time in the formation of conjugated dienes may provide a measure of the degree of protection. For example, in LDL-receptor-deficient rabbits, high doses of vitamin E prolonged the lag time of LDL from 200 min (in controls) to approximately 400 min, yet failed to reduce atherogenesis. In contrast, when tested under identical conditions probucol prolonged the lag time to more than 1000 min, and effectively reduced lesion formation.

Lipoprotein oxidation in new animal models of atherosclerosis

The advent of transgenic and gene-knockout techniques made it possible to generate murine strains overexpressing or lacking specific apoproteins, lipoprotein receptors, or enzymes involved in lipoprotein metabolism. Among these, apoE-deficient mice and LDL-receptor-negative mice may be of particular value, because the aortic predilection sites and the cellular composition of their lesions resemble those of other species [87–89]. Evidence for the pathogenic contribution of lipoprotein oxidation in apoE-deficient mice consists of the presence of oxidation-specific epitopes in their lesions [22], the presence of very high titers of

autoantibodies to epitopes of OxLDL in their circulation [22], and the fact that an antioxidant, DPPD, reduces lesion formation in this model [Tangirala R *et al.*, unpublished observations]. These murine models may be invaluable to study the *in vivo* relevance of factors involved in lipoprotein oxidation and the relevance of the hypothesized atherogenic mechanisms [90,91].

OxLDL and the immune system in atherogenesis

The involvement of immune mechanisms in atherogenesis is increasingly recognized [92]. Human atherosclerotic lesions contain immunoglobulins, complement and large numbers of immune-competent cells. These include not only macrophages, but also CD4+ and CD8+ T-lymphocytes [92]. Several observations indicate activation of the immune system [93–97]. Intimal T-lymphocytes and macrophages express IL-2 receptors. Major histocompatibility class II antigens are expressed on endothelial cells and on smooth muscle cells adjacent to T-lymphocytes, presumably as a result of γ-interferon secreted by activated T-cells. Furthermore, activation of the complement system is indicated by the presence of the terminal complement complex (C5b-9) in lesions.

OxLDL may contribute to these processes in several ways (Fig. 2). OxLDL is highly immunogenic [65], and autoantibodies recognizing epitopes of OxLDL are present in the circulation of several species [19,22,67]. In humans, higher titers of such autoantibodies correlated with increased atherosclerosis [98,99], suggesting that they may be an indicator of the extent of atherosclerosis. Furthermore, in murine models of atherosclerosis, the autoantibody titers correlated with the extent of aortic atherosclerosis, supporting the concept that oxidized lipoproteins in lesions may indeed be the immunogen inducing their formation [Palinski W, *et al.*, unpublished observations]. Antibodies against epitopes of OxLDL are also found in atherosclerotic lesions, in part as immune complexes with OxLDL [68]. Finally, OxLDL is chemotactic for T-lymphocytes [100] and may activate T-cells in the presence of monocytes [101].

Initially, we thought that the evocation of an immune response by OxLDL would increase atherogenesis by enhancing the uptake of immune complexes via Fc receptors or phagocytosis, and thus enhancing foam cell formation. However, we recently demonstrated that hyperimmunization of LDL receptor-deficient rabbits for six months with homologous malondialdehyde-modified LDL resulted in extremely high auto-antibody titers to an epitope of OxLDL and significantly reduced atherosclerosis [102]. Potential mechanisms responsible for this protective effect could include the removal of minimally oxidized LDL from the circulation, an increase in the number of immune competent cells in the intima or their increased activation. We may also have to reconsider the biological significance of the increased uptake of modified lipoproteins by macrophages. In view of the many pro-atherogenic properties of OxLDL and its byproducts, their rapid removal from the intima via scavenger receptors, Fc receptors or phagocytosis may be beneficial *a priori*, even though increased uptake results in the accumulation of cholesteryl esters in macrophages and eventually leads to necrosis of foam cells [103]. Evidence supporting a beneficial role of the immune system is also provided by the observation that the elimination of T-lymphocytes with monoclonal antibodies resulted in larger proliferative lesions in balloon-catheterized rat aortas [104]. Finally, several strains of immune-deficient mice have been reported to develop increased atherosclerosis, in particular class I MHC-deficient C57BL/6 mice, which lack cytolytic T-cells and have impaired natural killer cell activity [105].

Mechanisms increasing atherogenesis in diabetes mellitus

Although diabetes mellitus is generally accepted as a cardiovascular risk factor, the mechanisms responsible for increased atherogenesis have not been identified. Alterations of plasma lipids have been described in some populations, in particular in uncontrolled insulin-dependent diabetes, and increased triglyceride levels associated with a decrease in HDL cholesterol content are frequent in non-insulin-dependent diabetes mellitus [106]. These changes in lipoprotein levels alone cannot account for the increased risk of coronary artery disease. Nevertheless, changes in lipoprotein composition may reduce the binding of LDL to LDL receptors [107], and direct effects of insulin deficiency on the LDL receptor [108] may also prolong the half-life of LDL in the circulation. Changes in LDL metabolism could also result from impaired receptor recognition of glycated LDL. LDL from diabetic subjects is more extensively glycated than that of euglycemic subjects [109,110]. Turnover experiments with LDL glycated to various extents (3–6% of the lysine residues) indicated that even minor degrees of modification reduced the plasma clearance by 5–20% [111]. However, the lack of consistently elevated plasma LDL levels in diabetic subjects does not suggest that reduced plasma clearance of glycated LDL would be a significant contributor to atherogenesis in diabetics. The diverse mechanisms that may contribute to the increased atherogenesis in diabetic patients, particularly NEG, are examined in detail elsewhere in this book. In the following, we will only emphasize the analogies and possible interactions between oxidative modification and NEG.

Non-enzymatic glycation

NEG initially leads to the reversible formation of labile Schiff bases, followed by a chemical rearrangement to more stable Amadori products [27]. Regeneration of the protein amine and dehydration of the sugar may then result in the formation of highly reactive dicarbonyl compounds (e.g. deoxyglucosones) [112]. These intermediate products are either metabolically deactivated, or, in turn, propagate the reaction and lead to the irreversible formation of heterocyclic advanced glycation end products (AGE) [29]. To date, the chemical nature of many AGE remains controversial, and little is known about their occurrence *in vivo*. The common denominator — at least from a functional point of view — is probably their cross-linking property. By contrast, it is now apparent that many AGE do not fulfil the criteria of fluorescence or 'browning' [113].

Oxidative processes have long been implicated in the formation of AGE, and excellent reviews exist on this topic [113]. Initial support for the enhancement of NEG by oxidative processes was

provided by the observation that oxygen radicals and free metal ions increase AGE formation, and conversely, that reducing agents such as vitamin C inhibit NEG. More recently, three carbohydrate-derived compounds were identified as being formed by autoxidation (i.e. by reactive oxygen species). Two of these 'glycoxylation products', N^{ε}-carboxymethyl-lysine and N^{ε}-carboxymethyl-hydroxylysine, are generated by oxidation of Amadori products, a process that can be inhibited by antioxidants. The third, pentosidine, is a cross-link between lysine and arginine residues of proteins, and is inhibited by anaerobic conditions [113,114]. Collagen from diabetic subjects contains greater amounts of these glycoxylation products. Even though they occur only in minute amounts in AGE, these glycoxylation products may constitute models of proteins modified by oxidative processes independent of lipid peroxidation [113].

Interactions between oxidation and glycation

Hyperglycemia may enhance lipid peroxidation, in general. In fact, the lipoprotein fractions of diabetics contain increased lipid peroxide levels [115]. This increase may, in part, reflect processes subsequent to or unrelated to hyperglycemia, such as hypertriglyceridemia [116], increased content of small dense LDL particles more susceptible to oxidation, increased atherosclerosis or increased activation of the coagulation system. Nevertheless,

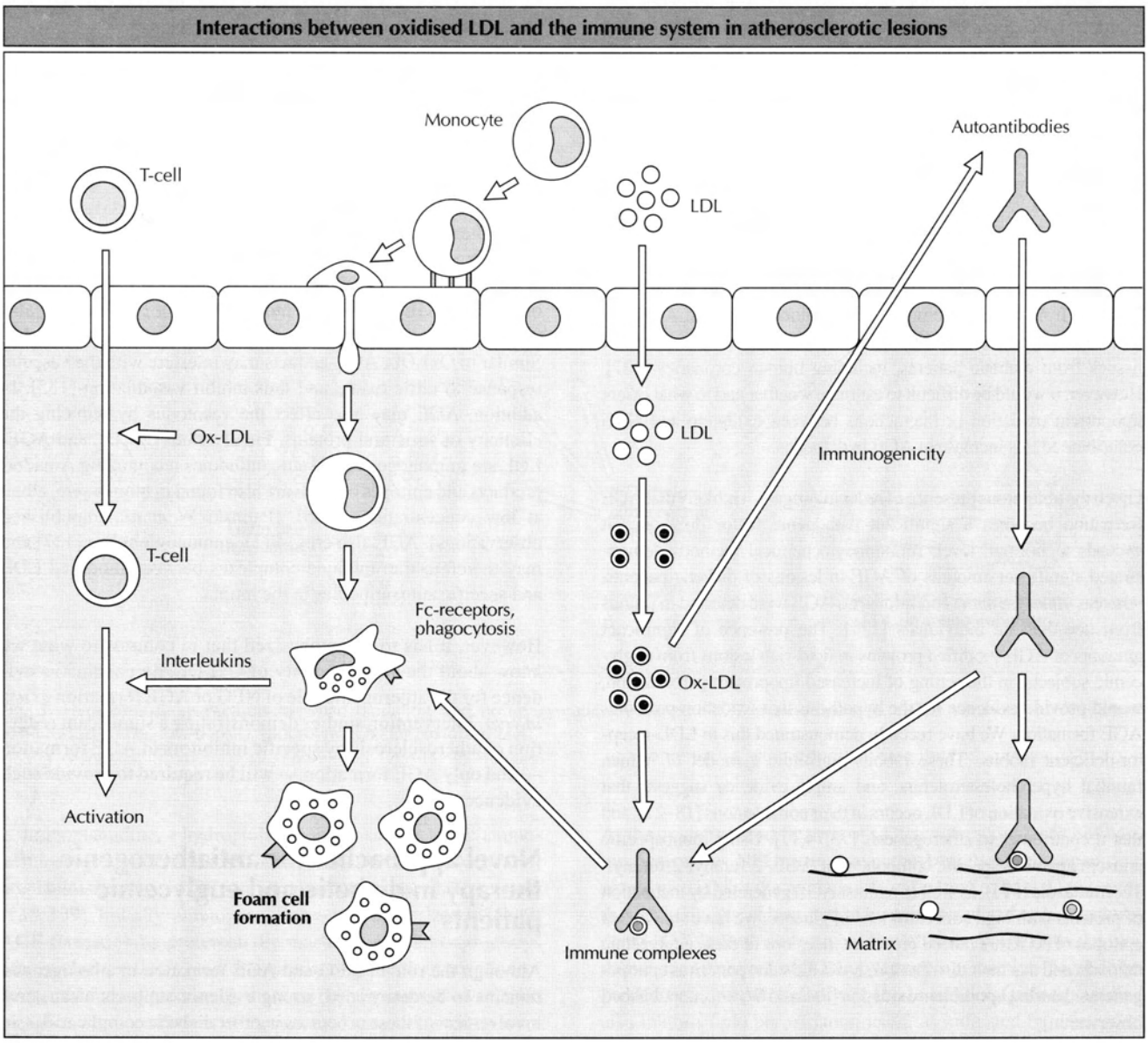

Fig. 2. Interactions between oxidized low-density lipoprotein (OxLDL) and the immune system in atherosclerotic lesions. OxLDL, oxidized low-density lipoprotein.

the demonstration of increased peroxidation of plasma LDL in diabetic rats and the prevention of this increase by antioxidants supports the assumption that NEG potentiates the oxidative modification of lipoproteins [117]. Furthermore, it was recently reported that LDL isolated from diabetic subjects was more susceptible to lipid peroxidation in general, and that plasma of diabetics has less antioxidant capacity [118].

Lipid peroxidation and NEG lead to similar modifications of lysine residues, and both processes may involve similar reactions and intermediate products. It is therefore conceivable that the two processes increase each other. Free radicals generated by autoxidation of glucose or early and intermediate glycation products may trigger or promote the peroxidation of lipids in lipoprotein particles, especially in hyperglycemic conditions [119–124]. Conversely, increased lipoprotein oxidation may promote cross-linking and AGE formation. For example, NEG could be increased by lipid hydroperoxides or other lipid peroxidation products [125,126]. In-vitro experiments suggest that the extent of the pro-oxidative effects is not correlated with the extent of protein glycation [124]. The determination of the extent of such an interaction *in vivo* is rendered difficult by the limitations of the methods available to detect oxygen radicals, lipid peroxidation products, and products of AGE.

If this mutual enhancement also occurs *in vivo*, increased lipoprotein oxidation in atherosclerotic lesions could further increase the formation of AGE in the arteries of diabetics, in addition to the increase resulting from hyperglycemia and NEG alone. Increased AGE formation has been detected by immunocytochemistry in a variety of tissues from diabetic patients, including human coronaries [127]. However, it would be difficult to estimate whether and to what extent lipoprotein oxidation or interactions between oxidation and NEG contribute to this increase in AGE in diabetics.

Given the ubiquitous presence of reducing sugars, it is likely that AGE formation becomes a significant pathogenic factor only once it exceeds a 'normal' level. Immunocytochemical methods demonstrated significant amounts of AGE in lesions of diabetic patients, whereas under the same conditions, no AGE were detected in lesions from non-diabetic individuals [127]. The presence of significant amounts of AGE-modified proteins in lipid-rich lesions from euglycemic subjects, in the setting of increased lipoprotein peroxidation, would provide evidence for the hypothesis that oxidation promotes AGE formation. We have recently demonstrated this in LDL-receptor-deficient rabbits. These rabbits constitute a model of human familial hypercholesterolemia, and ample evidence suggests that extensive oxidation of LDL occurs in their aortic lesions [18–21], and that it contributes to atherogenesis [73,74,77]. Using monospecific antisera against a specific component of AGE, 2-furanyl-2-furanyl-1H-imidazole (FFI), as well as against AGE generated by incubation of proteins with high concentrations of glucose, we have shown that epitopes of AGE are indeed prevalent in lesions of these euglycemic animals, and that their distribution shows the same pattern as epitopes generated during lipoprotein oxidation [Palinski W *et al.*, unpublished observation].

A reciprocal increase in oxidation and glycation is also conceivable in inflammatory processes in other lipid-rich environments. For example, oxidation of lipid accumulations in the retina of diabetic subjects might also be induced by pro-oxidant factors secreted by activated leukocytes [128].

Common atherogenic mechanisms

The above described potential interactions at the biochemical level are not the only analogies between oxidation and glycation. The mechanisms by which both processes could contribute to atherogenesis also show many similarities (Fig. 3). In addition to the hypothesized direct promotion of lipoprotein oxidation, NEG could increase the oxidation of lipoproteins by cross-linking them to structural proteins of the vascular matrix [129]. This may increase their intimal residence time [130], and expose them to increased oxidation by the factors previously discussed. Once formed OxLDL, AGE-proteins, and possibly AGE-OxLDL may increase lesion formation by common mechanisms.

First among these mechanisms would be the rapid uptake by macrophages. Macrophages possess not only several receptors that recognize OxLDL [131] but also receptors that recognize AGE-proteins [30–34]. Uptake of AGE-modified lipoproteins via these receptors could therefore contribute to the intracellular accumulation of cholesterol. Both OxLDL and AGE could enhance the accumulation of macrophages in the intima, either by promoting monocyte binding to specific receptors on endothelial cells [34], or by direct chemotactic effects [59,132,133]. Binding of AGE to AGE-specific receptors or binding proteins may also induce endothelial cells to generate a pro-oxidant state [134]. Similar to OxLDL, AGE-adducts may interfere with the vascular response to nitric oxide, and thus inhibit vasodilation [135]. In addition, AGE may also affect the vasotonus by reducing the elasticity of structural proteins. Finally, both OxLDL and AGE-LDL are immunogenic, and autoantibodies recognizing Amadori products and epitopes of AGE are also found in human sera, albeit at low concentrations [136], [Palinski W *et al.*, unpublished observations]. AGE also cross-links immunoglobulins [137] and may therefore trap immune complexes between modified LDL and specific autoantibodies in the intima.

However, it has to be emphasized that in contrast to what we know about the atherogenicity of OxLDL, no conclusive evidence for the atherogenic role of NEG or AGE formation exists *in vivo*. Intervention studies demonstrating a significant reduction of atherosclerosis by specific inhibitors of AGE formation — and only AGE formation — will be required to provide such evidence.

Novel approaches for antiatherogenic therapy in diabetic and euglycemic patients

Although the role of NEG and AGE formation in atherogenesis remains to be determined, strong evidence supports a causative involvement of these processes in other diabetic complications, in particular the microvascular pathology of the kidney and the retina. Several compounds are now available that effectively reduce the formation of AGE. The best known of these compounds

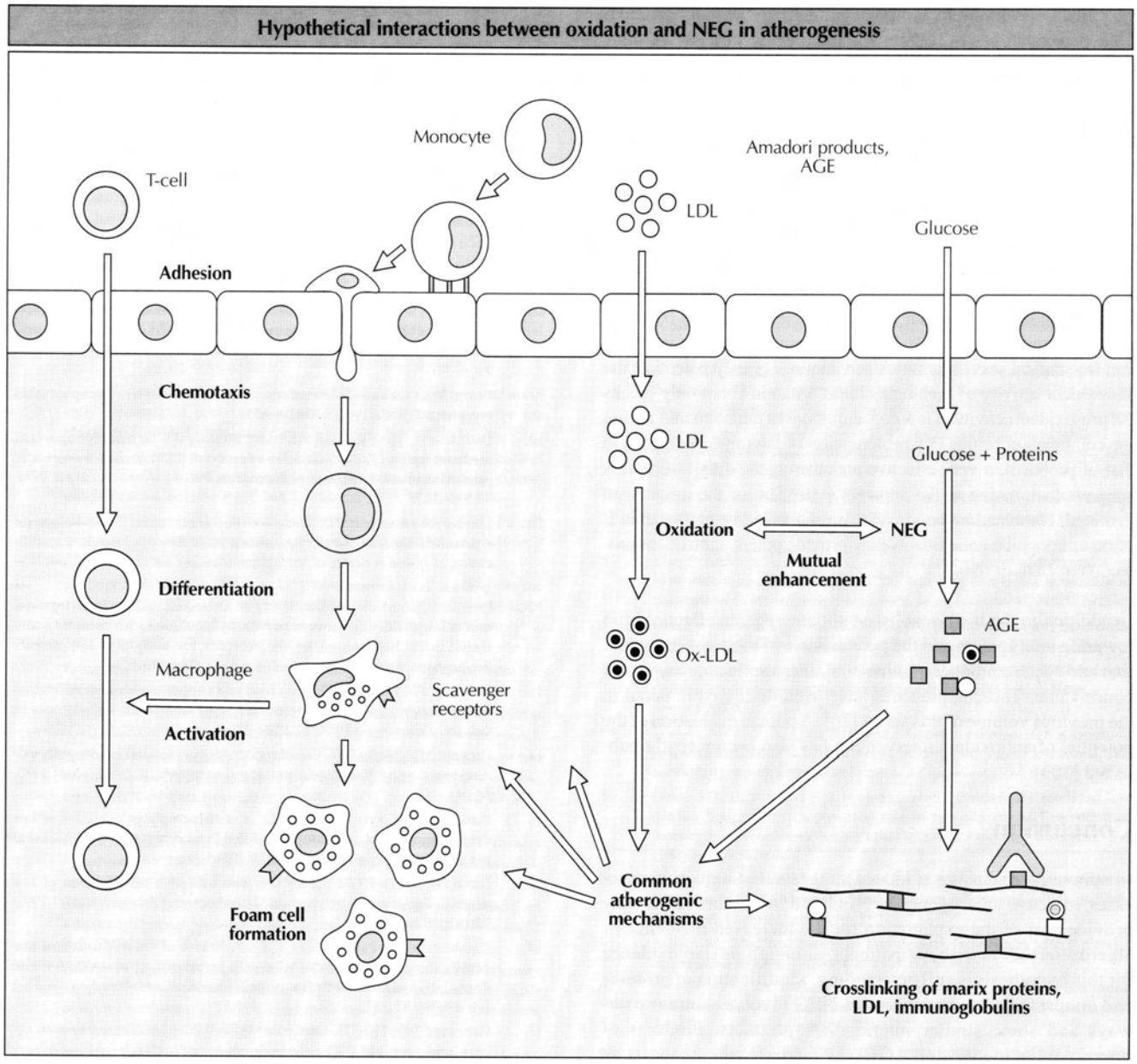

Fig. 3. Hypothetical interactions between oxidation and non-enzymatic glycation (NEG) in atherogenesis. AGE, advanced glycosylation end-products; LDL, low-density lipoprotein; OxLDL, oxidized LDL.

is aminoguanidine, a hydrophilic structure similar to the amino-terminal group of arginine. The mechanism by which aminoguanidine reduces AGE formation remains somewhat uncertain [138, 139]. Initially, aminoguanidine was hypothesized to inhibit AGE formation by preferentially reacting with carbonyl groups of Amadori products, and thus preventing the derivatization of lysine residues of proteins by these carbonyl groups. However, no significant concentrations of the assumed reaction products were detected *in vitro*. Alternate mechanisms include scavenging of the reducing sugar, and reactions with intermediate products generated during AGE formation, such as 3-deoxyglucosone [140].

As the oxidative modification of LDL involves similar Schiff base mechanisms as NEG, it was hypothesized that aminoguanidine would also inhibit the oxidative modification of LDL [141], even though aminoguanidine cannot be considered a classical antioxidant [113]. Indeed, aminoguanidine effectively prevents the oxidative modification of LDL by trapping reactive breakdown products of lipid peroxidation [141]. In addition, aminoguanidine also inhibits lipid peroxidation itself, as indicated by the loss of polyunsaturated fatty acids and the delay in the formation of conjugated dienes in LDL exposed to copper [141,142]. However, aminoguanidine has a bimodal effect on lipid peroxidation, and at

very low concentrations aminoguanidine may actually promote lipid peroxidation [142]. The antiatherogenic efficacy of aminoguanidine is currently under investigation in animal models.

If the 'threshold hypothesis' previously discussed also applies to hydrophilic antioxidants such as aminoguanidine, the protective effect of aminoguanidine on atherosclerosis would be doubtful, as the prolongation of lag times is comparable to that obtained with natural lipophilic antioxidants, such as vitamin E, which were ineffective in reducing lesion formation. In addition, the extent of protection conveyed by a hydrophilic compound on LDL particles in the arterial wall remains to be determined. The fact that we nevertheless propose to test aminoguanidine as an antiathero- sclerotic drug is based on another exciting observation. Recent experiments *in vitro* indicated that free radical scavengers such as vitamin E greatly potentiate the antioxidant activity of aminoguanidine. Vitamin E not only blocks the pro-oxidant activity of low concentrations of aminoguanidine, but the combination results in a prolongation of lag times analogous to that of probucol, a very effective antiatherogenic drug [142]. This suggests that, owing to the apparent synergism of the mechanism involved, combination therapy with aminoguanidine and vitamin E or other lipophilic antioxidants may provide potent antioxidant protection of LDL *in vivo*.

In addition to the well established antiatherogenic effect of lipid lowering intervention and the potential use of inhibitors of oxidation and NEG, a number of other anti-atherogenic approaches are conceivable. These approaches have been discussed in detail in the previous volume of this series [26]. A critical evaluation of the potential of antioxidant intervention has also been recently published [103].

Conclusion

In summary, extensive evidence suggests that oxidative processes increase atherogenesis. NEG and AGE formation may provide part of the explanation for the increased incidence of atherosclerosis in diabetic patients, although *in vivo* evidence for this hypothesis is still outstanding. Modification of proteins and apoproteins by oxidation and NEG involves similar pathways and some similar intermediate products. Products of lipoprotein oxidation and AGE may increase atherogenesis by common mechanisms, and a reciprocal increase in the two processes is likely. These analogies may indicate novel approaches for antiatherogenic intervention in both diabetic and euglycemic patients.

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