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Neutrophil-oxidized low density lipoprotein: generation in and clearance from the plasma

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Summary. The prevailing concept of an extremely rapid disappearance of 'modified' low density lipoprotein (LDL) from the circulation was reinvestigated. Rabbit LDL was 'modified' by homologous activated (phagocytosing) polymorphonuclear leucocytes (PMLN), radiolabelled with a non-degradable ligand (^{125}I-TC-LDL) and injected into rabbits. The plasma half-lives of 'modified' and native LDL were $T_{1/2} = 2.5$ and 5.75 h, respectively. Furthermore, the possibility of LDL oxidation in plasma by stimulated PMNL was investigated. Hirudin-anticoagulated human plasma was incubated with unstimulated or stimulated autologous PMNL. Chemiluminometry (reactants with microperoxidase) of the lipid extract of plasma after incubation showed lipid peroxidation to be induced by phagocytosing, but not by quiescent, leucocytes.

These findings show that in plasma, stimulated leucocytes can 'modify' LDL and the circulatory half-life of the latter enables its contribution to atherogenesis.

Keywords: lipid peroxides, low density lipoprotein, neutrophils, atherosclerosis

There is now substantial evidence that oxidized or 'modified' low density lipoprotein (LDL) plays an important role in atherogenesis (Steinberg *et al.* 1989; Palinski *et al.* 1989; Rosenfeld *et al.* 1990; Wissler 1991; Witztum & Steinberg 1991). Exposure of LDL to different cell types sets in motion a series of events beginning with peroxidation of the lipid components. Alteration may progress by conformational change of the tertiary structure, oxidation and degradation of (apo)lipoproteins. The resultant 'modified' LDL is highly atherogenic.

A strong view prevails that oxidized LDL does not form or exist in the circulation (Steinberg *et al.* 1989; Palinski *et al.* 1989; Rosenfeld *et al.* 1990). Rather, both generation and contribution to atherosclerosis by oxidized LDL occur within the arterial wall. This view is based on the extremely rapid clearance of oxidized LDL from the circulation (Nagelkerke *et al.* 1984), and the assumption that the powerful oxidant defence system of plasma safeguards against any oxidative process.

However, three lines of evidence suggest that lipids *can* be oxidized in the circulation, and their concentration *can* reach measurable plasma levels. Firstly, elevated plasma lipid peroxides were measured in patients with varying disorders, including severe atherosclerosis (Yagi 1982; Uysal *et al.* 1986; Szamosi *et al.* 1987; Stringer *et al.* 1989; Davies *et al.* 1990). Secondly, hyperlipidaemic sera from cholesterol-fed animals induced lipid accumulation in various cul-

tured cells and organs (St Clair *et al.* 1983; Laughton *et al.* 1988). Thirdly, unlike blood serum from healthy subjects, sera and isolated LDL of patients with coronary heart disease possessed high atherogenic potential in tissue cultures: enhanced cholesterol accumulation, proliferation and synthesis of matrix components (Orekhov *et al.* 1989, 1990).

As the plasma clearance rate of 'modified' LDL is a key issue in the above dispute, we set out to reinvestigate these phenomena. Relevant to physiology, LDL was 'modified' by exposure to stimulated leucocytes and the rate of disappearance from the circulation of this 'modified' LDL was measured. Furthermore, generation of lipid hydroperoxides by incubating plasma with quiescent or stimulated leucocytes was measured.

Materials and methods

Isolation and modification of LDL

LDL (density 1.019–1.063 g/ml) was prepared from freshly drawn rabbit plasma containing disodium EDTA (1.5 mg/ml) by differential ultracentrifugation. The lipoprotein was dialysed against 0.15 M NaCl for 24 h before modification and labelling.

Polymorphonuclear leucocytes (PMNL) were separated from heparin-anticoagulated human or rabbit blood by centrifugation over Mono-Poly Resolving Medium (ICN-Flow, UK). Cells were washed and resuspended in Hanks' balanced salt solution. Modification of LDL by PMNL phagocytosing beads (3 μm diameter, Polysciences, UK) was identical with our earlier studies using human monocytes (Görög & Kakkar 1987; Görög 1990).

Lipoprotein iodination

The original technique of preparing radioiodinated adduct of cellobiose and tyramine (tyramine cellobiose; TC) and the covalent attachment of the adduct to LDL was employed (Pittman *et al.* 1983). Specific activity and free iodine content of the preparation were 190 c.p.m. per ng LDL and 1.3%, respectively. Lipid peroxide content (nmol/mg LDL) of native (n-LDL) and ox-LDL was 0.8 and 7.7, measured as TBARS (Görög 1990), 3.2 and 47, respectively, as measured by iodometry (El-Saadani *et al.* 1989). Relative electrophoretic mobility of ox-LDL (native LDL = 1) was 1.22.

Clearance of ox-LDL from the circulation

Adult male New Zealand White rabbits, weighing between 3 and 3.5 kg, were used. Serial blood samples (60 μl) were taken from a pierced marginal ear vein and analysed for ^{125}I-TC-LDL radioactivity. After mixing the blood samples with the anticoagulant (4 μg r-hirudin), 10 μl blood was withdrawn into a microcapillary tube (whole blood sample). The rest of the blood was centrifuged at 12 000 r.p.m. for 2 min and then 10 μl clear plasma was drawn into another microcapillary tube (plasma sample). The radiolabelled LDL was injected through the marginal vein of the opposite ear. Blood sampling was started initially at 5 min after injection of LDL (0 min) and then at varying intervals thereafter. Radioactivities measured at the nominal 0 min (5 min after LDL) were >80% of the activities calculated for the real zero time, by dividing the injected radioactivities with the estimated total blood volume of 45 ml/kg.

Extraction of lipid peroxides from plasma

Plasma was prepared from hirudin (20 μg/ml) anticoagulated human blood. Plasma samples (3.5 ml) containing PMNL (3×10^7 cells/ml) were incubated at 37°C for 1 h. At the end of incubation with PMNL (with or without stimuli), cells (and beads) were sedimented by rapid centrifugation. An aliquot of plasma (200 μl) was used for the measurement of lipid peroxides by iodometry (El-Saadani *et al.* 1989). The bulk of plasma (3.0 ml) was mixed with ice-cold methanol

(3 ml) following the addition of 30 ml cold hexane. After shaking vigorously and centrifuging at 2000 r.p.m. for 10 min, 20 ml hexane layer was collected and dried under vacuum in a freeze-drier. The extract was dissolved by whirlmixing firstly in 0.2 ml methanol/*t*-butanol (50/50 by volume) and then adding 0.8 ml distilled water. An aliquot (200 μl) was used for the chemiluminometric measurement of lipid peroxides.

Chemiluminometry

The principle of the measurement is that hydroperoxides react with the enzyme microperoxidase in the presence of a light-amplifier (luminol), and the generated light is detected by a sensitive device (Olsson 1982; Yamamoto *et al.* 1987).

The reaction mixture (1.0 ml) containing 100 μM luminol (Sigma), 3 μM microperoxidase (Sigma), 10 μM Cu^{2+} as catalyst in 0.1 M phosphate buffer, pH 10 was placed into a cuvette and stirred with a rotating magnetic bar. After obtaining a steady background emission, 0.5 ml of lipid extract was injected into the cell through a port. Light emission was monitored for 2 min and the peak intensity was detected with a purpose built chemiluminometer. The latter consisted of an EMI photomultiplier, mounted in a refrigerated chamber (Products for Research Inc., Danvers, Mass.), an amplifier-counter (Panax-Reigate, UK) and a servorecorder (Watanabe) to monitor the peak of the deflection. This assay measures all of the hydroperoxides in the lipid extract but neither unoxidized lipids nor prostaglandin endoperoxides are detected (Yamamoto *et al.* 1987). The assay system was calibrated using hydrogen peroxide. In a concentration range between 2×10^{-9} M (detection limit) and 10^{-7} M, a linear relationship was observed between the amount of hydrogen peroxide added and the peak chemiluminescence response.

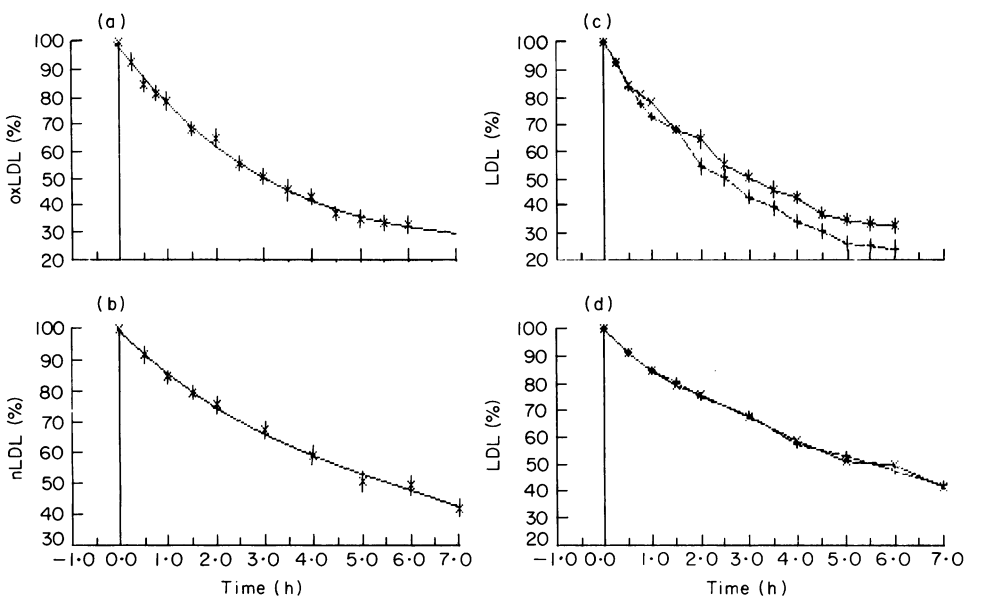

Fig. 1. Clearance of oxidized and native LDL from the circulation of rabbit ($n = 5$). a, Clearance of ox-LDL from whole blood (best-fit curve, vertical bars: SE). b, Clearance of n-LDL from whole blood. c, Clearance of ox-LDL. —✳—, Whole blood *vs* – — + plasma samples). d, Clearance of n-LDL: —✳—, Whole blood *vs* – — + plasma samples).

Table 1. Peroxidation of plasma lipids by polymorphonuclear leucocytes *in vitro**

Leucocytes	Lipid hydroperoxide (nmole/ml/1h)	
	Iodometry	Chemiluminometry
None	9 (8; 6; 13)†	1.5 (2.2; 1.6; 0.8)
Unstimulated	12 (17; 11; 9)	2.3 (3.7; 1.0; 2.1)
Phagocytosing	263 (340; 196; 254)	123.0 (125; 67; 176)

* Human plasma and autologous PMNL. For the experimental conditions, see Methods.

† Means of three parallel experiments with different plasma samples and PMNL (the individual measurements are in parentheses).

Results

The rates of disappearance of autologous native and ox-LDL from the circulation of rabbits are shown in Fig. 1. In the case of ox-LDL, the decay curves are clearly biphasic: rapid disappearance of about 60% of the injected lipoprotein is followed by clearance at a much slower rate. In the case of native LDL, the decay from plasma and whole blood were identical. In contrast, there was a small but significant ($P<0.01$) difference between clearance of ox-LDL from plasma and from whole blood. Results of the measurements of lipid peroxidation, when human plasma samples were incubated either with unstimulated or phagocytosing PMNL, are shown in Table 1. One-hour incubation of plasma with stimulated PMNL resulted in significant lipid peroxidation, as assessed both by iodometric assay performed directly from plasma or by chemiluminometry performed from the lipid extract. Quiescent PMNL did not induce significant peroxidation of plasma lipids.

Discussion

Our findings conflict with the extremely rapid decay of endothelial cell-modified (44h incubation) LDL in rats which has been reported elsewhere (complete disappearance from the circulation was 10 min) (Nagelkerke *et al.* 1984). This very great difference in the plasma half-life is difficult to explain, but the 'modification' of LDL in the two studies may offer an explanation. From the number of ways LDL can be 'modified', it is the modification induced by reactive oxygen species (superoxide, hydrogen peroxide) which is most likely to be of physiological relevance (Cathcart *et al.* 1985; Hiramatsu *et al.* 1987). Involvement of 5-lipoxygenase in the oxidation of LDL by macrophages has recently been questioned (Jessup *et al.* 1991). The reactive oxygen species produced by phagocytosing leucocytes or activated platelets (Aviram *et al.* 1990) play crucial roles in the modification of LDL. Endothelial cell-modified LDL differs from the preparations we studied in at least two respects. Firstly, superoxide is not involved, as resting endothelial cells do not generate superoxide (Rosen & Freeman 1984; Görög *et al.* 1988), and the 'modification' is not influenced by superoxide dismutase (Van Hinsbergh *et al.* 1986). Secondly, 'modification' of LDL by endothelial cells is also unaffected by lipoxygenase inhibitors (Van Hinsbergh *et al.* 1986). Also, the electrophoretic mobility of endothelial cell-modified LDL is considerably increased (Zhang *et al.* 1990), while monocyte (Görög 1990), or PMNL-modified LDL (present study) show very little increase in electrophoretic mobility.

Apart from the reasons for discrepancy, the plasma half-life of LDL, modified in a physiologically relevant manner, is long enough not to be dismissed. At biological half-lives of hours, it is the production rather than the disappearance of altered LDL, which is the determinant of the actual plasma level. Even at very low levels, this can play an important role in atherogenesis. A concentration of minimally modified LDL as low as 0.12 μg/ml (0.015% of the normal plasma LDL concentration) was shown to have significant biological effects (Berliner *et al.* 1990).

The clearance rate of native LDL from plasma was identical with that measured from whole blood. The short but significantly longer half-lives of ox-LDL in whole blood than in plasma suggests that blood cells, presumably leucocytes, take up and retain some ox-LDL in the circulation.

The effective antioxidant defence in plasma is another pillar of the argument which questions a prolonged existence of lipid peroxides in the circulation. The major source of oxidants in blood is activated PMNL capable of inducing peroxidation of lipids *in vitro* (Frei *et al.* 1988) and possibly *in vivo* (Ricevuti *et al.* 1989). However, of the various antioxidants in plasma, it is only the ascorbic acid which effectively prevents activated PMNL from forming lipid peroxides. Once plasma ascorbate has oxidized, peroxidation of lipid transported in lipoproteins becomes detectable (Frei *et al.* 1988). Despite the differences in the experimental conditions (hirudin-anticoagulated plasma, phagocytosing PMNL), our present results corroborate the above findings (Frei *et al.* 1988) and strengthen the evidence for the possibility of lipid peroxidation in plasma.

The present findings provide further evidence which indicates that the hypothesis that lipid hydroperoxides and 'modified' LDL are restricted to the arterial vessel wall needs revision. This demonstration that lipid peroxidation in plasma is possible could change current views relating to the pathogenesis and therapeutic approach to atherosclerosis.

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