Platelet Secretory Products Increase Low Density Lipoprotein Oxidation, Enhance Its Uptake by Macrophages, and Reduce Its Fluidity

Michael Aviram, Gertrud Dankner, and J. Gerald Brook

Oxidized low density lipoprotein (Ox-LDL) is considered to be involved in the atherogenic process. Factors influencing the formation of Ox-LDL are thus of importance. Oxidation of LDL in a cell-free system in the presence of copper ions was significantly increased (up to 60%) by the presence of platelet-conditioned medium (PCM) obtained from collagen-activated platelets for the duration of the oxidation period. The effect was time- and dose-dependent and was related to hydrogen peroxide and superoxide production, since PCM-induced enhanced LDL oxidation was inhibited by catalase and by superoxide dismutase, but not by protease treatments. PCM also reduced the fluidity of oxidized LDL by 45%. Upon incubation with a J-774 macrophage-like cell line, PCM-treated Ox-LDL enhanced cellular cholesteryl ester synthesis by 47% and lipoprotein degradation by 41%. Thus platelet secretory products appeared to enhance LDL oxidation through the involvement of oxidative agents. The resulting Ox-LDL demonstrated increased atherogenic properties.

Macrophage cholesterol accumulation and foam cell formation are early events in atherogenesis.1 Modified forms of low density lipoprotein (LDL), but not native LDL, are able to cholesterol-load macrophages.2 Recently,3,4 it was shown that the in vivo modified form of LDL is probably oxidized LDL (Ox-LDL), which accumulates in the arterial wall in areas of proximity to the atherosclerotic plaque. Blood platelets are also found in close association with the atherosclerotic lesion5-9 and have been shown to contribute to macrophage cholesterol accumulation either directly,9-10 via LDL modification,11-12,13 or by modulation of the LDL-macrophage interaction.14,15 The possible effect of platelet-conditioned medium (PCM) obtained from activated platelets on LDL oxidation was studied in a cell-free system. The consequent change in the properties of the Ox-LDL exposed to PCM was also explored.

Methods

Oxidized Low Density Lipoprotein

Blood was drawn from normolipidemic subjects into 1 mM of Na2 ethylenediaminetetraacetate (EDTA). LDL was separated from the plasma by discontinuous density gradient ultracentrifugation16 and was dialyzed against saline EDTA. LDL was iodinated by the iodine monochloride method as described elsewhere.17 LDL was diluted in Ham's F-10 medium to 300 μg of protein/ml and was dialyzed overnight against phosphate-buffer saline (PBS) at 4°C. The lipoprotein was then incubated in the presence of 10 μm of CuSO4 at 37°C for 24 hours. Oxidation was terminated by refrigeration and the addition of 0.1 mM of EDTA.

LDL oxidation was carried out in the absence (control) or presence of PCM usually added at a 25% volume concentration for the whole 24 hours of the oxidation period. Lipoprotein oxidation was determined by the thiobarbituric acid reactive substances (TBARS) assay17 with malondialdehyde (MDA) as the standard. This method measures MDA equivalents.

Platelet-conditioned Medium

Blood was drawn from healthy normal volunteers into acid citric dextrose (14 g/l citric acid, 25 g/l sodium citrate, and 20 g/l dextrose) in a volume ratio of 6:1. Platelet-rich plasma was prepared by low-speed centrifugation (200 g for 10 minutes at 23°C). Acetic acid (10 mM) was added to the platelet suspension, and the platelets were pelleted by centrifugation (1500 g for 10 minutes). The platelet pellet was then resuspended in HEPES medium (5 mM HEPES, 140 mM NaCl, 2.5 mM KCl, 1 mM MgCl2, 12 mM NaHCO3, 0.4 mM Na2HPO4, 5 mM glucose, pH 7.4) and was used for PCM preparation.

Washed platelets (106/ml) in HEPES medium were activated by the addition of collagen (3 μg/ml) for 5 minutes at 37°C while stirring. The platelet suspension was then centrifuged at 1500 g for 10 minutes at 4°C, and the supernatant (PCM) was collected. The control medium contained HEPES medium with collagen.

Lipoprotein Fluidity

The steady-state fluorescence polarization of diphenylhexatrien (DPH) incorporated into lipoprotein was measured.18,19 The analysis was carried out with a spec-
trofluorimeter equipped with polarizers. Lipoproteins (50 μg of protein/ml) were incubated with 0.1 mM DPH for 30 minutes at 37°C. Fluorescence polarization measurements were carried out at various temperatures, and the anisotropy was determined. The anisotropy parameter is inversely correlated with the fluidity and is given as:

\[
\left[\frac{(r_0 - r)}{r_0} \right]^{-1}
\]

where \( r \) is the fluorescent anisotropy obtained from the polarization analysis and \( r_0 \) is the upper theoretical limit of the anisotropy.19

Macrophase Cholesterol Esterification

Monolayer cultures of J-774A.1 murine macrophage-like cells were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum, penicillin (100 μg/ml), streptomycin (100 μg/ml), and glutamine (2 mM). The cells were fed twice every week. Ox-LDL (25 μg of protein/ml) was incubated with the cells for 24 hours at 37°C in the presence of 10 μCi/ml of \( \text{H} \)-oleic acid (0.27 mmol/l, 83 nmol oleate/mg albumin). At the end of the incubation, cellular lipids were extracted with hexane/isopropanol (3:2, vol/vol) and the cholesteryl ester was separated by thin-layer chromatography (hexane/ether/acetic acid, 130:30:1.5, vol/vol/vol), scraped into vials containing 15 ml of scintillation fluid and counted in a beta scintillation counter.13

Macrophase Degradation of Oxidized Low Density Lipoprotein

LDL degradation was measured after incubation of \( ^{125} \text{I} \)-LDL (150 to 250 cpm/mg) with cells for 5 hours at 37°C. The hydrolysis of LDL protein was assayed in the incubation medium by measurement of trichloroacetic acid-soluble noniodide radioactivity.20 Cell-free LDL degradation was minimal and was subtracted from the total degradation. The cell layer was washed three times with PBS and was extracted by a 1-hour incubation at room temperature with 0.5 ml of 0.1 N NaOH for the measurement of protein.

Results

Oxidation of LDL in vitro in the presence of copper resulted in a time-dependent elevation in TBARS concentration (Figure 1A). The addition of PCM (25% volume concentration obtained from collagen-activated platelets) to LDL for the duration of the oxidation period resulted in a significant (\( p<0.01 \)) and substantial elevation in lipoprotein oxidation (up to 47%) as measured by its MDA content at all time points (Figure 1A). No TBARS activity was found in PCM alone (data not shown). The addition of increasing concentrations of PCM during the oxidation period resulted in a dose-dependent response; there was up to 60% elevation in MDA concentration (Figure 1B). PCM-treated Ox-LDL demonstrated increased mobility on lipoprotein electrophoresis in comparison to Ox-LDL not exposed to PCM (39.6±0.3 vs. 38.0±0.2 mm, \( p<0.02, \) n=4). Native LDL mobility was 30.1±0.2 mm in this study. PCM by itself did not initiate LDL oxidation in the absence of copper (Figure 2).

To determine the component of PCM that was involved in the enhancement of LDL oxidation, PCM was pretreated for 15 minutes at 37°C with either antioxidants or proteases. Both superoxide dismutase (SOD) and catalase reduced LDL oxidation by 41% and 30%, respectively, but catalase pretreatment of the PCM had a more marked inhibitory effect than did SOD (75% vs. 60%) on PCM-mediated LDL oxidation (Figure 2). Vitamin E pretreatment of PCM, as well as protease, trypsin, and heat treatments (100°C, 15 minutes) did not alter the ability of PCM to enhance LDL oxidation (Figure 2).

The fluidity of Ox-LDL was markedly reduced (by 64%) in comparison to native LDL (Figure 3). The addition of PCM to LDL during oxidation reduced lipoprotein fluidity by a further 45%. PCM addition after termination of lipoprotein oxidation, however, had only a minimal effect on the fluidity of Ox-LDL (Figure 3).

Upon incubation of the oxidized lipoproteins with a J-774 macrophage-like cell line for 24 hours at 37°C, the PCM-treated Ox-LDL enhanced cellular cholesterol esterification rate by 47% in comparison to Ox-LDL obtained in the absence of PCM (Table 1). Furthermore, cellular
The Influence of platelet-conditioned medium (PCM) pretreatment on low density lipoprotein (LDL) oxidation. PCM was pretreated for 15 minutes at 37°C with vitamin E (20 μM), superoxide dismutase (SOD, 20 μg/ml), catalase (250 μg/ml), protease (10 μM), trypsin (20 μg/ml), or heat (100°C for 15 minutes followed by centrifugation at 1500 g for 10 minutes to remove denatured precipitated proteins). LDL oxidation was then carried out in the presence of the pretreated PCM preparations as described in the Methods section. The open bars represent controls incubated without PCM but with the various substances for the whole LDL oxidation period. The results are the means±SD of three experiments.

Figure 3. Fluidity of oxidized low density lipoprotein (Ox-LDL) prepared in the presence of platelet secretory products. LDL was oxidized in the absence or presence of platelet-conditioned medium (PCM) (25% volume concentration) before analysis of lipoprotein fluidity. T is the absolute temperature. The anisotropy parameter is calculated from the fluorescence anisotropy and is inversely related to the lipoprotein fluidity. Ox-LDL+PCM represent Ox-LDL to which PCM was added at the end of the oxidation, in contrast to Ox-LDL-PCM that represents LDL that was oxidized in the presence of PCM. The results are representative of three similar experiments.

degradation of 125I-Ox-LDL (25 μg of protein/ml) was similarly increased when 125I-LDL was oxidized in the presence of PCM (25% volume concentration), but not when PCM was added at the end of the oxidation.

Discussion

Circulating platelets have an important role in atherogenesis.20 Among the effects already described is the contribution of the platelet to enhanced coagulation21 and the promotion of smooth muscle cell proliferation.22 The presence of LDL enhances platelet activation with a consequent increase in atherosclerotic-promoting effects.23,24 Moreover, platelets have been shown to affect lipoprotein composition and biological activity, rendering the lipoproteins more atherogenic.11-12-13 Platelet secretory products can cholesterol-load macrophages9,10 and can modify LDL so that it is more avidly taken up by macrophages.11-12,13 Some of the characteristics of the macrophage lipoprotein receptors were markedly affected by these products.14,15 The present study highlights a further effect of platelets, which may have considerable relevance in atherogenesis. In the presence of PCM, the oxidation of LDL resulted in the evolution of an Ox-LDL, which differed markedly from Ox-LDL not exposed to PCM in terms of TBARS content, fluidity of the lipoprotein, and its biological activity.

Since it is thought that Ox-LDL is involved with and important in macrophage cholesterol accumulation and foam cell formation in vivo,3,20 the extent of LDL oxidation
might be an indicator of the degree of its atherogenicity. Certainly, in our study PCM obtained from activated platelets caused a profound enhancement in the extent of LDL oxidation in vitro.

Although Ox-LDL has been shown to result mainly from superoxide action on the lipoprotein lipid moiety, with subsequent apolipoprotein fragmentation, the present study demonstrated that, in the presence of PCM, hydrogen peroxide was also involved in the oxidation process. The net effect of PCM on LDL oxidation was the result of superoxide and even more of hydrogen peroxide action, since both catalase and superoxide dismutase inhibited the PCM effect. Vitamin E, which is a potent antioxidant, did not prevent LDL oxidation in the absence or presence of PCM, probably because LDL oxidation is associated with a substantial depletion of vitamin E.

Proteins released by activated platelets were not involved in PCM-mediated LDL oxidation since pretreatment of PCM with protease, trypsin, or heating did not hamper the PCM effect.

As a measure of the atherogenicity of the PCM-affected Ox-LDL, both the fluidity of the lipoprotein and its biological activity in macrophages were determined. The fluidity of lipoproteins plays an important role in their interaction with cells. Lipoprotein fluidity is considered to be inversely correlated with the atherogenicity of the lipoprotein. Fluidity is determined by the ratio of cholesterol/phospholipid as well as by the fatty acid composition of the LDL. In a previous study, no significant differences in the cholesterol/phospholipid ratio between native and Ox-LDL were found. Ox-LDL, however, contains fatty acids that have undergone oxidation, and these oxidized fatty acids are known to reduce fluidity and to cause rigidity in membranes. This is the likely explanation for the reduction in the fluidity of Ox-LDL in parallel to its enhanced oxidation.

A further result of the enhanced oxidative state of the Ox-LDL exposed to PCM was the increased cellular cholesterol esterification rate and lipoprotein degradation induced in macrophages incubated with the PCM-treated Ox-LDL, which was indicative of enhanced macrophage uptake.

Both parameters that were investigated were indicative of the increased degree of atherogenicity that characterized the Ox-LDL exposed to PCM. It is unclear to what extent this in vitro study can be extrapolated to the situation in vivo and how far platelet releasate may modify the oxidation of LDL in vivo.

Although the release of platelet secretory products into the arterial wall may occur frequently, the local concentration of antioxidants can influence the net effect of PCM on LDL oxidation. If Ox-LDL is important in atherogenesis in vivo, then the action of platelets found in abundance in the proximity of every atherosclerotic lesion in enhancing LDL oxidation can be of relevance. Our study, then, has further implicated platelets in atherogenesis and has elaborated a hitherto undescribed role.

Acknowledgments

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References


Table 1. Thioarbituric Acid Reactive Substances Content and Macrophage Uptake of Oxidized Low Density Lipoprotein Prepared in Absence or Presence of Platelet-conditioned Medium

<table>
<thead>
<tr>
<th>Additions</th>
<th>TBARS (nmol MDA/mg protein) (n=4)</th>
<th>Cholesterol esterification (nmol/mg cell protein) (n=4)</th>
<th>125I-Ox-LDL degradation (ng/mg cell protein) (n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>15.2 ± 1.3</td>
<td>0.83 ± 0.11</td>
<td>425 ± 43</td>
</tr>
<tr>
<td>PCM</td>
<td>21.1 ± 1.7*</td>
<td>1.18 ± 0.09*</td>
<td>599 ± 51*</td>
</tr>
<tr>
<td>PCM added after termination of oxidation</td>
<td>16.8 ± 1.3*</td>
<td>0.93 ± 0.09</td>
<td>451 ± 53</td>
</tr>
</tbody>
</table>

Macrophages (J-774 A1) were incubated for 24 hours at 37°C with oxidized low density lipoprotein (Ox-LDL) or 125I-Ox-LDL (25 µg protein/ml) prepared from LDL or 125I-LDL in the absence (control) or presence of a 25% volume concentration of platelet-conditioned medium (PCM) or with Ox-LDL to which PCM was added at the end of the oxidation. *p < 0.01 vs. control.

TBARS = thioarbituric acid reactive substances.


Index Terms: oxidized low density lipoproteins • platelet secretory products • macrophages • fluidity
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