Oxygen Radicals and Atherosclerosis

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Summary. There is increasing evidence that lipids, especially those in low density lipoprotein, may be oxidised during the development of atherosclerotic lesions. The lipid-laden "foam cells" of atherosclerosis are macrophages, which are known to produce oxygen radicals in their microbicidal role. The same process could result in oxidation of lipid or lipoprotein in atherosclerosis. In human atherosclerotic lesions, many of the macrophage foam cells also contain ceroid, an insoluble polymer formed by oxidation of mixtures of lipid and protein. Using in vitro systems, we have studied the possibility that macrophages may be responsible for the oxidation of lipid and/or lipoprotein. Experiments are described in which mouse peritoneal macrophages and human monocyte-derived macrophages have been shown to oxidise cholesteryl linoleate, added to the cultures in the form of an artificial lipoprotein, with the production of soluble oxidised lipids, including oxidised sterols, and, in the case of mouse peritoneal macrophages, abundant ceroid. The oxidation was inhibited by radical scavengers. Oxidised sterols are cytotoxic. It is thus conceivable that oxidised sterols produced by monocyte-macrophages may lead to necrosis and progression of the lesion. Possibilities for prevention of this oxidation are discussed.

Abbreviations: AX/ORO = alcohol-xylene/Oil red O; BHA = butylated hydroxyanisole; BHT = butylated hydroxytoluene; BSA = bovine serum albumin; C/BSA = cholesterol-bovine serum albumin artifical lipoprotein; CA/BSA = cholesteryl arachidonate-bovine serum albumin artifical lipoprotein; CD = cholest-5-en-3β,7β-diol; CL = cholesteryl linoleate; CL/BSA = cholesteryl linoleate-bovine serum albumin artifical lipoprotein; CO = cholesteryl oleate; CO/BSA = cholesteryl oleate-bovine serum albumin artifical lipoprotein; DMEM = Dulbecco's modification of Eagle's medium; EDTA = ethylenediamine-tetraacetic acid; FACS = Fluorescence-activated cell sorter; GC = gas chromatography; GC-MS = gas chromatography-mass spectrometry; HMGCoA = 3-hydroxy-3-methyl-glutaryl-coenzyme A; HMM = human monocyte-macrophages; HETEs = hydroxyeicosatetraenoic acids; HODEs = hydroxyoctadecadienoic acids; LDL = low density lipoprotein; LPD-FCS = lipoprotein-deficient foetal calf serum; LPD-HS = lipoprotein-deficient human AB serum; mRNA = messenger ribonucleic acid; MPM = mouse peritoneal macrophages; ORO = oil red O; USP = United States Pharmacopeia; WHHL = Watanabe heritable hyperlipidemic

Macrophages are well-known for their ability to produce oxygen radicals and other reactive oxygen species in response to a variety of stimuli (Fantone and Ward 1982; Nakagawara et al. 1981), including phagocytosis. However, the possible connection of this phenomenon with atherosclerosis did not come to the fore until, in the mid-1980's, evidence was obtained, by immunohistochemistry, that the lipid-laden "foam cells" of human atherosclerotic lesions were macrophages (Aqel et al. 1984, 1985; Klurfeld 1985; Jonasson et al. 1986; Gown et al. 1986). Prior to this, most researchers believed that foam cells were smooth muscle cells. Earlier, Mitchinson (1983) had hypothesised that foam cells were macrophages and that their microbicidal oxidative mechanism was responsible for the formation of ceroid in atherosclerotic lesions.

Ceroid is a polymer, insoluble in lipid solvents, which is believed to be formed from oxidation of mixtures of lipid and protein (Pearse 1972). Ceroid is found within foam cells, in early lesions, and both intracellular and extracellular in more advanced lesions (Mitchinson et al. 1985). Indeed, the observation of ceroid in atherosclerotic lesions had led Porta and Hartroft (1969) to the suggestion that peroxidation of polyunsaturated lipid might be involved in atherosclerosis, well in advance of the evidence for foam cells being macrophages.

Returning to the last decade, evidence for the involvement of oxidative processes in atherosclerosis was growing from another angle – that of modification of low density lipoprotein (LDL). It was shown by Goldstein and Brown (Goldstein et al. 1979; Brown and Goldstein 1983) that macrophages could take up large amounts of LDL only when the LDL had been chemically modified, e.g. by acetylation. Other workers found that several

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cell types, including macrophages, could modify LDL in a way which in many respects resembled LDL artificially oxidised by copper (II) salts (Cathcart et al. 1985; Parthasarathy et al. 1986; Hiramatsu et al. 1987). Both macrophage-modified and Cu^{2+}-oxidised LDL were avidly taken up by macrophages. Modified LDL can be taken up via the macrophages' so-called "scavenger receptors" (Brown and Goldstein 1983), but aggregation of LDL or of modified LDL can also lead to uptake by phagocytosis (Khoo et al. 1988; Hoff et al. 1989).

There are several other pieces of evidence that oxidative processes are involved in the progression of atherosclerosis. Oxidised derivatives of cholesterol and of polyunsaturated fatty acids have been found in human atherosclerotic plaques (Brooks et al. 1971). Cholesteryl linoleate is depleted in foam-cell-rich lesions, in comparison to plasma LDL (Smith et al. 1967). Similarly, polyunsaturated fatty acids such as linoleic and arachidonic acids are depleted in the ceroid-rich insoluble fraction of human atherosclerotic plaques (Ball et al. 1987a) and in Cu^{2+}-oxidised LDL (Ball et al. 1986; Esterbauer et al. 1987). In addition, modified LDL, which is probably oxidised LDL, has been identified, using monoclonal antibodies, in human atherosclerotic lesions (Yla-Herttuala et al. 1989). Auto-antibodies to ceroid are found in patients with advanced atherosclerosis, and cross-react with artificially-oxidised LDL (Parums et al. 1990).

We therefore developed model systems for studying the possibility that macrophages might be responsible for the oxidation of lipid and/or lipoprotein in atherosclerosis. As LDL itself contains a variable mixture of lipids, natural antioxidants and other substances, we adopted the approach of utilising artificial lipoproteins containing a single, defined lipid. We chose cholesteryl linoleate (CL) as the test lipid for the majority of our experiments as it is commonly the major single lipid species in human LDL. We emulsified CL with bovine serum albumin (BSA) to make an artificial lipoprotein with diameter of ca. 0.5–1.0 μm (Werb and Cohn 1972). These particles are avidly taken up by mouse peritoneal macrophages (MPM) in vitro to result in foam cells packed with lipid. This CL/BSA partially competes with acetyl-LDL for binding and uptake by MPM in vitro (Goldstein et al. 1981). CL/BSA may have a naturally-occurring counterpart in the large cholesterol ester-rich, albumin-rich particles isolated from human atherosclerotic plaques by Hoff and Clevidence (1987), but it may also serve as a crude model of CL in LDL.

Methods

(1) Artificial Lipoproteins

Artificial lipoproteins were made as described by Ball et al. (1987b) to give an artificial lipoprotein with a nominal mole ratio of CL:BSA of 60:1. In some experiments, radical scavengers were incorporated into the artificial lipoprotein to give, typically, a ratio of CL:BSA:scavenger of 60:1:2.7 (Ball et al. 1987b). Probucol was used at half the molar concentration of the other radical scavengers since it contains two phenolic groups per molecule instead of one. This represents a final concentration of scavenger in the culture medium of approximately 20 μM (in the case of probucol, 10 μM).

(2) Experiments Using Cultures of Mouse Peritoneal Macrophages

Resident MPM were obtained from male Balb/c mice, and cultured on glass coverslips in DMEM plus 10% lipoprotein-deficient foetal calf serum (LPD-FCS) plus antibiotics, as described by Ball et al. (1987b), or in RPMI-1640 plus 10% LPD-FCS plus antibiotics (Carpenter et al. 1990a). Artificial lipoprotein was added to the medium, and the medium plus artificial lipoprotein renewed daily. At the end of the incubation period (typically 1–3 d) the cells were fixed in formal saline and stained with Oil red O (ORO) to reveal neutral lipid, and with ORO after ethanol-xylene treatment (AX/ORO) to reveal ceroid. Ceroid was assessed visually by counting the percentage of AX/ORO-positive cells. Ultrastructural studies were also carried out (Ball et al. 1988). In a later development, ceroid was quantitated by means of its autofluorescence, using a Fluorescence-activated Cell Sorter (FACS) (Carpenter et al. 1990a). In addition, analyses of soluble lipids and soluble oxidised lipids were also carried out on MPM cultured similarly (Carpenter et al. 1988), using the analytical method described below.

(3) Experiments Using Cultures of Human Monocyte-Macrophages

Human monocyte-macrophages (HMM) were prepared from defibrinated venous blood, from healthy adult volunteers. The cells were then cultured, in glass dishes, in RPMI-1640 plus 20% lipoprotein-deficient human AB serum (LPD-HS) or in DMEM plus 20% LPD-HS. Typically 10 × 10^6 cells were used in each dish. CL/BSA was added to the cultures and incubated for 24 h (Carpenter et al. 1990b).

Analyses of soluble lipids (including soluble oxidised lipids) in the extracellular medium (and, in some cases, in the cells) were carried out as follows (Carpenter et al. 1990b). The samples were extracted with chloroform-methanol. The extract was treated with sodium borohydride to reduce any peroxides and hydroperoxides to the more stable alcohols (this procedure also reduces aldehydes and ketones to alcohols), and then saponified with potassium hydroxide. The resulting fatty acids and sterols were converted to methyl esters and trimethylsilyl ethers prior to analysis by capillary gas chromatography (GC) and capillary gas chromatography-mass spectrometry (GC-MS).

Results

(1) Mouse Peritoneal Macrophages

(a) Lipid Uptake and Ceroid Accumulation. MPM cultured for 1 d and 3 d with CL/BSA avidly took up the artificial lipoprotein, apparently by phagocytosis, to give cells packed with lipid droplets and ceroid, as shown by ORO and AX/ORO staining respectively. If cholesteryl oleate/BSA (CO/BSA) or cholesterol/BSA (C/BSA) was used instead of CL/BSA, no ceroid accumulated, though the cells still contained many lipid droplets (Ball et al. 1987b).

The ceroid formed in MPM varied according to the lipid species. In cells cultured with CL/BSA it was in the form of rings. When cholesteryl arachidonate/BSA (CA/BSA) was used instead, the ceroid was in small granules (Ball et al. 1987b). These different forms of ceroid are both seen in human atherosclerosis, by both light and electron microscopy (Mitchinson et al. 1985; Ball et al. 1988).

The formation of ceroid rings from CL/BSA could be almost completely inhibited by the phenolic radical scavenging antioxidants butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA) and probucol (Ball et al. 1987b; Carpenter et al. 1990a). The experiments were repeated using FACS measurement of ceroid, giving similar results (Carpenter et al. 1990a).

Thus in this system the production of ceroid from polyunsaturated lipid is inhibited by radical scavengers. Vitamin E (in the form of dl-alpha-tocopherol) also inhibited ceroid formation, when added to the cultures as CL/BSA/vitamin E, but its effect at comparable concentrations (20 μM) was visibly weaker.

(b) Soluble Oxidised Lipids. The oxidation products of CL, hydroxyoctadecadienoic acids (HODEs), cholest-5-en-3β,7β-diol (CD) and cholest-5-en-3β,7α-diol, were identified by GC and GC-MS both in the cells and the extracellular medium of cultures of MPM with CL/BSA (Carpenter et al. 1988 and unpublished results). Production of the cholest-5-en-3β,7-diols was inhibited by BHT and by probucol (unpublished results). The above oxidised lipids were not seen when CO/BSA was used in place of CL/BSA. When CA/BSA was used, hydroxyeicosatetraenoic acids (HETEs) were identified in addition to CD (Carpenter et al. 1988).

(2) Human Monocyte-Macrophages

(a) Lipid Uptake and Ceroid Accumulation. Lipid uptake by HMM incubated 24 h from day 0 to day 1 with CL/BSA (N.B. the day of isolation and plating of cells is designated day 0) was lower than in MPM, but considerably more lipid was taken up by more mature HMM (e.g. 7 d in culture, followed by 24 h exposure to CL/BSA), to result in a foam-cell-like appearance (Carpenter et al. 1990b), though the cells were seldom quite as bloated as MPM cultured with CL/BSA.

In contrast to MPM, comparatively little ceroid appeared to accumulate in HMM incubated with CL/BSA, even when they had been allowed to mature in culture for 4–7 d before addition of CL/BSA. The ceroid, visualised by AX/ORO staining, was in the form of a diffuse cytoplasmic blush and fine granules. It seemed that more ceroid accumulated in HMM from older volunteers, though data from more subjects in this age group are needed before this can be stated with certainty (Carpenter et al. 1990b).

(b) Soluble Oxidised Lipids. The following results on the production of soluble oxidised lipids by HMM are summarised from Carpenter and colleagues (1990b).

In spite of the low lipid content of the HMM incubated 24 h from day 0 to day 1 with CL/BSA, CD was detected, at around 10% of the level of cholesterol, in the cells. Amounts of CD were much greater in the extracellular medium, and in all but a few cases were significantly higher than in controls in which CL/BSA was incubated in the absence of cells, and all the following results on HMM refer to the extracellular medium. Considerable variations were observed in CD production from CL/BSA by HMM from different blood donations. Other lipid oxidation products so far identified in the extracellular medium include cholest-5-en-3β,7α-diol and several isomeric HODEs. Cholest-5-en-3β,7α-diol was produced at levels of ca.

one-third or less relative to the amount of CD, and was greater in the presence of cells than in no-cell controls. The total amount of HODEs were variable relative to controls.

CD production from CL/BSA by HMM was almost completely inhibited by the radical scavengers BHT, probucol and alpha-tocopherol, and was partially inhibited by the metal chelator EDTA (200 μM).

The production of CD from CL/BSA was accompanied by a decrease in linoleic acid. CD was negligible in incubations of CO/BSA or C/BSA with HMM. Ability to produce CD from CL/BSA appeared to increase with age in culture of HMM, when CL/BSA was incubated with them for 24 h. Cultures up to 7 days old showed a progressive increase in CD production, whilst control incubations of CL/BSA without cells showed no such increase.

Discussion

The above results show that macrophages in vitro are able to oxidise polyunsaturated cholesteryl esters to produce ceroid, oxidised fatty acids, and oxidised sterols.

The mechanisms of the oxidations are not yet known, but free radicals appear to be involved since radical scavengers inhibit both processes. The respiratory burst production of reactive oxygen species, triggered by phagocytosis of the artificial lipoprotein particles is one obvious possibility. The partial inhibition by EDTA of CD formation suggests that metal ions might play a role. EDTA can inhibit iron-stimulated lipid peroxidation in vitro (Gutteridge et al. 1979). In many other systems the important oxidising agent is thought to be the hydroxyl radical or some other highly reactive oxidising species generated by the reaction of superoxide and/or hydrogen peroxide with transition metal, such as ferryl radicals (Halliwell and Gutteridge 1989), and it may be that such a process is at least partly responsible for the oxidations observed in our systems.

The possible involvement of the enzyme lipoxygenase in these oxidative processes must also be considered, as monocytes and macrophages are known to possess lipoxygenase activity (Pawlowski et al. 1983; Rabinovitch et al. 1981). Indeed, cholest-5-en-3β-ol-7-hydroperoxides are reported to be produced in vitro (in the absence of cells) by co-oxidation of a mixture of ethyl linoleate and cholesterol by soybean lipoxygenase (Teng and Smith 1973). Evidence for lipoxygenase involvement in the atherosclerosis of Watanabe Heritable

Hyperlipidemic (WHHL) rabbits (the animal model of familial hypercholesterolemia), comes from the studies of Yla-Herttuala and colleagues (1990). They found colocalization of 15-lipoxygenase mRNA with protein possessing the immunostaining properties of oxidised LDL, in macrophage-rich atherosclerotic lesions in these rabbits. Lipoxygenase has also been implicated in monocyte oxidation of LDL in vitro (McNally et al. 1990), in addition to the apparent involvement of superoxide in the process (Hiramatsu et al. 1987; Cathcart et al. 1989).

A possible mechanism for formation of cholest-5-en-3β,7-diols from CL/BSA in our systems can be envisaged in which oxidation is initiated by abstraction (e.g. by hydroxyl radical or lipoxygenase) of a hydrogen atom from the bisallylic methylene group of linoleate, leading to formation of peroxides, which then proceed to oxidise the cholesterol moiety as part of a radical chain reaction (Carpenter et al. 1990b). This would explain why CD is not produced when CO/BSA is used instead of CL/BSA, as oleate has no bisallylic methylene group, and thus is much less susceptible to peroxidation.

A number of findings suggest these oxidative processes may be important in atherogenesis. Oxidised sterols have been found in human atherosclerotic lesions, together with HODEs, in a polar sterol esters fraction of the lipid extract (Brooks et al. 1971). Auto-oxidation impurities in USP cholesterol were more angiotoxic than cholesterol itself, when given orally to rabbits (Imai et al. 1976), and also several individual cholesterol oxidation products, injected intravenously, showed similar effects, again in rabbits (Imai et al. 1980). In vitro, oxidised sterols were cytotoxic (Taylor et al. 1979; Baranowski et al. 1982). Oxidised sterols inhibited cholesterol biosynthesis by inhibiting HMGCoA reductase, demonstrated in cell cultures (Kandutsch et al. 1973). They also increased cholesterol esterification (Brown et al. 1975), and stimulated both arachidonic acid release and prostaglandin biosynthesis (Lahoua et al. 1988), in cells in vitro. Thus it is conceivable that oxidised sterols produced from polyunsaturated cholesterol esters by monocyte-derived macrophages within the atherosclerotic lesion may have a number of damaging effects, in addition to those of the peroxides, aldehydes, etc. resulting from the peroxidation of the polyunsaturated fatty acid chains, which could lead to necrosis and progression of the lesion. Oxidised LDL is cytotoxic (Cathcart et al. 1985) and positively chemotactic for monocytes (Quinn et al. 1987).

The formation of ceroid itself may also contribute to the development of the lesion. Skins of insoluble ceroid surrounding droplets of soluble lipid could hinder the dispersion of the lipid, and thus prevent regression of the lesion (Ball et al. 1987a).

There is thus considerable evidence to support the theory that lipid and lipoprotein oxidation are occurring in atherosclerosis. This begs the question of whether the progression of atherosclerosis can be slowed by means of inhibiting this oxidation. Already some studies using radical scavengers suggest that this may be the case. Probucol dramatically slowed the progression of atherosclerosis in WHHL rabbits, apparently independently of its mild hypocholesterolemic effect (Kita et al. 1987; Carew et al. 1987). Similarly, BHT was anti-atherogenic in cholesterol-fed rabbits, even though it exerted a hypercholesterolemic effect (Björkhem et al. 1991). An epidemiological study of European middle-aged men showed that plasma vitamin E levels were inversely correlated with population mortality from ischaemic heart disease (Gey et al. 1991). A clinical study of the effect of probucol on femoral artery atherosclerosis is currently in progress (Walldius et al. 1988).

Probucol, BHT and vitamin E are all general radical scavengers. They are all strongly lipophilic, and are believed to act by breaking the chain of radical reactions involved in the propagation of lipid peroxidation. This is likely to be the way in which these radical scavengers inhibit oxidation in our experiments. Their apparent efficacy against atherosclerosis tells us little about the agents which initiate lipid/lipoprotein oxidation. Answering this question might lead to development of more specific ways of controlling atherosclerosis. Both superoxide and lipoxygenase appeared to be involved in oxidation of LDL by activated monocytes (McNally et al. 1990). More studies are needed in our own systems to shed light on the mechanism(s) of lipid oxidation and ceroid formation, which may in turn suggest ways of attempting to combat atherosclerosis in vivo.

The Western diet, high in lipid and low in natural antioxidants, may be responsible for the epidemic of atherosclerosis in urbanised populations. Dietary supplements of, for example, vitamin E may provide protection. Vitamin E oral supplements have recently been shown to protect LDL against in vitro oxidation in some human subjects (Esterbauer et al. 1990). For LDL from normal humans having no oral supplements, resistance to in vitro oxidation appears to be determined by the combined effects of tocopherols with other natural antioxidants (e.g. carotenoids), in relation to the fatty acid composition (Esterbauer et al. 1991).

It is also possible that Vitamin E (and other naturally-occurring antioxidants) may lower the macrophages' oxidative capacity, and thus exert an additional protective effect against atherosclerosis. The apparent variations in oxidative capacity (as judged by CD production from CL/BSA in vitro) shown by monocyte-macrophages obtained from different individuals on different occasions (Carpenter et al. 1990b) might be partly due to variations in the antioxidant status of the cells. Oral vitamin E supplements are reported to modify the respiratory burst of neutrophils, giving slightly elevated superoxide, impaired release of hydrogen peroxide, enhanced phagocytosis, and impaired microbicidal activity (Baehner et al. 1977). Similar studies need to be done on macrophages, to help assess the advisability of using Vitamin E supplements to protect against atherosclerosis.

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