Oxidized LDL Induces an Increase in the Relative Collagen Synthesis of Rabbit Aortic Smooth Muscle Cells

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Cell degeneration and collagenosis are the main features in atherosclerotic plaque. We examined the effects of human low density lipoprotein (LDL) on cultured rabbit aortic smooth muscle cells (SMCs). Copper oxidized LDL (LDL+Cu) injured the SMCs more than did native LDL. Cytotoxicity of oxidized LDL was prevented by the simultaneous addition of butylated hydroxytoluene (BHT) and ethylenediamine tetraacetic acid. Collagen synthesis increased up to 6 fold after incubation with 200 µg protein/ml of both native and oxidized LDL compared with that incubated in bovine serum albumin. Noncollagen protein synthesis was significantly reduced by oxidized LDL when compared to that by native LDL. Therefore, oxidized LDL increased the relative collagen synthesis (3.33%) to a greater extent than did native LDL (0.72%). By adding BHT to LDL+Cu, the elevated relative collagen synthesis was reversed due to the restoration of noncollagen protein synthesis while it also inhibited LDL peroxidation as evaluated by the formation of malondialdehyde (MDA). However, sodium MDA (up to 200 µM) did not induce either cytotoxicity or an increase of relative collagen synthesis. We therefore conclude that oxidized human LDL enhanced the relative collagen synthesis coinciding with the induction of injury in cultured aortic SMCs, however free MDA may not be the component responsible for these effects. J Atheroscler Thromb, 1994 ; 1 : 53-59.

Key words : Collagen synthesis, Low density lipoprotein, Malondialdehyde, Lipid peroxidation, Smooth muscle cell

Atherosclerosis involves intimal thickening as a cellular response to a proliferation of smooth muscle cells (SMCs). Thereafter, collagenosis occurs in the deeper intima accompanied by the disappearance of SMCs. However, it is still unclear as to why the lesions become acellular and why SMCs suffer degenerative changes which often result in cell fragmentation or cell death in the fibrous plaque. It has been reported that arterial SMCs synthesize the components associated with the arterial extracellular matrix including collagen (1-3) elastin (1), and glycosaminoglycans (1, 4).

Hypercholesterolemia is believed to be one of the key factors for foam cell formation in atherosclerosis (5, 6).

Since hypercholesterolemic or hyperlipoproteinemic serum contains many components other than low density lipoprotein (LDL), both positive and negative effects on collagen production in cultured cells have been reported (7, 8). However, ascorbic acid (9), estrogen (10), platelet-derived growth factor (11), transforming growth factor (11) and dimethylsulphoxide-soluble particles from cigarette smoke (12) have been identified as stimulating factors for collagen synthesis in cultured cells.

Oxidized LDL can be generated by several kinds of cells such as endothelial cells (13), SMCs (14) and macrophages (15). Thus, one of the most plausible candidates for the presence of oxidized LDL in vivo is thought to be the arterial wall (16, 17). Oxidized LDL is recognized by the scavenger receptor of macrophages (14, 15, 18, 19) and SMCs (20), and leads to lipid accumulation in the cells. Oxidized LDL also has independent injurious or toxic properties that affect vascular SMCs (21). One cause of these LDL actions is supposed to be the lipid peroxidation...
in the LDL. Chojkier et al. (1989) (22) reported that following the addition of ascorbic acid, malondialdehyde (MDA), as a lipid peroxidation product, was formed in human fibroblasts, therefore MDA accumulation in the cells was an important step for subsequent collagen production.

In this study, we examined the relationship between collagen synthesis and cytotoxicity in rabbit aortic SMCs incubated with oxidized human LDL.

Materials and Methods

Cell culture

Aortic SMCs, derived from explants of the aortic media of New Zealand white rabbits, were grown at 37°C in Eagle’s minimum essential medium (MEM) (SIGMA) containing 20% new born calf serum (SIGMA) in a humidified atmosphere of 95% air/5% CO2 as previously described (23). The SMCs used in this study were all in the fifth to seventh passage.

LDL isolation and lipoprotein deficient serum

LDL (d=1.019-1.063 g/ml) was isolated from normal human plasma containing 1 mg/ml ethylenediamine tetraacetic acid (EDTA) by ultracentrifugation according to the method described by Ferrel (24). Lipoprotein deficient serum (LPDS) (d=1.25 g/ml) was prepared by ultracentrifugation. LDL and LPDS were dialyzed against phosphate-buffered saline (PBS) containing 0.01/0 EDTA.

LDL oxidation and assay for thiobarbituric acid reacting substances

LDL in PBS was oxidized by incubating LDL with 5 μM CuSO4 for 24 hours at 37°C(18). The extent of lipid peroxidation in LDL was estimated by the amount of thiobarbituric acid reactive substances (TBARS)(25). LDL in MEM (50 μg protein in 500 μl) was mixed with 1.5 ml of 0.65% TBA and 20% trichloroacetic acid (TCA). After heating at 80°C for 1 hour, the reaction product was assayed fluorometrically using a Perkin-Elmer spectrophotofluorometer (model 650-10s), with excitation at 515 nm and emission at 553 nm. Freshly diluted tetramethoxypropane was used as the standard. The results are expressed as nano moles TBARS/mg of LDL protein.

Morphological examination

Confluent SMCs in 18 mm culture dishes were incubated for 24 hours in MEM in the presence of 200 μg BSA/ml or LDL at a concentration of 100 or 200 μg protein/ml. In some cases, 5 μM copper ions were also added to the cultures. At the end of the incubation, the cells were gently rinsed with PBS, and then fixed with 1.4% buffered glutaraldehyde. SMCs were stained with 0.1% toluidine blue and examined by light microscopy.

Collagen and protein synthesis

SMCs were initially seeded at 5×10^4 cells/well in 24 well plates (Corning) and incubated in MEM containing 20% new born calf serum. After overnight incubation, the medium was changed to MEM containing 10% LPDS. The cells were then incubated for 48 hours to eliminate any factors which could affect collagen and/or protein production. SMCs were then incubated for 24 hours in the presence of either 200 μg BSA/ml, 200 μg LDL/ml or 200 μg LDL/ml plus 5 μM copper ions. Alternatively, some cultures were incubated with 100 μM or 200 μM sodium malondialdehyde (MDA) prepared using the method described by Saslaw and Waravdekar (26). The medium also contained 0.1 M L-ascorbic acid and 10 μCi/ml of [5-3H] proline (35 Ci/mmol) (Amersham). Collagen synthesis was determined by the method used by Webster and Harvey (27), with some modification for use with the 0.5 ml/well culture medium in 24-well plates. Both the cellular and medial newly synthesized collagen, which were extracted overnight with 1 mg/ml pepsin containing 1 M acetic acid at 4°C, were then purified by successive salt precipitations in acid and neutral pH at a concentration of 1.2 and 4.0 M NaCl, respectively, using carrier collagen. To check for the purity of collagenous protein, the radiolabeled collagen was separated by electrophoresis on a sodium dodecyl sulphate-polyacrylamide gel containing 8% acrylamide (28). The extracted protein was fluorometrically identified and contained types I, III, IV and V collagen. Radiolabeled proline associated with those collagens was approximately 95% of the total by a densitometric analysis.

The measurement of noncollagen protein synthesis was performed as follows. After 24 hours of incubation with [3H] proline, both cellular and medial proteins were precipitated twice at 100°C by the addition of 10% TCA. The precipitated protein was then washed 4 times with TCA and was dissolved in 500 μl of 1N NaOH. The solution was neutralized with 1N HCl and was adjusted to a final volume of 1 ml. Noncollagen synthesis was calculated by subtracting the count for collagen from the count for total protein incorporated with proline. Relative collagen synthesis was also calculated using the formula proposed by Peterkofsky et al. (29) as follows : relative collagen synthesis = collagen synthesis/(noncollagen synthesis × 5.4 : collagen synthesis).

Dye exclusion assay

A dye exclusion assay was performed to estimate the injurious effect of native and oxidized LDL on cells using 0.4% trypan blue solution (SIGMA). Dye accepting SMCs were counted under a light microscope and were expressed as a percentage of a total of 1,000 cells.

DNA quantification and protein assay

DNA in SMCs was extracted and quantified by the fluorometrical method(25) using Hoechst 33258 dye
Oxidized LDL and Collagen Synthesis

Table 1. LDL oxidation by copper ions and protective effect of antioxidants. LDL (200 μg protein/ml) in MEM was incubated with or without 5 μM Cu²⁺ for 24 hours in the presence of SMCs. Effects of antioxidants, BHT and EDTA were examined under several conditions.

<table>
<thead>
<tr>
<th>Condition</th>
<th>LDL in MEM (TBARS nM/mg LDL protein)</th>
<th>5 μM Cu²⁺ (TBARS nM/mg LDL protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ BHT (μM)</td>
<td>2.19</td>
<td>34.80</td>
</tr>
<tr>
<td>+ 50</td>
<td>2.63**</td>
<td></td>
</tr>
<tr>
<td>+ 100</td>
<td>2.26**</td>
<td></td>
</tr>
<tr>
<td>+ 200</td>
<td>1.78</td>
<td>2.31**</td>
</tr>
<tr>
<td>+ EDTA (μM)</td>
<td>25</td>
<td>15.35*</td>
</tr>
<tr>
<td>+ 50</td>
<td>2.47</td>
<td>5.38**</td>
</tr>
</tbody>
</table>

All data are the mean of triplicate samples.

*: p < 0.05, **: p < 0.01 vs LDL + Cu²⁺

Results

As shown in Table 1, the incubation of LDL at 37 ºC for 24 hours with 5 μM copper ions resulted in extensive oxidation of LDL (p < 0.01). The values for TBARS increased more than fifteen fold when LDL was incubated with 5 μM copper ions in the presence of SMCs (34.80 nM TBARS/mg LDL protein). However, the increase in TBARS was minimal when LDL was incubated in the absence of copper ion (2.19 nM TBARS/mg LDL protein). Two types of antioxidants were used, butylated hydroxytoluene (BHT) as a free radical scavenger and EDTA as a metal chelator. BHT effectively prevented the oxidation of LDL by copper ions. The concen tration of TBARS in the LDL incubated with 50, 100 or 200 μM BSA/ml was nearly the same in all cases, and were also comparable to the concentration in native LDL. EDTA also decreased the concentration of TBARS in the LDL incubated with copper ions, but was less effective than BHT.

To check the cytotoxic effects of the different LDL conditions on SMCs, a trypan blue dye exclusion assay was used. Exposure to copper oxidized LDL resulted in the highest value for the dye accepting cells (13.6%) as compared to that in the native LDL (5.4%) (p < 0.05, Table 2). When antioxidants were added to the cultures in the presence of LDL with copper, cytotoxic effects were prevented relative to the decrease in TBARS levels (p < 0.05, Table 2).

In the cultures with 5 μM copper ions and 200 μM BSA/ml, there were no changes in cellular morphology after 24 hours of incubation. Light microscopy of the control cultures incubated in media without LDL showed confluent growth (Figs. 1A, B). The cultures with 100 or 200 μg LDL/ml showed minimum changes, but still maintained either spindle or angular shapes (Figs. 1C, E). However, the same amount of LDL in the presence of copper ions induced cell damage in a dose-dependent manner (Figs. 1D, F). The cells condensed with a shrunken cytoplasm and were rounded.

The protein concentration in cells incubated with copper oxidized LDL was significantly lower than that in the cells with either BSA or LDL alone (Table 3). However, no statistical differences were observed in the DNA contents of the cells incubated with 200 μg BSA/ml, 200 μg LDL/ml or 200 μg LDL/ml with 5 μM copper ions.

Collagen synthesis by SMCs after 24 hours incubation is shown in Table 4. No differences in collagen production were observed for incubation in MEM only, or after adding 5 μM copper ions and 200 μg BSA/ml (0.78, 0.85, 1.56 x 10³ cpm/ng DNA, respectively). When cells were incubated with 200 μg LDL/ml, the collagen production was dramatically increased (7 fold) over that of cells in BSA (p < 0.01). In addition, copper oxidized LDL induced almost the same degree of collagen production as native LDL.

Table 4 shows the noncollagen protein synthesis in SMCs. The level of noncollagen protein synthesis in native LDL was similar to that of the BSA control. However, by incubating LDL with copper ions the non-collagen protein synthesis declined substantially (p < 0.01). When relative collagen synthesis was adopted...
Fig. 1. Rabbit aortic SMCs were incubated for 24 hours in the presence of 200 μg/ml BSA (a, b), 100 μg/ml LDL (c, d) or 200 μg/ml LDL (e, f). The cultures shown in b, d and f contained 5 μM copper ions. The cells were fixed with 1.4% buffered gluteraldehyde and stained with 0.1% toluidine blue.
Oxidized LDL and Collagen Synthesis

Table 3. DNA and protein contents in SMCs. After SMCs were incubated with 200 µg/ml BSA, native LDL and copper oxidized LDL, DNA and protein contents of SMCs were measured.

<table>
<thead>
<tr>
<th>200 µg/ml</th>
<th>ng DNA/well</th>
<th>µg protein/well</th>
<th>Protein/DNA (ng/ng)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BSA</td>
<td>359.8±115.6</td>
<td>86.2±1.4</td>
<td>239.6</td>
</tr>
<tr>
<td>LDL</td>
<td>373.3±65.7</td>
<td>94.7±3.3</td>
<td>253.7</td>
</tr>
<tr>
<td>LDL + Cu²⁺</td>
<td>319.8±46.3</td>
<td>63.4±5.6</td>
<td>198.2</td>
</tr>
</tbody>
</table>

mean±SD (n=3) *: p<0.05, **: p<0.01, ***: p<0.001

Table 4. Noncollagen protein and collagen synthesis in SMCs. SMCs were incubated with the indicated agents in MEM for 24 hours, and noncollagen and collagen synthesis were measured as described in the text.

<table>
<thead>
<tr>
<th>200 µg/ml</th>
<th>Noncollagen protein synthesis (×10⁵ cpm/ng DNA)</th>
<th>Collagen synthesis (10 cmp/ng DNA)</th>
<th>Relative collagen synthesis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(MEM only)</td>
<td>1.97±0.08</td>
<td>0.78±0.11</td>
<td>0.07</td>
</tr>
<tr>
<td>BSA</td>
<td>2.26±0.13</td>
<td>1.56±0.45</td>
<td>0.13</td>
</tr>
<tr>
<td>LDL</td>
<td>2.79±0.28</td>
<td>11.00±0.13</td>
<td>0.72</td>
</tr>
<tr>
<td>LDL + Cu</td>
<td>0.60±0.04</td>
<td>11.14±2.43</td>
<td>3.33</td>
</tr>
<tr>
<td>LDL + Cu +100 µM BHT</td>
<td>2.35±0.11</td>
<td>9.69±0.34</td>
<td>0.76</td>
</tr>
<tr>
<td>LDL + Cu +25 µM EDTA</td>
<td>—</td>
<td>10.88±0.43</td>
<td>—</td>
</tr>
<tr>
<td>LDL + Cu + 50 µM EDTA</td>
<td>—</td>
<td>10.07±1.03</td>
<td>—</td>
</tr>
</tbody>
</table>

Cu: 5 µM Cu²⁺, mean±SD (n=3), *: p<0.01

Table 5. Effects of MDA on noncollagen protein and collagen synthesis in SMCs. After sodium MDA was added to the SMC cultures, noncollagen and collagen synthesis in a 24 hour incubation were measured as described in the text.

<table>
<thead>
<tr>
<th>200 µg/ml</th>
<th>Noncollagen protein synthesis (×10⁵ cpm/ng DNA)</th>
<th>Collagen synthesis (10 cmp/ng DNA)</th>
<th>Relative collagen synthesis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM</td>
<td>1.97±0.07</td>
<td>0.78±0.11</td>
<td>0.07</td>
</tr>
<tr>
<td>MEM + 100 µM MDA</td>
<td>—</td>
<td>0.70±0.19</td>
<td>—</td>
</tr>
<tr>
<td>MEM + 200 µM MDA</td>
<td>1.66±0.07</td>
<td>0.89±0.11</td>
<td>0.10</td>
</tr>
</tbody>
</table>

mean±SD (n=3)

instead of collagen synthesis, copper oxidized LDL induced a more than 4 fold higher value (3.33%) than native LDL (0.72%) or BSA alone (0.13%). BHT (100 µM) was then added to the media containing LDL and copper ions. There were no differences in collagen synthesis when compared to the native or copper oxidized LDL, however due to the restoration of noncollagen protein synthesis by BHT, the relative collagen synthesis decreased by 0.76% which was comparable to that in native LDL.

Discussion

It has been believed that hyperlipidemic serum carries more oxidation products than normolipidemic serum (30). The role of oxidized LDL in atherogenesis has been receiving increased attention (6, 17, 18). However, one study claims that the oxidation product, MDA, stimulates collagen synthesis in vitro (22). There has been no prior report examining the stimulatory effect of oxidized LDL on collagen synthesis.
Oxidized LDL has been shown to be injurious or cytotoxic to several kinds of cells including SMCs (21). In this study, both native and oxidized LDL caused morphological damage in the cultured SMCs. However, the effects of the oxidized LDL were consistently more extensive. This was also supported by a dye exclusion assay which indicated a stronger cytotoxicity for oxidized LDL. The possibility must also be considered that some changes caused by the native LDL were due to the oxidation effects resulting from the interaction between the cell and LDL after LDL had been endocytosed.

Noncollagen protein synthesis in SMCs incubated with copper oxidized LDL showed a significant decrease (21%) of the control, which resulted in a decreased total protein content (67% of the native LDL). The reduction in non-collagen protein synthesis was not likely due to a shifting of the substrate to collagen synthesis since both the native and oxidized LDL treated SMCs increased collagen synthesis, however, only the oxidized LDL treated SMCs had greatly reduced noncollagen protein synthesis.

Chojkier et al. (22) reported that ascorbic acid induced lipid peroxidation-stimulated collagen production in cultured human fibroblasts. Collagen synthesis in these cells was also elevated by the direct addition of 200 μM MDA as a peroxidation end product. They thus concluded that the formation of aldehyde in the cells was necessary for the stimulation of collagen gene expression. In our study, both native and oxidized LDL stimulated collagen production more strongly as compared with the BSA control, while no such difference was found between the LDLs. When MDA, which had a maximum concentration (200 μM) 28.7 fold higher than that in the oxidized LDL, was directly added to the cultures, no effect such as oxidized LDL, was noted on either collagen synthesis or cytotoxicity in the rabbit aortic SMCs. Thus, it appears that either peroxidation or the peroxidation products carried in LDL, other than free MDA, are involved in the subsequent cytotoxicity and depression of noncollagen protein synthesis in SMCs. However, the exact causes of these differences, especially regarding MDA, could not be defined.

When relative collagen synthesis was adopted, in contrast to total protein synthesis, the oxidized LDL induced an increase in the relative collagen synthesis (3.32%) as compared with that of native LDL (0.7%). This increase was reversed by the addition of BHT accompanied by the prevention of cytotoxicity. A reciprocal effect has also been reported for cigarette smoke which is also known to be a risk factor for atherosclerosis. A component of cigarette smoke extracted by dimethylsulfoxide increased both collagen synthesis and cytotoxicity in SMCs (12). These actions were also reversed by the addition of protease inhibitors. Therefore, the toxic components in the blood caused cell degeneration, and the injured SMCs thus responded by increasing collagen production. An accumulation of oxidized LDL has been demonstrated in advanced fibrous plaque in humans and rabbits (17, 31). There are two distinct characteristics of this plaque, one is the substantial accumulation of the extracellular matrix (1-3), while the other is tissue necrosis where fragmented and dying SMCs are present (32). Either endogenous or exogenous regulatory mechanisms on SMCs must be involved to become a cell in the advanced collagenous plaque. Our results suggest that oxidized LDL is a component which allows for these two major changes seen in atherosclerotic plaque. Therefore, oxidized LDL might play a role in the formation of not only lipid-laden foam cells, but also advanced stages of atherosclerosis by causing both collagenosis and cell degeneration.

References

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