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Evidence for Oxidised Low Density Lipoprotein in Synovial Fluid from Rheumatoid Arthritis Patients

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The oxidative modification of human LDL has been implicated in atherosclerosis, but the mechanisms by which such modification occurs *in vivo* are not fully understood. In the present study, we have isolated LDL from knee-joint synovial fluid of patients with rheumatoid arthritis. We demonstrate that such LDL is oxidatively modified as evidenced by an increased negative charge, distorted particulate nature and more rapid degradation by cultured macrophages. These results indicate that formation of oxidised LDL is associated with the local inflammatory response. Because the cellular interactions in rheumatoid arthritis have analogies with those in atherogenesis, we suggest that the rheumatoid joint is a useful model of atherosclerosis in which the *in vivo* process of LDL oxidation may be readily studied.

Keywords: Oxidised LDL, rheumatoid arthritis, atherosclerosis, inflammation

INTRODUCTION

The oxidative modification of human low density lipoprotein (LDL) has been implicated in atherogenesis.[1,2] The formation of oxidised LDL generates atherogenic epitopes, such as malondialdehyde (MDA)–lysine adducts. LDL oxidation results in increased negative surface charge and aggregation of LDL particles.[3,4] Oxidised LDL is taken up by "scavenger receptors" expressed on the surface of macrophages, leading to the conversion of these cells to cholesterol-laden "foam cells". Oxidised LDL may also contribute to inflammation by causing endothelial cell damage, infiltration of monocytes and lymphocytes into the arterial wall, smooth muscle cell proliferation, and cellular adhesion molecule expression.[1]

Despite the recent advances in our understanding of the role of oxidised LDL in atherosclerosis,

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there is controversy as to the mechanism by which oxidised LDL is formed. This chemical process may be initiated by reactive oxygen species (ROS). It has been suggested that the interior of advanced human atherosclerotic lesions represents a pro-oxidant environment: extracts from such lesions can generate highly-reactive hydroxyl radicals from hydrogen peroxide and promote lipid oxidation, including peroxidation of LDL.[5] The proposed candidates for catalysing LDL oxidation within arterial walls include transition metal ions[6] and radical-generating enzymes such as NADPH oxidase,[7] lipoxygenase[8] and myeloperoxidase.[9]

The inflamed joints of rheumatoid arthritis (RA) patients are sites at which oxidants are generated and in which transition metal ions, lipoxygenase, and myeloperoxidase are present.[10,11] Both protein and lipid oxidation products have been found in knee joint synovial fluid from patients with RA, and are generally considered to result from inflammation. Because the cellular interactions in RA have analogies with those in atherogenesis,[2] it is of interest to see if LDL particles from the rheumatoid joint carry the hallmarks of oxidative modification. Particles of oxidised LDL within the RA joint might contribute to the inflammatory response there. We showed earlier that the rheumatoid synovial membrane contains foam cells that give positive immunohistochemical staining with an antibody to oxidised LDL.[12] We also reported the presence of LDL with increased negative charge in RA synovial fluid.[13] In this paper we present further evidence that LDL from RA synovial fluid is oxidatively modified to a form which is potentially atherogenic. We propose that the rheumatoid joint is a useful model of atherosclerosis in which the *in vivo* process of LDL oxidation may be studied.

MATERIALS AND METHODS

Patients and Sample Collection

Blood and synovial fluid were taken from patients with definite or classical RA (American College of Rheumatology criteria) and osteoarthritis (OA). Synovial fluid was aspirated for genuine therapeutic purposes and each synovial fluid sample was paired with a blood sample from the same patient. Samples were immediately mixed with Na_2EDTA (0.2% final concentration) and centrifuged at 500 g for 15 min at 4°C to remove erythrocytes/cellular debris. Bovine hyaluronidase (100 μg/ml; Sigma, Poole, Dorset, UK) was added to synovial fluid samples which were then incubated at room temperature with continuous gentle mixing for 15 min. Subsequently, synovial fluid samples were centrifuged a second time and the pellet discarded.

LDL Isolation

LDL from paired samples of human plasma and synovial fluid was isolated by density gradient ultracentrifugation as described.[13] Briefly, potassium bromide (KBr) was added to plasma or synovial fluid such that the final density was 1.019 g/ml. Ultracentrifugation was carried out using an NVT-65 rotor (Beckman, High Wycombe, UK) for 5 h at 4°C and 55,000 rpm, and the resulting single chylomicron/VLDL layer at the top of the tube was discarded. The density of the infranatant was adjusted to 1.063 g/ml with KBr and spun a second time under identical conditions. The orange LDL band at the top of the tube was aspirated and dialysed against three changes of PBS containing 1.0 mM Na_2EDTA over 18 h. The protein concentration was determined by a modified Lowry procedure.[14] In preliminary experiments, immunoelectrophoresis was employed to confirm the purity of the isolated LDL using anti-LDL and anti-HDL antisera (Sigma, Poole, Dorset, UK). The LDL samples were kept at 4°C in the presence of 1.0 mM Na_2EDTA, and measurements of LDL samples were carried out within two weeks after isolation. Thus, EDTA was present throughout in order to prevent the oxidation of LDL.[15]

Electrophoresis of Isolated LDL

Agarose electrophoresis (Paragon Lipogel, Beckman, High Wycombe, UK) was performed to assess the net negative charge of isolated LDL samples. Electrophoresis was carried out for 30 min (100 V, pH 8.6) and staining for lipid was carried out with Sudan Black. Relative electrophoretic mobility (REM) was expressed as: [migration distance of the second band]/[migration distance of the first band], the first band corresponding to native LDL and having the smaller migration distance.

Transmission Electron Microscopy

Transmission electron microscopy was carried out on isolated RA plasma and synovial fluid LDL ($n = 5$ matched samples) according to the method of Morris and Walton.[16] One drop of LDL (10–20 mg protein/ml) was placed on a piloform-coated nickel grid (Agar Scientific, Halstead, Essex, UK) for 20 s and then negatively stained with 2.0% (w/v) phosphotungstate. Quantitative image analysis was performed using a Seescan Symphony Analyser and Task Programme Language IV (Cambridge, UK). The longest and shortest diameters of individual LDL particles were acquired from the photographic negatives (100–300 LDL particles counted per negative).

Determination of the Rate of Macrophage Degradation of LDL

Synovial fluid or plasma LDL was radioiodinated[17] and diluted to a protein concentration of 10 μg/ml in Dulbecco's Modified Eagle's Medium (DMEM) containing foetal calf serum (20%, v/v), gentamycin (10 μg/ml), streptomycin (10 μg/ml), penicillin (10 IU/ml) and amphotericin B (5 μg/ml) (Gibco, Middlesex, UK). The diluted LDL samples were then incubated for 20 h with J774 cells, a macrophage-like cell line (2×10^5 cells/well), or cell-free wells at a volume of 1 ml/well at 37°C under 5% CO_2. The radioactive non-iodide, trichloroacetic acid-soluble degradation products released into the medium were measured as described previously.[18] The degradation products in the cell-free wells were subtracted from those in wells containing cells. The cells were washed three times in Dulbecco's phosphate buffered saline containing Ca^{2+} and Mg^{2+}, lysed in 2 ml of NaOH (0.2 M) for 1 h and assayed for protein by a modified Lowry procedure.[14]

RESULTS

Electrophoretic Mobility of LDL Derived from Plasma and Synovial Fluid

Agarose electrophoresis of LDL isolated from all samples of matched plasma and synovial fluid gave a strong band with an electrophoretic mobility similar to normal plasma LDL. In all eight of the matched OA plasma and synovial fluid LDL samples analysed, this was the only band present. However, some samples of LDL from RA synovial fluid (6 of the 20 samples studied) gave a second band of higher mobility and relatively weak staining intensity. A typical gel is shown in Figure 1. The mean relative electrophoretic mobility (REM ± SD) for the second band was 2.2 ± 0.2,

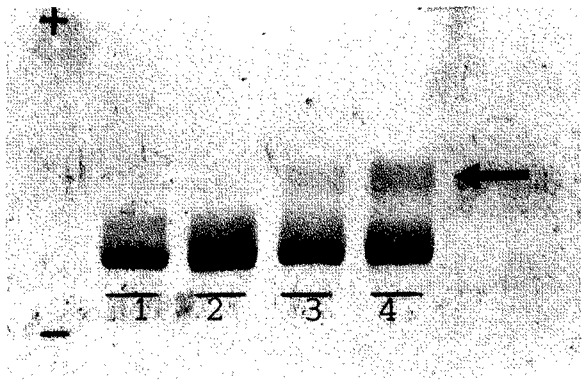

FIGURE 1 Agarose gel electrophoresis of LDL isolated from human plasma and synovial fluid. Lane 1, native LDL; Lane 2, plasma LDL from a patient with RA; Lanes 3 and 4 synovial fluid LDL from the knee and shoulder, respectively, of the RA patient whose plasma LDL was applied to Lane 2. Both synovial fluid LDL samples show a second, more electronegative, band (arrow).

range 2.0–2.4 and $n = 6$. In the case of 1 of the 6 patients where a second band was present, it was possible to obtain synovial fluid on a second occasion about eight months later. On repeat isolation and agarose electrophoresis of the LDL, the second band was again present (data not shown).

We also looked at the clinical details of the RA patients. A significant difference in the mean erythrocyte sedimentation rate (ESR), a measure of the acute phase response associated with inflammation, was seen when comparing the patients with the second band in their synovial fluid LDL samples and those having one (native) LDL band (61.2 ± 33.8 mm/h vs. 40.2 ± 22.1 mm/h, $p < 0.05$, Student's t-test). The total white blood cell count in synovial fluid showed no such difference ($p = 0.09$). There was no obvious relationship between the presence of the second band and the drugs being administered to these patients.

Electron Microscopic Appearance of LDL Particles

Representative electron micrographs of RA plasma and synovial fluid LDL are shown in Figure 2, panels A and B, respectively. In agreement with earlier studies,[16] the plasma-derived LDL particles consisted of spheres with similar diameters. In contrast, the synovial fluid LDL particles were a heterogeneous population of shapes and sizes. Quantitative image analysis was performed to calculate LDL particle asymmetry (the difference between the longest and shortest diameters of individual LDL particles). Figure 2, panels C and D, shows the distribution of particle asymmetry of the plasma and synovial fluid LDL samples corresponding to Figure 2, panels A and B. Five paired samples of RA plasma and synovial fluid LDL were analysed. The mode values of particle asymmetry of the plasma samples were 5.8, 6.5, 6.7, 6.5, and 4.7 nm, and the mode values for the synovial fluid samples were 9.7, 7.5, 11.5, 7.7, and 5.6 nm. There was a statistically significant difference in particle asymmetry between the plasma and synovial fluid LDL samples ($p < 0.05$, Wilcoxon test). These results indicate that LDL preparations from synovial fluid contain an abnormal population of particles which do not conform in shape to native LDL particles from human plasma.

Degradation of LDL by Macrophages

Table I shows the rate of degradation of each LDL sample (expressed as the mean of three determinations) isolated from rheumatoid synovial fluid,

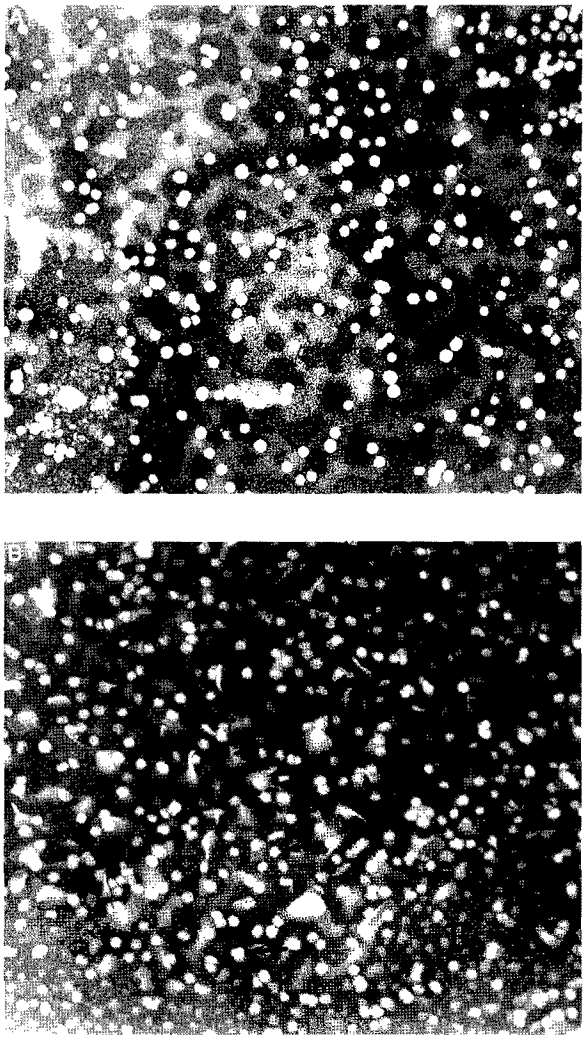

FIGURE 2A and B

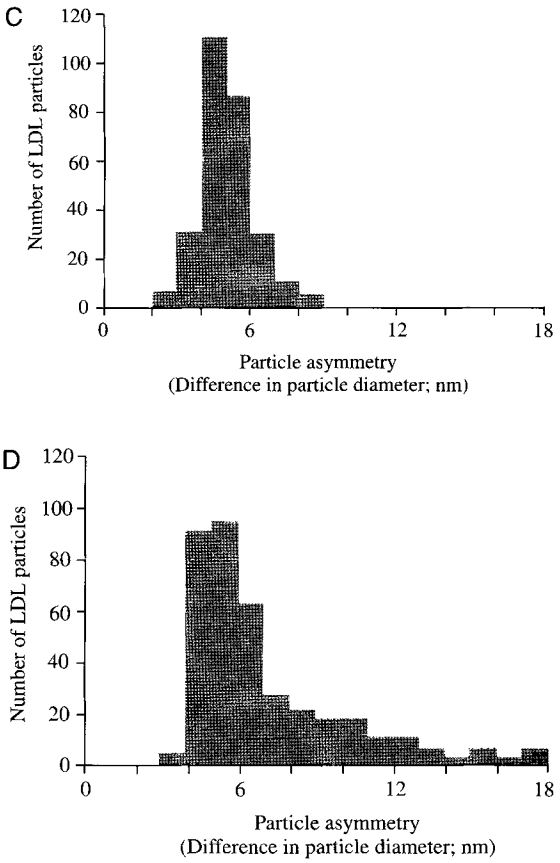

FIGURE 2C and D

FIGURE 2 Transmission electron micrographs (×110,000), together with histograms showing the corresponding image analysis data, for LDL from paired samples of RA plasma (A, C) and synovial fluid (B, D). The plasma LDL particles in (A) are evenly shaped and have a similar appearance to native LDL (see arrow). Some, but not all, of the particles in (B) have an aberrant shape (see arrows). Panels C and D show the distribution of particle asymmetry (difference between the longest and shortest diameter) for these LDL samples from plasma and synovial fluid, respectively. Each bar represents LDL particles with a specific range of particle asymmetries.

rheumatoid plasma, osteoarthritic synovial fluid and osteoarthritic plasma. The table also shows the ratio of synovial fluid LDL degradation/plasma LDL degradation for each RA and OA patient. The mean synovial fluid/plasma ratio for LDL degradation in the RA patients (1.37 ± 0.51, mean $\pm$ SD for 11 patients) was significantly higher ($p < 0.01$, Wilcoxon test) than the corresponding ratio for the OA patients (0.80 ± 0.21, $n = 5$ patients). It can be seen that there is considerable variation between patients in the absolute amount of LDL degraded, particularly within the RA synovial fluid group, and in this respect there was no statistically significant difference ($p = 0.11$) for the comparison of synovial fluid and plasma LDL degradation in RA patients. The degradation of the LDLs by macrophages was assessed using a number of different macrophage cultures (but always the same cultures for the comparison of plasma and synovial fluid LDL from each patient) and these may have added to the variability.

DISCUSSION

There is substantial evidence for the presence of oxidised LDL in atherosclerotic lesions. However, fewer studies have attempted to identify oxidised LDL in the rheumatoid joint. We previously reported foam cells in the rheumatoid synovial membrane,[12] and an increased negative charge of LDL in rheumatoid synovial fluid.[13] Recently, James *et al.*[19] reported the presence of mildly oxidised LDL in synovial fluid from the inflamed joint. We show here that about 30% of RA synovial fluid samples yielded detectable amounts of a modified form of LDL with a substantially increased negative charge, as detected by agarose electrophoresis. Some of the LDL particles isolated from RA synovial fluid had a distorted non-spherical shape, as assessed by electron microscopy, whereas those from RA plasma were normal. Furthermore, we provide evidence that the relative degradation by macrophages of synovial fluid LDL compared to plasma LDL is significantly greater for RA patients than the OA patients.

The purity of the isolated LDL was confirmed by immunoelectrophoresis using anti-LDL and anti-HDL antisera. No HDL was detectable. It is unlikely that the LDL preparation was

TABLE I Degradation of ^{125}I-labelled LDL samples from RA and OA patients

RA				OA			
Patient	Plasma LDL	Synovial fluid (SF) LDL	SF LDL/ Plasma LDL	Patient	Plasma LDL	Synovial fluid (SF) LDL	SF LDL/ Plasma LDL
1	0.365	0.257	0.704	1	0.340	0.290	0.853
2	0.818	1.74	2.13	2	0.400	0.412	1.03
3	0.858	0.782	0.911	3	0.590	0.339	0.575
4	0.300	0.500	1.67	4	0.291	0.174	0.598
5	0.380	0.610	1.60	5	0.241	0.231	0.959
6	0.300	0.540	1.80				
7	0.340	0.660	1.94				
8	0.097	0.140	1.44				
9	0.648	0.429	0.662				
10	0.789	0.958	1.21				
11	0.458	0.438	0.956				
Mean ± SD	0.487 ± 0.252	0.641 ± 0.429	1.37 ± 0.510		0.372 ± 0.135	0.289 ± 0.092	0.800 ± 0.210

Degradation of ^{125}I-labelled LDL by macrophages in 20 h (μg protein/mg cell protein). Values for each patient represent the mean values of triplicate determinations.

contaminated with VLDL fraction, because rheumatoid synovium is rather impermeable to VLDL.[20] The possibility of artefactual oxidation of LDL samples was minimised by addition of Na_2EDTA (final concentration of 1.0 mM) throughout the isolation/analysis procedures.[15,19] However, rheumatoid synovial fluid is known to contain a variety of biomolecules, including neutrophil elastase and hyaluronic acid. We cannot rule out the possibility that the synovial fluid LDL may have been modified by other unknown mechanisms, although previous work showed that LDL digested by elastase[21] or pre-incubated with hyaluronic acid (L. Dai, unpublished observation) did not have an altered electrophoretic pattern on agarose gels. Although the synovial fluid LDL from the RA patients was degraded by macrophages faster on average than their plasma LDL, it appeared that occasionally it was degraded more slowly than the corresponding plasma LDL in particular patients (Table I). Synovial fluid LDL from the OA patients also sometimes appeared to be degraded more slowly than the corresponding plasma LDL. One possible explanation of this is that there may have been a modest oxidation of the synovial fluid LDL. This would decrease its recognition by the LDL receptor but may not have been sufficient to have caused its recognition by scavenger receptors.[22,23] Our results indicate that the formation of oxidised LDL (or possibly some other form of modified LDL) is associated with the local inflammatory response present in RA joints. In rheumatoid arthritis, the inflammatory response is the dominating feature and it is both local (to joints) and systemic. The inflamed rheumatoid joint combines prominent features of both acute and chronic inflammation. In contrast, osteoarthritis is often referred to as a "non-inflammatory", degenerative joint disease, although features of secondary inflammation resulting from joint damage may be present.[24]

The pathophysiology of the rheumatoid joint is characteristic of a chronic inflammatory response and the inflamed joint is thought to be a site of oxidative stress. The concentration of LDL in rheumatoid joints is increased due to increased permeability of the synovial membrane to high molecular weight components of plasma.[25] LDL is a likely target molecule for attack by ROS or reactive nitrogen species (RNS) if the generation of ROS/RNS is excessive, if the antioxidant capacity is depressed or if the pH falls.[26,27] It is known that cells present in the inflamed joint,

such as neutrophils, macrophages, endothelial cells, and fibroblasts, all have the ability to produce ROS, whilst synovial fluid from RA patients is a potent stimulant for ROS generation by cells.[28] The potential mechanisms of ROS/RNS generation by these cells involve the activities of NADPH oxidase, myeloperoxidase, lipoxygenase, and inducible NOS.[11] These cell-derived ROS or RNS could be responsible for the oxidation of LDL in joints. On the other hand, it has been suggested that movement of the rheumatoid joint causes hypoxic–reperfusion cycles within the joint, providing an alternative source of ROS generation.[29]

There are increased amounts of transition metal ion binding proteins, including ferritin and caeruloplasmin, in rheumatoid joints.[30,31] Exogenously added caeruloplasmin stimulates the oxidative modification of LDL by cells,[32,33] although in other systems caeruloplasmin was shown to have an antioxidant effect.[34] Ascorbate-mediated iron release from ferritin also effectively increases the oxidative modification of LDL.[35] It could be that, under certain conditions, perhaps following proteolytic degradation, metalloproteins may be involved in the catalysis of LDL oxidation within the rheumatoid joint.

Oxidised LDL may play many roles in atherogenesis. The existence of oxidised LDL in the inflamed joint of RA patients indicates that there might be common mechanisms of LDL oxidation in RA and atherosclerosis. We suggest that the rheumatoid joint may constitute a system in which the ability of anti-atherogenic drugs to inhibit LDL oxidation *in vivo*, in humans, may be tested.

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