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Transition metal ions within human atherosclerotic lesions can catalyse the oxidation of low density lipoprotein by macrophages

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Abstract The oxidation of low density lipoprotein (LDL) in the arterial wall may contribute to atherogenesis. The oxidation of LDL by cells usually requires catalytically active transition metal ions. We show here some that gruel samples from human advanced atherosclerotic lesions are capable of catalysing the oxidation of LDL by macrophages as measured by thiobarbituric acid-reactive substances, enhanced electrophoretic mobility and increased macrophage uptake. This catalysis could be inhibited by pretreatment of the gruel with Chelex-100, which binds transition metal ions. The presence of catalytically active transition metal ions in atherosclerotic lesions may help to explain why LDL oxidation occurs at these sites.

Key words: Atherosclerosis; Gruel; Low density lipoprotein; Macrophage; Oxidised low density lipoprotein; Oxidized low density lipoprotein

1. Introduction

The local oxidation of low density lipoprotein (LDL) in the arterial wall may result in its increased uptake by macrophages and foam cell formation. Oxidised LDL may also play other roles in atherogenesis, including increasing the migration of monocytes into the arterial wall and cytotoxicity [1,2].

The mechanisms by which LDL becomes oxidised in the arterial wall remain uncertain. It has been demonstrated in vitro, however, that LDL can be oxidised by the main cells present in atherosclerotic lesions, namely endothelial cells [3], smooth muscle cells [4], monocyte-macrophages [5–7] and lymphocytes [8]. Most workers find that LDL oxidation by cells requires the presence of transition metal ions, usually iron [7,9], but copper ions will also catalyse the oxidation [9,10]. A sufficiently high concentration of iron [7,9] or copper [10,11] can catalyse the oxidation of LDL in the absence of cells.

Free iron and copper ions capable of catalysing free radical reactions are not normally found in the plasma, but are firmly bound to proteins, namely transferrin and ferritin for iron [12–14] and caeruloplasmin for copper [15,16]. It has been demonstrated by Smith et al. [17], however, that gruel extracted from advanced atherosclerotic lesions from human aortas can contain significant amounts of catalytically active copper (0–28 μM) or iron ions (0–7 μM). This finding has very recently been confirmed by Swain and Gutteridge [18], who also showed that lesions can contain ferrioxidase I activity, due to caeruloplasmin, and immunologically-detectable caeruloplasmin. Ferroxidase 3 activity, due to xanthine oxidase, was also present in some lesions. Homogenates of some lesions, but not all, could stimulate the oxidation of linoleic acid micelles in the presence of ascorbate [18]. In addition, catalytically active iron and copper have been shown to be present in mechanically disrupted early lesions and the normal arterial wall [19]. We have demonstrated here that gruel from atherosclerotic lesions is capable of catalysing the oxidation of LDL by macrophages and that this catalytic activity is due to the presence of transition metal ions.

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Abbreviations: BHT, butylated hydroxytoluene; DMEM, Dulbecco's modified Eagle's medium; LDL, low density lipoprotein; MDA, malondialdehyde; TBARS, thiobarbituric acid-reactive substances.

2. Materials and methods

2.1. Reagents

All reagents and media were treated with Chelex-100 (Sigma, Poole, Dorset, UK) prior to use, to remove any contaminating transition metal ions.

2.2. Isolation and radioiodination of LDL

LDL (1.019–1.063 g/ml) was isolated from normal pooled human plasma by sequential density ultracentrifugation in KBr solutions at 4°C as described elsewhere [20] and radiolabelled with $Na^{125}I$ using iodine monochloride [7].

2.3. Gruel extraction

Gruel was extracted from advanced atherosclerotic lesions in the abdominal aorta of cadavers within 8–125 h after death by washing the artery with water to remove blood, then incising the lesion with a scalpel and scraping out the yellow semi-solid gruel with the blunt edge of the scalpel. Pooled gruel from several lesions in the same aorta was frozen at −70°C. Storage at −70°C did not exceed 1 month which, normally does not result in oxidative deterioration of biological samples [21].

2.4. Oxidation of LDL by macrophages

Known masses of gruel (0.26g ± 0.12; mean ± S.D.) were homogenised in a Potter homogeniser in 4.8 ml of Ham's F-10 medium (ICN Flow, Thame, Oxfordshire, UK) specially formulated to be deficient in $FeSO_4$, $CuSO_4$, $ZnSO_4$ and L-cysteine (the absence of detectable amounts of Fe and Cu in the Ham's F-10 medium was confirmed by us by atomic absorption spectroscopy). Half of the homogenate (and also Ham's F-10 medium containing either 1 μM $CuSO_4$ or 6 μM $FeSO_4$) was treated with 0.8 g Chelex-100 for 1 h, the remaining half being untreated. Each was centrifuged at $1500 \times g$ for 1 h at 4°C and the supernatant was removed.

Resident mouse peritoneal macrophages were isolated by peritoneal lavage of female Swiss T.O. mice as described elsewhere [7]. ^{125}I-Labelled LDL (100 μg protein/ml) was incubated at 37°C under 5% CO_2 in air in triplicate with intact macrophages (1.0×10^6 peritoneal cells/well) or in cell-free wells with 0.4 ml per well of the gruel supernatants containing 100 μM cystine. The purpose of adding the cystine was to allow the macrophages to take it up, reduce it to cysteine and release this into the medium, thereby promoting the oxidation of LDL in the wells containing cells [22,23] but not in the wells without cells. The oxidation of LDL was stopped after 18 h with 20 μM butylated hy-

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droxytoluene (BHT; from a stock solution of 2 mM in ethanol) and 1 mM EDTA.

2.5. Determination of oxidised LDL degradation by a J774 macrophage-like cell line

Oxidised or control ^{125}I-labelled LDL was diluted to 10 μg protein/ml in DMEM containing 20% (v/v) foetal calf serum, 10 IU penicillin/ml, 10 μg streptomycin/ml and 5 μg amphotericin B/ml (Gibco, Uxbridge, Middlesex, UK). It was then incubated for 20–22 h with J774 cells, a macrophage-like cell-line, (0.2×10^6 cells/well) or cell-free wells at 1 ml per well. The radioactive noniodide, trichloroacetic acid-soluble degradation products released into the medium were measured as described previously [19]. The degradation products in the cell-free wells were subtracted from those in the wells containing cells. The cells were washed in PBS containing Ca^{2+} and Mg^{2+}, and lysed in 2 ml of 0.2 M NaOH as described elsewhere [7]. The lysate was assayed for protein by a modified Lowry procedure [24] and the radioactivity determined as a measure of cell-associated LDL. This cell-associated radioactivity was added to the degradation products in the medium as a measure of total ^{125}I-labelled LDL uptake.

2.6. Thiobarbituric acid-reactive substances (TBARS) assay

Samples (100 μl) of oxidised and control LDL and standards of tetramethoxypropane were taken in triplicate and to each was added 1 ml of 0.335% (w/v) thiobarbituric acid in 10% (w/v) trichloroacetic acid [25]. They were heated at 95°C for 15 min, centrifuged at $1500 \times g$ for 5 min at 4°C, and the absorbance measured at 535 nm. The results were expressed as nmol of malondialdehyde (MDA) equivalents per mg of LDL protein.

2.7 Determination of LDL electrophoretic mobility

Samples (5 μl) were loaded on to a Beckman Paragon agarose gel and electrophoresed at 100 V for 30 min, fixed, dried and stained with the lipid stain Sudan Black. The distance of LDL migration was then measured to the centre of the band.

3. Results

^{125}I-Labelled LDL was incubated with mouse peritoneal macrophages or in cell-free wells in the presence of homogenised gruel supernatants in Ham's F-10 medium deficient in iron and copper ions. The TBARS generated, the electrophoretic mobility of the LDLs and the rate of uptake of these LDLs by a macrophage-like cell line (J774) were then determined.

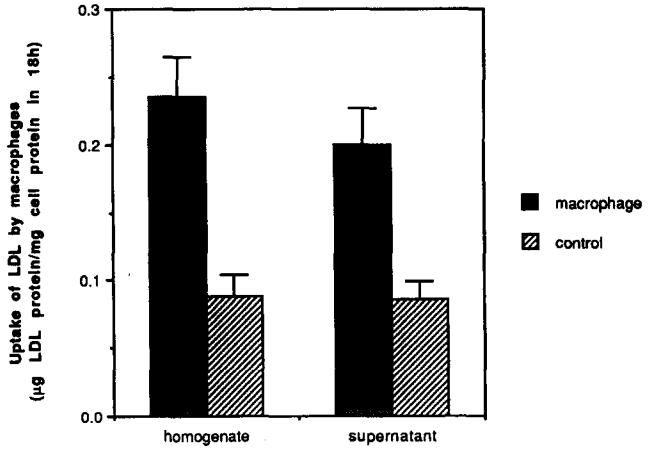

Fig. 1. The effect of complete or centrifuged gruel homogenate on LDL oxidation by macrophages. ^{125}I-Labelled LDL (100 μg protein/ml) was incubated for 18 h with complete gruel homogenate or a supernatant of gruel homogenate in the presence (macrophage) or absence (control) of macrophages. The rate of uptake of these LDLs by the macrophage-like cell-line J774 was then determined. Each bar is the mean ± S.E.M. of three wells. These results are representative of 2 independent experiments.

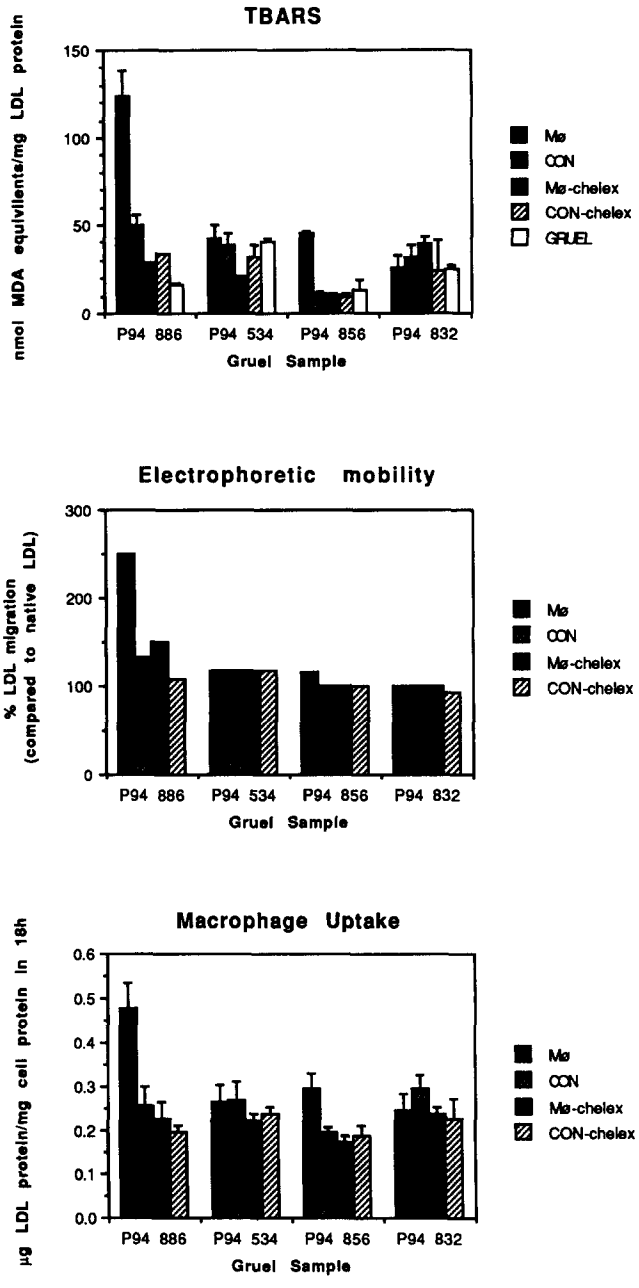

Fig. 2. The effect of treating gruel from human atherosclerotic lesions with Chelex-100 on LDL oxidation by macrophages. ^{125}I-Labelled LDL (100 μg protein/ml) was incubated with gruel supernatants (Mϕ, CON) or Chelex-100 treated gruel supernatants (Mϕ-Chelex, CON-Chelex) in the presence (Mϕ, Mϕ-Chelex) or absence (CON, CON-Chelex) of macrophages for 18 h. The TBARS generated from these LDLs were then determined, including TBARS in gruel samples alone (GRUEL). Each bar is the mean ± S.E.M. of three wells. The electrophoretic mobility of these LDLs was also determined and expressed as the percentage migration compared to native LDL migration. The rate of uptake of these LDLs by a J774 macrophage-like cell line was also determined. Each bar is the mean ± S.E.M. of three wells.

The result of centrifugation of the gruel homogenates was a large pellet of gruel debris and a buoyant layer of lipid. The supernatant was taken so as not to disturb either the pellet or lipid layer. Fig. 1 shows the effect of incubating either complete gruel homogenate or a supernatant of gruel homogenate with macrophages or in cell-free wells on the oxidation of LDL as

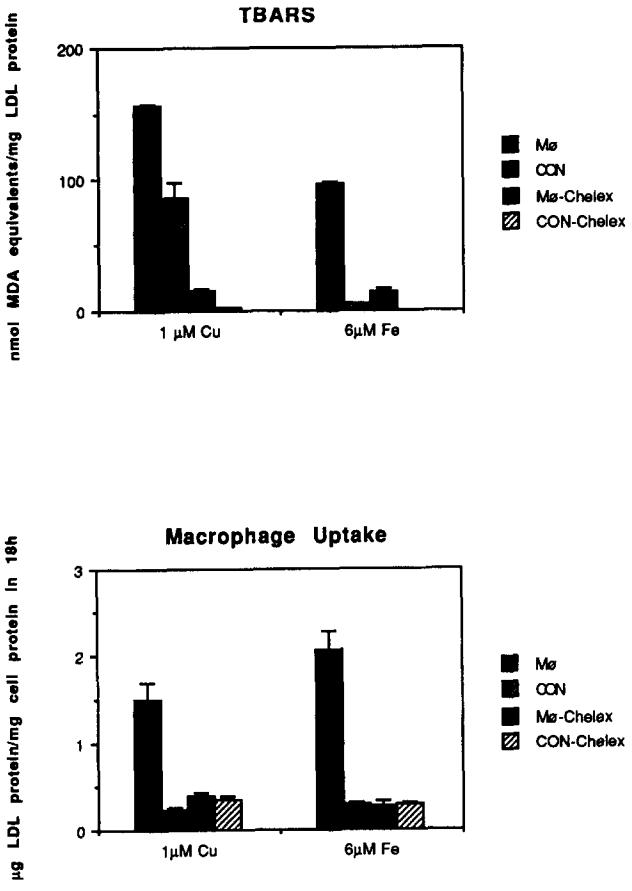

Fig. 3. The effect of Chelex-100 treatment of Ham's F-10 medium containing either copper or iron ions on LDL oxidation by macrophages. ^{125}-Labelled LDL (100 μg protein/ml) was incubated in Ham's F-10 medium (specially formulated to be deficient in $CuSO_4$ and $FeSO_4$) containing either 1 μM $CuSO_4$ or 6 μM $FeSO_4$ (Mφ, CON) or in Chelex-100 treated Ham's F-10 medium (Mφ-Chelex, CON-Chelex) in the presence (Mφ, Mφ-Chelex) or absence (CON, CON-Chelex) of macrophages for 18 h. The TBARS and the rate of uptake of these LDLs by the macrophage-like cell line J774 were then determined. Each bar is the mean ± S.E.M. of three wells. The TBARS in the Chelex-100 pretreated Ham's F-10 medium containing control LDL and $FeSO_4$ were too low to measure. These results are representative of 3 independent experiments.

determined by macrophage uptake. There was no great difference between incubating with a complete homogenate or a homogenate supernatant on the oxidation of the LDL by macrophages. The remaining experiments were therefore conducted with supernatants of gruel homogenates to avoid having the large particles of debris or the floating lipids present.

Table 1 shows the macrophage uptake of macrophage-modified LDL and cell-free control LDL that had been incubated with homogenised gruel supernatants, and also the macrophage uptake of native LDL which had not been previously incubated. Of the 19 gruel samples that had been incubated with LDL in the presence or absence of macrophages, 13 supported a statistically significant increase in the uptake of the LDL by macrophages compared to the uptake of the native LDL. Incubation of the gruel and LDL with macrophages resulted in a statistically significant increase in the uptake of LDL compared to the LDL that had been incubated in cell-free wells in 7 of the 19 samples.

The 19 samples of gruel were examined in 5 experiments which yielded similar results, although the absolute rate of uptake of the native, control and macrophage-modified LDL varied considerably between experiments probably because of variations in the endocytic activity or receptor expression of the J774 'target' macrophage cultures. Fig. 2 shows the TBARS, relative electrophoretic mobility and macrophage uptake data from one of these experiments. One sample (P94 886) stimulated oxidation of the LDL in the presence of macrophages so that over 100 nmol TBARS/mg LDL protein were generated over and above the TBARS already present in the gruel sample. This oxidation also resulted in the LDL migrating 2.5 times faster on an agarose gel than the native LDL and in a doubling in its rate of uptake by 'target' macrophages. This oxidation, measured by all three parameters, was much less in the absence of macrophages and was inhibited by pretreatment of the gruel by Chelex-100 both in the presence and absence of macrophages. Another sample (P94 856) stimulated oxidation modestly in the presence of macrophages as measured by all three parameters. There was no oxidation in the absence of macrophages, and the macrophage-mediated oxidation was again inhibited by pretreatment of the gruel with Chelex-100. Some gruel samples (e.g. P94 534 and P94 832) appeared to stimulate little or no oxidation in the presence or absence of macrophages as measured by TBARS, although there appeared to be a marginal increase in electrophoretic mobility for all conditions for gruel P94 534 compared to the native (non-incubated) LDL. There was an increase in macrophage uptake compared to native LDL for both gruel samples, although there was little difference between macrophage-modified and control LDL (Table 1).

Table 1
The effect of gruel samples from human atherosclerotic lesions on LDL oxidation by macrophages

Gruel sample	Macrophage uptake (μg LDL protein/mg cell protein in 18 h)		
	Macrophage-modified	Control	Native
P92 662	0.130 ± 0.018*	0.031 ± 0.012	0.036 ± 0.021
P92 664	0.279 ± 0.044*	0.197 ± 0.026*	0.036 ± 0.021
P92 684	0.093 ± 0.006*	0.067 ± 0.006	0.045 ± 0.009
P92 744	0.074 ± 0.002	0.040 ± 0.002	0.045 ± 0.009
P92 749	0.104 ± 0.014*	0.096 ± 0.016	0.045 ± 0.009
P92 773	0.043 ± 0.006	0.065 ± 0.015	0.045 ± 0.009
P92 774	0.085 ± 0.008*	0.081 ± 0.006*	0.045 ± 0.009
P92 790	0.149 ± 0.012*	0.092 ± 0.013	0.045 ± 0.009
P94 779	1.097 ± 0.121*	0.404 ± 0.051	0.263 ± 0.006
P94 798	0.152 ± 0.018	0.182 ± 0.006	0.263 ± 0.006
P94 534	0.266 ± 0.037*	0.271 ± 0.042	0.229 ± 0.029
P94 832	0.246 ± 0.039	0.302 ± 0.015	0.229 ± 0.029
P94 856	0.296 ± 0.033*	0.197 ± 0.012	0.229 ± 0.029
P94 886	0.477 ± 0.059	0.259 ± 0.042	0.229 ± 0.029
P95 248	0.628 ± 0.004*	0.234 ± 0.031	0.211 ± 0.024
P95 290	0.490 ± 0.033*	0.210 ± 0.015	0.211 ± 0.024
P95 400	0.302 ± 0.034	0.242 ± 0.011	0.211 ± 0.024
P95 473	0.906 ± 0.133*	0.445 ± 0.031*	0.211 ± 0.024
P95 474	0.642 ± 0.025*	0.511 ± 0.051*	0.211 ± 0.024

^{125}I-Labelled LDL (100 μg protein/ml) was incubated with gruel supernatants in the presence (macrophage-modified) or absence (control) of macrophages for 18 h. The rate of uptake of macrophage-modified, control and native (non-incubated) LDLs by the macrophage-like cell line J774 was then determined. Each entry is the mean ± S.E.M. of three wells.
*Denotes a statistically significant increase in uptake compared with the native LDL ($P < 0.05$).

These increases are unlikely to be due to oxidation because of the lack of increase of TBARS, but more likely a direct result of an interaction of the LDL with one of the many components of the gruel.

Fig. 3 shows that Chelex-100 treatment was effective in removing copper or iron ions from Ham's F-10 medium. Macrophages oxidised LDL well in Ham's F-10 medium specially formulated to be deficient in $FeSO_4$ or $CuSO_4$ to which either 1 μM $CuSO_4$ or 6 μM $FeSO_4$ had been added, as measured by either TBARS or enhanced macrophage uptake. There was also a modest increase in TBARS in the presence of 1 μM $CuSO_4$ alone, although this was insufficient to modify the apolipoprotein B-100 enough to result in increased uptake by macrophages, an event that occurs later than the rise in TBARS. Oxidation of LDL in the presence of absence of macrophages was inhibited by pretreatment of the $CuSO_4$ or $FeSO_4$ containing Ham's F-10 medium with Chelex-100.

4. Discussion

Our results show that the gruel from human advanced atherosclerotic plaques is capable of modifying LDL resulting in its increased uptake by macrophages. By centrifuging the gruel homogenates and using the supernatants below the floating lipid layer, we were able to show that the modification of LDL by macrophages was not mediated by either the large particulate content of the gruel or by the lipid content that floated upon low-speed centrifugation.

The modification of LDL sometimes occurred both in the presence and absence of 'modifying' macrophages and to a similar degree in both (for example P92 749 and P95 474). Because the 'modifying' macrophages were unable to enhance the degree of LDL modification in such cases, it is unlikely that this modification was oxidative and probably resulted from a direct interaction between the LDL and the gruel contents. Hoff and O'Neil [26] showed that the contents of atherosclerotic plaques were capable of modifying LDL so that it was taken up faster by macrophages, but were unable to show that this modification was oxidative.

Other gruel samples (for example P94 856 or P94 886) catalysed macrophage-mediated LDL modification so that the LDL was taken up by macrophages faster than control LDL (incubated in the absence of macrophages). This modification was likely to be oxidative because macrophages are well known to enhance the oxidation of LDL [6,7] and we confirmed this by showing enhanced TBARS and electrophoretic mobility in these macrophage-modified LDLs. Furthermore, pretreatment of the gruel with Chelex-100, which binds transition metal ions, could prevent the oxidation of the LDL catalysed by the gruel sample. As expected, Chelex-100 removed either copper or iron ions that had been added to Ham's F-10 medium and prevented them from catalysing LDL oxidation. These observations are supported by the work of Smith et al. [17] and Swain and Gutteridge [18], who showed that gruel from atherosclerotic lesions contained significant levels of catalytically active copper and iron ions that were able to catalyse the oxidation of rat liver microsomes or linoleic acid micelles.

The presence of catalytic iron or copper ions in human advanced atherosclerotic lesions may explain why LDL becomes oxidised in these areas of the body, but does not explain how these catalytically active ions become available. There is circumstantial evidence to suggest that the interior of atherosclerotic lesions may be acidic (discussed in [27]). We have shown that at acidic pH both caeruloplasmin [10] (the plasma copper-carrying protein) and transferrin [28] (the plasma iron-carrying protein) can generate catalytically active copper or iron that can oxidise LDL. It has also been shown that caeruloplasmin can oxidise LDL in phosphate buffered saline [29] and that peroxynitrite can make the copper in caeruloplasmin catalytically active so that it can catalyse lipid oxidation at pH 7.4 [30]. Furthermore, free radical attack on ferritin [31] or haem proteins [32,33] may release iron ions and in fact oxidised LDL has been shown to induce iron release from activated myoglobin [34].

In conclusion, these studies show that the contents of human advanced atherosclerotic lesions contain transition metal ions that can support the macrophage-mediated oxidation of LDL. This may help to explain why LDL becomes oxidised in these advanced lesions but it does not explain why LDL becomes oxidised in early lesions or the normal arterial wall.

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