Lipoprotein Oxidation and Lipoprotein-Induced Cell Injury in Diabetes

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There is ample evidence that oxidized lipoproteins exist in vivo, not only in atherosclerotic lesions, but also associated with some experimental models of diabetes. Whether the lipoprotein oxidation is an epiphenomenon of other atherogenic or diabetogenic agents or processes or whether it is causally related to lesion formation in atherosclerosis or other forms of tissue damage in people with diabetes is unresolved. Intense interest in testing these ideas derives from in vitro observations of the ways in which oxidized lipoproteins interact with cells that are unlike the interactions with native lipoproteins. Many of these altered interactions suggest known features of atherosclerotic lesions, and recent data show that antioxidant treatment reduces the progression of vascular lesions. There are reasons to believe that hyperglycemia may worsen lipid and lipoprotein oxidation. If this observation is the case in vivo, and if it is ultimately proved that lipoprotein oxidation facilitates lesion development, these events may help explain the accelerated atherosclerosis suffered by diabetic patients. The multiple pathways for which there is evidence that hyperglycemia may contribute to oxidative events—for example, by enhancing free radical production in stimulated inflammatory cells or by forming glycation products that can propagate free radical events—suggest avenues for further research and may ultimately indicate points for intervention in the various manifestations of the disease. *Diabetes* 41 (Suppl. 2):61–66, 1992

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LDL, low-density lipoprotein; VLDL, very-low-density lipoprotein; MCP-1, monocyte chemotactic protein-1; TBA, thiobarbituric acid; F_c, crystallizable immunoglobulin fragment; STZ, streptozocin; HDL, high-density lipoprotein.

In recent years, the study of atherosclerosis has been influenced by the hypothesis that lipoproteins can become modified in vivo by free radical–mediated oxidation and may initiate atherosclerotic lesion development or worsen its course. In part, this notion emanated from observations that human LDLs could be cytotoxic to vascular endothelial cells and vascular smooth muscle cells grown in culture (1), and that this cytotoxicity followed the formation of potent cell-injuring agents during free radical–mediated oxidation of the lipoprotein (2,3). Related ideas developed in parallel with studies by Henriksen et al. (4–6) showing that LDL could be modified by cultured endothelial cells in such a way that the lipoprotein became a ligand for macrophage scavenger receptors, thus suggesting a mechanism for the formation of macrophage-derived foam cells, an early cellular component of atherosclerotic lesions. This modification by vascular endothelial cells was later shown to be caused by free radicals produced by the endothelial cells (7,8), and LDL oxidation was thus readily demonstrable both in cell culture and in cell-free systems that supported lipid peroxidation. Collectively, these investigators proposed that oxidized forms of LDL could injure vascular cells, perhaps accounting for early endothelial injury that putatively accompanies atherosclerosis and the appearance of dead cell debris in later lesions, and that oxidized LDL could be the in vivo counterpart of chemically modified forms of LDL (for example, malondialdehyde-treated LDL and acetylated LDL), previously shown to be taken up in vitro by macrophages in unregulated fashion.

OXIDIZED LIPOPROTEINS AND ATHEROSCLEROSIS

Since 1984, additional tenets of a hypothesis for atherosclerosis involving oxidation-mediated lipoprotein alterations have fluorished as experimental support for

numerous cell function changes mediated by oxidized lipoproteins has been obtained in vitro. The possible cellular interactions of oxidized LDL now go far beyond the early observations above (9–13).

Oxidized LDL alters the gene expression for and secretion of several growth factors and cytokines by macrophages and endothelial cells grown in culture. Treatment of macrophages with oxidized LDL inhibits the gene expression of tumor-necrosis factor–α, interleukin 1α, and interleukin 1β that otherwise follows stimulation (14,15). After incubation with oxidized LDL, endothelial cell production of colony-stimulating factors is enhanced (16), yet platelet-derived growth factor activity from both endothelial cells and macrophages is reduced (17,18). Oxidized LDL is a chemoattractant to monocytes (19,20) and, at moderate levels of oxidation, induces the expression on endothelial cell surfaces of a specific monocyte-binding protein (21). The oxidatively modified lipoprotein also stimulates production of monocyte chemotactic protein–1 by endothelial cells (22). Oxidized LDL also interferes with endothelial cell–induced relaxation of vascular tissue (23–26).

These actions are only a sampling of the reported influences of oxidized lipoproteins on cultured vascular cells; however, the extent to which any of these effects have pathophysiological counterparts in vivo is unknown and will depend, of course, on the focal presence of oxidized LDL in vivo and the local concentration. At least as important, however, is the similarity of the in vivo form of the modified lipoprotein to the forms resulting from experimental LDL oxidation in vitro, which includes the use of metal ions, cultured cells, and ultraviolet irradiation as mediators of the free radical oxidation. There is ample evidence supporting the existence in vivo of oxidized forms of VLDL and LDL. Antibodies that recognize oxidized LDL, but not native LDL, including those that recognize certain lipid peroxidation products linked to polypeptides, were used to demonstrate these epitopes in the vascular lesions of experimental animals and humans (27–31). Lipoprotein fractions extracted from lesions have properties similar to those of oxidized LDL (32,33) and react with these antibodies (33). Antibodies recognizing oxidized but not native LDL were reported circulating in human plasma (33), and a subfraction of human LDL was isolated that has properties similar to those of LDL after oxidation (34). Finally, lipoprotein fractions with elevated levels of thiobarbituric acid reactivity, an indirect indication of lipid peroxidation, were demonstrated in diabetic animals and humans (35–38). Collectively, the above evidence suggests strongly that oxidized lipoproteins occur in vivo.

Various hypotheses can be proposed to explain the initiation and further development of vascular lesions based on lipoprotein oxidation, and what follows is an example of such a hypothesis. The first step could be elevated levels of VLDL or LDL in plasma, which are known to enhance the levels of these lipoproteins in the interstitial space of the arterial intima (39). The increased concentration and resulting increase in lipoprotein residence time (40) increases the probability of opportunistic oxidation by the adjacent endothelial or smooth muscle cells (7,8,41). This moderately oxidized LDL may injure proliferating endothelium (1,42), increasing the permeability to large molecules (43–45). The oxidized LDL may also promote monocyte recruitment to the lesion site by causing endothelial cells to produce MCP-1 and express monocyte-binding molecules on their luminal surface (21,22). Phagocytic action of the invading monocyte-derived macrophages could worsen lipoprotein oxidation in the interstitum by promoting free radical mechanisms (46–49). Once oxidized further, the lipoprotein could recruit more monocytes due to its inherent chemoattractant activity (19,20). The altered lipoprotein can then be taken up by these resident macrophages: by scavenger receptors that recognize highly oxidized LDL (50–52), by nonspecific phagocytosis after oxidation-induced aggregation of the lipoprotein (53), or by F_c-receptor–mediated uptake of complexes of oxidized LDL and antibodies that recognize it (54). Oxidized LDL may also recruit smooth muscle cells from the media to the intima (55) and injure the proliferating cells (1,42), leading to the accumulation of dead cell debris.

Although evidence supporting the existence of oxidized LDL in vivo is strong, the evidence that lipoprotein oxidation may be involved in lesion development is only indirect. It consists of data indicating that certain antioxidants impede the progression of arterial lesions. Probucol reduced fatty streak lesion development in the Watanabe heritable hyperlipemic rabbit (56,57) and the cholesterol-fed rabbit (58), separate from its lipid-lowering effects (57,58). Butylated hydroxytoluene inhibited atherosclerosis in cholesterol-fed rabbits, despite elevated lipids in the drug-treated animals (59). Three years of vitamin E supplementation to an atherogenic diet led to diminished arterial lesions in primates with diet-induced atherosclerosis compared with untreated control animals (60). β-Carotene supplements to humans reduced cardiovascular disease events significantly (61). However, the above studies need to be interpreted cautiously because each of these antioxidants has numerous cellular actions.

LIPID AND LIPOPROTEIN OXIDATION IN DIABETES

Diabetes is a strong risk factor for atherosclerosis. Therefore, it is logical to consider ways in which diabetes may complicate or accelerate lipoprotein oxidation (62,63). There is evidence that diabetes is accompanied by enhanced lipid peroxidation or lipoprotein oxidation and that hyperglycemia and accelerated oxidation may be related.

The possible role for oxidized lipids in the pathogenesis of diabetes is supported by the findings that the oxidation of LDL in vitro is enhanced in the presence of glucose. Hunt et al. (64) demonstrated that human LDL incubated with high glucose and cupric ion yielded higher levels of TBA reactivity and lipid peroxide formation than LDL exposed to copper without glucose. Sakurai et al. (65) found similar results with glycated LDL and iron. They also showed that antioxidants such as α-tocopheral and probucol were able to suppress the iron-mediated production of TBA reactivity in the glycated

LDL (65). Cutler (66) demonstrated increased iron storage in people with poorly controlled diabetes, whereas Howard et al. (67) showed increased urinary excretion of iron in people with diabetic nephropathy. This difference invites the speculation that iron-dependent oxidative reactions may be one mechanism for enhanced oxidation of lipids in the plasma and tissue of diabetic subjects. Kawamura et al. (68) found increased conjugated dienes, lipid peroxides, and TBA reactivity in LDL incubated with glucose compared with the control case; these effects were inhibited by superoxide dismutase.

Although LDL incubated with glucose was shown to increase lipid peroxide production, there are other factors related to diabetes that could further increase lipid peroxidation, such as hypertriglyceridemia. Hiramatsu and Arimori (69) demonstrated significant increases in stimulated superoxide production in mononuclear cells obtained from diabetic patients with hypertriglyceridemia and nondiabetic hypertriglyceridemic patients compared with control groups. This production significantly correlated with plasma triglyceride levels.

The observation that increased concentrations of glucose result in an increased likelihood of oxidation of LDL leads one to conclude that there should be an increased likelihood of finding oxidized lipids circulating in the plasma and deposited in the tissues in diabetic animal models. Lipid oxidation products were reported in the plasma, in particular in the lipoprotein fractions, of rats made diabetic with injections of streptozocin. The levels of TBA reactivity can be reduced by treatment with the antioxidants vitamin E or probucol (35,36,38). For example, using the STZ rat model, we found increased TBA reactivity in the VLDL + LDL fractions of plasma for 2 mo after STZ injection (35). Treatment with vitamin E, probucol, or insulin resulted in decreased TBA reactivity. The two antioxidants were administered after the hyperglycemia had developed, and neither caused a decrease in plasma glucose. Somova et al. (70) also measured lipid peroxide levels in the lipoprotein fractions of rats 2.5 mo after treatment with STZ. They also found a significant increase in lipid peroxide levels in the STZ rats compared with controls. Furthermore, they found that the measured lipid peroxide levels correlated with triglyceride content of LDL. Jain et al. (71) quantified TBA reactivity in diabetic erythrocyte membranes 2 and 4 mo after STZ injection. They found increases in the TBA reactivity of membranes from STZ-treated animals that did not receive insulin compared with nondiabetic controls, whereas measurements made from erythrocytes obtained from STZ-treated animals that received insulin were not significantly different from the controls. Yeh and Ashton (72) found increased TBA reactivity in the lenses from rats 2 wk after STZ treatment; however, the increases were not found in diabetic animals treated with insulin or sorbinil.

Findings of increased levels of oxidized lipids circulating in the plasma as well as deposited in the tissue of diabetic humans were also reported. Plasma lipid peroxides were reported in human subjects with diabetes (37,73), and were found to be particularly elevated in patients with poorly controlled diabetes and with angiopathy (73). Mooradian (74) quantified conjugated dienes in the serum of 45 male diabetic patients >60 yr of age (22 with complications, 23 without complications) and 24 healthy control subjects and found increased conjugated dienes in the diabetic subjects with complications compared with the healthy control subjects. They found the conjugated dienes to be significantly correlated with plasma triglyceride levels. No increase above control levels was noted in the diabetic patients without complications. Similarly, Jennings et al. (75) found that in diabetic people with microangiopathy, serum diene conjugates were double the levels found in people with uncomplicated diabetes and control subjects. Jain et al. (76) found an increase in TBA reactivity in the membranes of erythrocytes from diabetic subjects compared with nondiabetic control subjects. This increase correlated with the levels of glycosylated hemoglobin found in the erythrocytes. Simonelli et al. (77) compared the TBA reactivity measured in the lenses from 15 diabetic patients with cataracts with that in nondiabetic patients with cataracts and clear-lens patients and found a significant increase in tissue from the diabetic patients.

Both the animal and human studies outlined above are consistent with increased oxidized lipids and lipoproteins in the plasma and tissues of certain categories of diabetic subjects. The in vitro studies discussed above suggested that these increased levels may occur because lipids are more readily oxidized in the presence of increased glucose concentrations. This hypothesis is supported by the continued observations, in both animal models and humans, that in subjects with well-controlled diabetes (for example, in animals that receive insulin), lower levels of circulating lipid peroxides are found. The association between elevated lipid peroxidation and diabetes is likely to be a complex one, and not all instances of diabetes result in elevated oxidation. For example, Parinandi et al. (78) found lower TBA reactivity and elevated glutathione levels in the hearts and kidneys of rats with alloxan-induced diabetes.

OXIDIZED LIPOPROTEINS AND CELL INJURY

The reported incidence of increased lipid peroxidation with diabetes invited the speculation that certain pathological manifestations of diabetes may be initiated or worsened by the participation in cell and tissue damage of the elevated levels of free radicals or lipid peroxides. As indicated earlier, lipoproteins oxidized in vitro are potent toxins to cultured cells. Because diabetic lipoproteins appear to be oxidized in certain experimental models and human patient populations, it is reasonable to examine whether diabetic lipoproteins carry cell-injuring moieties. The lipoproteins of the streptozocin diabetic rat appear to be cytotoxic. Arbogast et al. (79) showed that the VLDL fraction from these animals, unlike that from control rats, injured cultures of endothelial cells. In a later study, we were able to correlate the in vitro toxicity of a VLDL + LDL fraction from STZ rats with the level of lipid peroxidation products (35). That this correlation implied cause and effect was supported by the decrease in oxidation levels and in toxicity of the lipoprotein fraction

after treatment of the animals with lipophilic antioxidants. The HDL fractions in these animals were not significantly oxidized, nor were they cytotoxic. The in vivo oxidized lipoproteins appeared analogous to the toxic human LDL oxidized in vitro by various free radical–mediated mechanisms.

We and others characterized the way in which oxidized LDL injures cells with the hope of ultimately identifying the mechanism of cell injury. The toxic activity appears to be nonspecific; a variety of cells are vulnerable to oxidized LDL–mediated cell injury (1,3). LDL-receptor recognition is not required for cells to be vulnerable to the toxicity (80,81), although there is evidence that if LDL is oxidized only moderately, such that it is still recognized by the LDL receptor, the toxic effect is enhanced in cells expressing this receptor (82). If LDL is oxidized extensively, such that it is no longer recognized by the LDL receptor, but becomes a ligand for the scavenger receptor (4,52), its toxicity to cells expressing scavenger receptors (52) may also be enhanced by receptor uptake, although this hypothesis has not been rigorously tested.

The toxic activity of oxidized LDL resides in the lipid phase; organic solvent extracts of oxidized, but not native, LDL will kill fibroblasts grown in culture (2). The characteristics of the principal toxic activity are consistent with those of an oxysterol (unpublished observations), but numerous toxic lipid oxidation products produced on oxidation of LDL (for example, 4-hydroxynonenal) are also cytotoxic. The mechanism by which oxidized LDL kills cells can be examined by studying the inhibition of its injurious effects. Cell death is inhibited by forcing target cells to arrest their progression through the cell cycle (1,42). Fibroblasts in the DNA-synthesis phase of the cell cycle appear most vulnerable (42). In addition, native HDL reduces the toxic effect (1,2,83).

With LDL oxidized in vitro with metal ions, but from which the bound metal has been removed after oxidation, we demonstrated that certain antioxidants (vitamin E, probucol) inhibit the toxic effects (unpublished observations). This result suggests that cellular oxidation reactions facilitate cellular injury. Analogous inhibition was observed for cellular injury caused by LDL oxidized by ultraviolet irradiation (84) and by the potent toxic form of oxidized LDL containing bound metal ions (85–87). The pattern of antioxidants that inhibit LDL-induced cell injury suggests that lipid hydroperoxides may be responsible for the injury through a mechanism analogous to that suggested for tertiary butylhydroperoxide injury to hepatocytes (88). Finally, although lipoproteins from diabetic rats appear to be oxidized (35,36) and toxic to cells in culture (35,79), their injurious actions in vivo have yet to be demonstrated.

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