Native and Oxidized LDL Enhances Production of PDGF AA and the Surface Expression of PDGF Receptors in Cultured Human Smooth Muscle Cells

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Animal studies have demonstrated that hypercholesterolemia leads to the development of fibromuscular atherosclerotic lesions that are characterized by the intracellular accumulation of cholesterol esters in macrophage foam cells and focal proliferation of smooth muscle cells (SMCs). There is now convincing evidence that formation of foam cells occurs as a result of macrophage uptake of oxidized low density lipoprotein (LDL), but the processes linking hypercholesterolemia to activation of SMC growth are less clear. In the present study, we demonstrated that native as well as oxidized LDL stimulates DNA synthesis in cultured human SMCs. Both native and oxidized LDL enhances the expression of platelet-derived growth factor (PDGF) A-chain transcripts in the cells, suggesting that the mitogenic effect of the lipoprotein preparations may be due to activation of autocrine or paracrine PDGF loops. Preincubation of SMCs with native and oxidized LDL also increased the expression of PDGF α- and β-receptors on SMCs and enhanced the responsiveness of the cells to exogenous PDGF. The maximal stimulatory effect of oxidized LDL occurred at a concentration of 3 μg/ml, whereas that of native LDL occurred at 10 μg/ml, but otherwise no difference was observed between the native and oxidized LDL preparations. The mitogenic effects of LDL disappeared if the cells were exposed to the lipoprotein preparations for more than 4 hours and was also effectively inhibited by superoxide dismutase. The present results suggest that LDL may influence the growth of SMCs by modulating the expression of growth-regulatory genes in the cells. (Arteriosclerosis and Thrombosis 1992;12:1099-1109)

KEY WORDS • low density lipoproteins • oxidation • smooth muscle cells • growth factors • atherosclerosis

Hypercholesterolemia due to elevated levels of cholesterol-rich low density lipoprotein (LDL) is a well-established risk factor for atherosclerosis and coronary heart disease in humans. Several animal species also develop atherosclerosis in response to diet-induced hypercholesterolemia. In animals fed a cholesterol-enriched diet, intimal accumulation of cholesterol-filled macrophages (foam cells) occurs within 1 month. The mechanism responsible for cholesterol uptake in intimal macrophages is still unclear. However, cultured macrophages have been shown to take up different forms of modified LDL, such as acetylated or oxidized LDL, by a specific scavenger receptor. Oxidized LDL has also been found to act as a chemoattractant for peripheral blood monocytes; to promote monocyte differentiation into tissue-bound macrophages, and to be cytotoxic to endothelial cells and may thus contribute to the development of atherosclerosis by several mechanisms.

In animals exposed to a high-cholesterol diet for longer periods of time, fibrous lesions arise as smooth muscle cells (SMCs) migrate from the media into the intima, modulate from the contractile to the synthetic phenotype, proliferate, and produce large amounts of tissue matrix. It has been suggested that intimal proliferation of SMCs occurs in response to platelet-derived growth factor (PDGF) released from platelets, endothelial cells, macrophages, or from the SMCs themselves. Human platelet PDGF is a 30-kd dimeric molecule composed of two disulfide-linked, partly homologous chains (A and B). SMCs appear to express only the A chain of PDGF and to synthesize PDGF AA homodimers, whereas endothelial cells also express the B chain. Earlier studies of cultured adult rat arterial SMCs have suggested that PDGF synthesis in these cells leads to an autocrine stimulation of cell growth. Two separate types of PDGF receptors were identified, the α-receptor, which preferentially binds PDGF A-chains, and the β-receptor, which binds preferentially PDGF B-chains. The mechanisms linking hypercholesterolemia to the development of intimal hyperplasia are unknown. Sev-
eral studies have demonstrated increased growth of arterial SMCs cultured in hypercholesterolemic serum.\textsuperscript{18,19} However, LDL appears not to function as a true mitogen but rather by augmenting the effect of other factors such as PDGF and fibroblast growth factor.\textsuperscript{20} The present study was designed to investigate the molecular mechanisms involved in LDL-induced increase in SMC sensitivity to growth factor stimulation.

\textbf{Methods}

\textbf{Materials}

Plastic culture dishes were from Costar (Cambridge, Mass.); sera and media from Gibco BRL; and \textsuperscript{3}Hthymidine, \textsuperscript{32}P, and the PDGF assay system from Amer sham (England). Antibodies against smooth muscle-specific actin were kindly provided by Dr. G. Gabbiani\textsuperscript{21} (University of Geneva, Geneva, Switzerland) and by Dr. A. Gown\textsuperscript{22} (University of Washington, Seattle, Wash.). The following probes were used: the 1.3-kb cDNA fragment of human PDGF A-chain clone D1 and human \textit{c}-sis clone PDGF-B-17 provided by Dr. C. Betsholtz (University of Uppsala, Uppsala, Sweden); the 1.5-kb cDNA fragment of human PDGF \textit{\alpha}-receptor clone 15.3 pUC and the 1.0-kb \textit{Pst} I cDNA fragment of human PDGF-\textit{\beta}-receptor provided by Dr. L. Claesson-Welsh (Pasteur Institute, France). Pure PDGF AA, PDGF BB, \textsuperscript{125}I-PDGF AA, and \textsuperscript{125}I-PDGF BB were kindly supplied to us by Dr. C.-H. Heldin (University of Uppsala, Uppsala, Sweden). Paul a-actin pAM 91 provided by Dr. M. Buckingham (Pasteur Institute, France). The following antibodies were used: \textit{\alpha}-smooth muscle actin (SMA) antibodies clone 15.3 pUC and the 1.0-kb \textit{Pst} I cDNA fragment of human PDGF-\textit{\beta}-receptor provided by Dr. L. Claesson-Welsh (University of Uppsala, Uppsala, Sweden); and mouse \textit{\alpha}-actin pAM 91 provided by Dr. M. Buckingham (Pasteur Institute, France). Pure PDGF AA, PDGF BB, \textsuperscript{125}I-PDGF AA, and \textsuperscript{125}I-PDGF BB were kindly supplied to us by Dr. C.-H. Heldin (University of Uppsala, Uppsala, Sweden).

\textbf{Cell Culture}

Human arterial SMCs were isolated from the renal artery of a 58-year-old woman undergoing nephrectomy, from the mesenteric artery of a 38-year-old man undergoing bypass surgery, and from the iliac artery of a 57-year-old male heart transplant donor. Samples of human peripheral veins were obtained from patients undergoing bypass surgery. The intima and the adventitia were carefully stripped off under a dissecting microscope. The media of the renal artery was cut into small pieces and digested with 0.1% collagenase in medium F-12 containing 10% FCS and 50 \( \mu \)g/ml gentamicin for 16 hours. The freed cells were suspended in medium F-12 containing 10% FCS and 50 \( \mu \)g/ml gentamicin sulfate. A confluent density was reached after 4–6 weeks of primary culture. The media of the mesenteric and iliac arteries and the veins were cut into small pieces that were allowed to attach to the bottom of 12-well plates. Cells began to migrate out from the explants within 2–4 weeks, and the primary cultures reached confluence within 6–8 weeks. Rat aortic SMCs were isolated and cultured as described earlier.\textsuperscript{10} In secondary cultures, the cells grow in a hill-and-valley pattern characteristic for SMCs. The cells were identified as SMCs by immunofluorescence using two different \textit{\alpha}-actin–specific antibodies.\textsuperscript{21,22} In accordance with earlier studies of \textit{\alpha}-actin expression in cultured SMCs, confluent cells showed a more intense staining than subconfluent cells.\textsuperscript{23} Cells were used during the eighth through the 16th passages. The cell doubling time of the cultured SMCs was approximately 72 hours. To determine whether the cells had transformed in culture, analysis of their ability to grow in 2% agarose gels was performed. For time periods of up to 3 weeks, SMCs were unable to form colonies in this gel, whereas mouse 3T3 cells formed abundant colonies within 1 week.

\textbf{Lipoprotein Preparations}

LDL (density range, 1.025–1.050 kg/l) was isolated from human plasma by sequential ultracentrifugation in a fixed-angle rotor in a Beckman L-8-55 ultracentrifuge\textsuperscript{24} and dialyzed against phosphate-buffered saline (PBS) containing 0.15 M NaCl and 1 mM EDTA for 24 hours. The LDL preparations could be identified as a single band on agarose gel electrophoresis. The protein concentration of LDL was determined according to Lowry et al.\textsuperscript{25} All LDL concentrations specified are expressed as amount of LDL protein per milliliter. Oxidation of LDL was performed by dialyzing LDL at a concentration of 0.2 mg/ml against 5 \( \mu \)M CuSO\textsubscript{4} in PBS for 24 hours. The efficacy of the oxidation procedure was determined by analyzing the presence of malondialdehyde and thiobarbituric acid–reactive substances (TBARS) expressed as malondialdehyde equivalents (MDA).\textsuperscript{26} LDL exposed to Cu\textsuperscript{2+} was found to contain between 35 and 45 nmol/MDA equivalent \textsuperscript{1}mg LDL\textsuperscript{1}, whereas the TBARS content of the native LDL preparations varied between 2 and 4 nmol/MDA equivalent \textsuperscript{1}mg LDL\textsuperscript{1}. No lipid peroxides were present in the native LDL preparations as determined by the spectrophotometric assay of El-Saadani et al.\textsuperscript{27} The oxidative modification was also demonstrated by enhanced mobility of the oxidized LDL during agarose gel electrophoresis (data not shown). Iodination of LDL and oxidized LDL was performed essentially as described by McFarlane.\textsuperscript{28} The \textsuperscript{125}I-LDL preparations were dialyzed against 0.15 M NaCl/1 mM EDTA (pH 7.4) overnight, filtered through a 0.45-\( \mu \)m filter, and stored at 4°C. The specific activity of the preparations ranged from 400 to 600 cpm/ng protein. More than 95% of the activity was protein bound as determined by trichloroacetic acid precipitation. The amount of endotoxin present in the lipoprotein preparations was analyzed by using the Limulus assay (Kabi, Stockholm, Sweden). All endotoxin levels were below 0.5 ng/ml in the stock solutions and below 1 pg/ml in the test samples. There was no difference in endotoxin levels between native and oxidized LDL. Addition of \textit{Escherichia coli} endotoxin (Sigma) at concentrations up to 5 pg/ml did not affect the rate of DNA synthesis in SMCs, whereas a minor inhibitory effect was observed in SMCs exposed to 10 pg/ml endotoxin.

\textbf{Lipoprotein Degradation}

Confluent cultures of SMCs grown in 12-multiwell plates were incubated with either 10 \( \mu \)g/ml \textsuperscript{125}I-LDL or \textsuperscript{125}I-oxidized LDL for 5 hours at 37°C. The degradation process was arrested by cooling the cells to 4°C. The medium was removed (1 ml) and mixed with 100 \( \mu \)l bovine serum albumin (BSA) (10 mg/ml) and 200 \( \mu \)l 50% trichloroacetic acid. The precipitated protein was removed by centrifugation for 10 minutes at 2,000g. Then 0.5 ml of 5% AgNO\textsubscript{4} was added to 1 ml of the supernatant to precipitate free iodide. After centrifugation at 2,000g for 10 minutes, the trichloroacetic acid–soluble \textsuperscript{125}I in the supernatant was determined in an LKB gamma counter. The cells were lysed through
incubation with 0.25 M NaOH, and protein content was determined according to Lowry et al.\textsuperscript{25}

**Isolation and Analysis of mRNA Expression**

Confluent 100-mm plates of human arterial SMCs were serum starved in Ham’s medium F-12 for 2x24 hours and then exposed to F-12 with 10 \( \mu \)g/ml native LDL or 10 \( \mu \)g/ml oxidized LDL for 1 hour. Total RNA was isolated from the cells by the guanidine isothiocyanate/lithium chloride/phenol method.\textsuperscript{29} RNA concentration measurements were performed and normalized by UV spectrophotometry and ethidium bromide-agarose plates. Total RNA was denatured, fractionated through formaldehyde/agarose (1.1%) gels, transferred to nylon membranes, and hybridized to \(^3\)P-labeled DNA probes as described.\textsuperscript{11} The DNA probes were labeled to 1-5x10\(^6\) dpm/\( \mu \)g by nick translation or oligonucleotide primer extension. Hybridized filters were washed twice in 2x standard saline citrate (SSC) and 0.5% sodium dodecyl sulfate (SDS) at room temperature for 25 minutes, once in 2x SSC and 0.5% SDS at 55°C for 1 hour, and twice in 0.1x SSC and 0.5% SDS at 55°C for 1 hour. Sizes of the transcripts were determined relative to human 18S and 28S rRNA. Relative intensities of hybridization signals were obtained by densitometer scanning (Shimadzu) of autoradiograms exposed within the linear range of the film (Fuji RX-L).

**Binding of PDGF AA and BB**

SMCs were grown to confluence in 24-well culture plates and were serum starved by transfer to Ham’s medium F-12 for 2x24 hours. The cells were then exposed to medium F-12, and with addition of 10 \( \mu \)g/ml native LDL or 10 \( \mu \)g/ml oxidized LDL for 1 hour, were rinsed and kept in Ham’s medium F-12 at 37°C for another 16 hours. They were then cooled to 4°C for 2 hours and rinsed with binding buffer (PBS containing 0.1% BSA). For determination of ligand specificity the cells were incubated with 2 \( \mu \)g/ml \(^{125}\)I-PDGF AA (specific activity, 62 cpm/pg) or \(^{125}\)I-PDGF BB (specific activity, 75 cpm/pg) with different concentrations of unlabeled PDGF AA or BB in 0.5 ml binding buffer for 2 hours at 4°C. The cells were rinsed six times in binding buffer and lysed in 1% Triton X-100/10% glycerol/20 mM N-hydroxethylpiperazine-N’-2-ethanesulfonic acid (HEPES; pH 7.4). To analyze saturation binding, cells were exposed to increasing concentrations of \(^{125}\)I-labeled PDGF AA or BB with or without a 20-fold excess of unlabeled ligand in 0.5 ml binding buffer for 2 hours at 4°C. The cells were then rinsed and lysed as described above. The cell-associated radioactivity was determined in an LKB gamma counter. Specific binding was calculated by subtracting binding in the presence of excess unlabeled PDGF from binding without addition of unlabeled PDGF.

**Collection of Conditioned Media**

SMCs grown in 12-well plates were serum starved by transfer to medium F-12 for 48 hours and exposed to 10 \( \mu \)g/ml native or oxidized LDL for 1 hour. The cultures were then rinsed and kept in medium F-12/0.1% BSA for 24 hours at 37°C. The conditioned medium was collected and centrifuged at 100g for 5 minutes to remove cellular debris.

**Determination of PDGF Release**

The presence of PDGF AA and BB in the conditioned media was determined by analyzing the ability of the medium to compete with \(^{125}\)I-labeled PDGF AA and BB to cultured rat SMCs. These cells were found to have functionally active PDGF receptors of both the \( \alpha \) and \( \beta \) type (data not shown). Confluent secondary cultures of rat aortic SMCs were grown in serum-free F-12 medium for 48 hours to upregulate PDGF receptors. The cells were then washed in binding buffer and kept in this buffer for 1 hour at 4°C. They were subsequently incubated with conditioned medium for 2 hours at 4°C, rinsed three times with binding buffer, and incubated with 2 \( \mu \)g/ml \(^{125}\)I-PDGF AA or BB for 1 hour at 4°C. Finally, they were rinsed five times in binding buffer and lysed as described above. The amount of PDGF AA and BB present in the conditioned medium was calculated from a standard curve.

**Figure 1.** Panel A: Northern blot analysis of platelet-derived growth factor (PDGF) A- and B-chain and PDGF \( \beta \)-receptor (rec) mRNA expression in human renal artery smooth muscle cells (SMCs). Confluent 100-mm-diameter plates of human arterial SMCs were serum starved in Ham's medium F-12 for 2x24 hours and then exposed to F-12 (lane 1), 10 \( \mu \)g/ml native low density lipoprotein (LDL, lane 2), or 10 \( \mu \)g/ml oxidized LDL (lane 3) for 1 hour, and Northern blot analysis was performed as described in "Methods." Fifty micrograms of total RNA was analyzed in each lane. The same filter was sequentially hybridized to the various DNA probes. Sizes of transcripts are shown in kilobases. Autoradiograph exposure times were 16 hours for PDGF A-chain and \( \alpha \)-actin, 72 hours for the PDGF \( \beta \)-receptor, and 120 hours for PDGF B-chain. Panel B: Scanning densitometric analysis of the Northern blot. Numbers on x axis correspond to lanes in panel A.
obtained with pure PDGF AA and BB. The application of this assay is complicated by the fact that PDGF BB binds to both the α- and β-receptor. Consequently, this assay may give incorrectly high values for PDGF BB. However, in the present study no presence of PDGF BB in the conditioned media was indicated.

![Graph](image)

**FIGURE 2.** Panel A: Northern blot analysis showing time course of platelet-derived growth factor (PDGF) α- and β-receptor (rec) and PDGF A-chain mRNA expression in human iliac artery smooth muscle cells (SMCs). Confluent 100-mm-diameter plates of human arterial SMCs were serum starved for 48 hours in Ham's medium F-12 and then exposed to 10 μg/ml native low density lipoprotein (LDL) or oxidized (ox) LDL for 1 hour. At the indicated times the cells were washed in phosphate-buffered saline, scraped off the dishes with a rubber policeman, and analyzed by Northern blotting. Fifty micrograms of total RNA was analyzed in each lane. The same filter was sequentially hybridized to the various DNA probes. Sizes of transcripts are shown in kilobases. Autoradiograph exposure times were 96 hours for the α- and β-receptor and 144 hours for the PDGF A-chain. Ethidium bromide-stained aliquots (1 μg) of the samples, separated on an agarose minigel, are illustrated in the uppermost portion (rRNA). Panel B: Scanning densitometric analysis of the Northern blot.

**TABLE 1. Release of Platelet-Derived Growth Factor and Mitogenic Activity From Human Renal Artery Smooth Muscle Cells Exposed to Native or Oxidized LDL**

<table>
<thead>
<tr>
<th></th>
<th>PDGF A</th>
<th>PDGF B</th>
<th>Mitogenic activity</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>by RRA (ng/ml)</td>
<td>by RIA (ng/ml)</td>
<td>(% labeled nuclei)</td>
</tr>
<tr>
<td>Control</td>
<td>0.67±0.11</td>
<td>0.56±0.58</td>
<td>10.4±1.6</td>
</tr>
<tr>
<td>Native LDL</td>
<td>0.62±0.21</td>
<td>0.71±0.31</td>
<td>10.6±2.6</td>
</tr>
<tr>
<td>Oxidized LDL</td>
<td>1.50±0.13†</td>
<td>3.85±0.91*</td>
<td>12.1±4.7</td>
</tr>
</tbody>
</table>

Smooth muscle cells grown in 12-well plates were serum starved by transfer to medium F-12 for 2x24 hours and exposed to 10 μg/ml native or oxidized low density lipoprotein (LDL). The cultures were then rinsed and kept in medium F-12/0.1% bovine serum albumin for 24 hours at 37°C. The conditioned medium was centrifuged at 100g for 5 minutes to remove cellular debris and analyzed for the presence of platelet-derived growth factor (PDGF) A- and B-chains and mitogenic activity. Each value represents mean±SD of triplicate cultures. RRA, radioreceptor assay; RIA, radioimmunoassay.

* p<0.05, † p<0.005 vs. control.
This finding may be explained by the fact that PDGF BB has a lower affinity for the α-receptor than does PDGF AA. The presence of PDGF in the conditioned media was also analyzed by radioimmunoassay (Amersham). Briefly, 100 μl of the conditioned medium was mixed with 100 μl of goat anti-human PDGF serum and incubated overnight at 37°C. One hundred microliters of [125I]-PDGF (recombinant c-sis) was then added followed by addition of anti-sheep antiserum. The samples were then centrifuged at 2500g for 15 minutes at 4°C, and radioactivity in the pellet was determined.

Analysis of DNA Synthesis and Cell Growth

Subconfluent SMCs grown on glass coverslips in 24-well culture plates were growth-arrested by transfer to Ham's medium F-12 for 2x24 hours. The cells were then exposed to medium F-12 and 10 μg/ml native LDL or 10 μg/ml oxidized LDL for 1 hour at 37°C, rinsed, and kept in medium F-12 for different periods. They were then incubated with increasing concentrations of PDGF AA or PDGF BB, 0.1% BSA, and 2 μCi/ml [3H]thymidine for 24 hours and fixed in 3% buffered glutaraldehyde. The specimens were dehydrated in ethanol, mounted on glass slides, dipped in Kodak NTB2 emulsion, exposed for 3 days, developed, and stained with methylene blue. The fraction of labeled nuclei was determined by counting a minimum of 200 randomly selected cells per glass. Each value represents the mean of triplicate cultures. The variation coefficient of values from triplicate cultures is usually between 20% and 30%.

Results

Uptake of native and oxidized LDL in human SMCs was determined by incubating the cells with [125I]-labeled lipoproteins and analyzing the amount of trichloroacetic acid-soluble [125I] in the culture medium after 5 hours at 37°C. Cells incubated with 10 μg/ml [125I]-labeled native LDL degraded 398±16.1 ng LDL protein/mg cell protein. Addition of a 10-fold excess of unlabeled native LDL reduced the rate of degradation by 52.5±0.1% (p<0.005), indicating that native LDL was taken up in the cells by a specific receptor-mediated process. Incubation of the cells with 10 μg/ml [125I]-labeled oxidized LDL resulted in a degradation of 244.6±10.8 ng LDL protein/mg cell protein. Addition of a 10-fold excess of unlabeled oxidized or native LDL reduced the rate of degradation by 52.5±0.1% (p<0.005), indicating that native LDL was taken up in the cells by a specific receptor-mediated process. Incubation of the cells with 10 μg/ml of [125I]-labeled oxidized LDL resulted in a degradation of 244.6±10.8 ng LDL protein/mg cell protein. The degradation of [125I]-oxidized LDL was not significantly reduced by addition of a 10-fold excess of unlabeled oxidized or native LDL, suggesting that the uptake of oxidized LDL occurred as a result of a nonspecific, non–receptor-mediated process. However, incubation of oxidized LDL in cell-free dishes reduced the rate of degradation by more than 75% compared with incubation in the presence of SMCs, demonstrating that most of the degradation occurred as a result of interaction between oxidized LDL and SMCs.
Earlier studies have demonstrated transcription of the PDGF A-chain in human arterial SMCs, whereas it has not been possible to identify transcription of the PDGF B-chain in these cells.30 Accordingly, the SMCs used in this study were found to contain mRNA hybridizing with the PDGF A probe but not with the probe for PDGF B (c-sis; Figure 1). To investigate if LDL influences the transcription of the two PDGF chains, confluent cultures of SMCs were serum starved for 48 hours and exposed to 10 μg/ml native or oxidized LDL for 1 hour at 37°C. A small increase in PDGF A transcripts was observed in cells exposed to native and oxidized LDL (Figure 1). No expression of PDGF B-chain was found in cells exposed to native or oxidized LDL (Figure 1). In controls, no expression of mRNA hybridizing with the probe for the PDGF B-receptor could be identified, whereas both native and oxidized LDL induced expression of this gene (Figure 1). Exposure of serum-starved SMCs to 10% FCS for 1 hour resulted in a small decrease in the expression of PDGF A-chain mRNA, whereas the level of PDGF B-receptor transcripts was essentially unchanged (data not shown), indicating that the effects on mRNA levels observed after incubation with lipoproteins were not due to a nonspecific effect of any protein.

To analyze the kinetics involved in lipoprotein-induced gene expression, the cells were harvested at different times after a 1-hour exposure to 10 μg/ml native or oxidized LDL (Figure 2). In this experiment, analysis of PDGF α-receptor gene expression was also performed. Both native and oxidized LDLs were found to enhance the number of PDGF α-receptor transcripts in the cells. The increase in PDGF α-receptor transcripts was more prominent than that of PDGF β-receptor transcripts. Furthermore, the onset of gene expression was slightly faster in cells exposed to oxidized LDL than in cells exposed to native LDL.

Secretion of PDGF-like material by the cells was investigated by analyzing conditioned medium for material competing with binding of [125I]labeled PDGF AA and BB to cultured rat arterial SMCs using unlabeled PDGF AA and BB as controls. Small amounts of PDGF A–containing material were found in medium from control cultures and from cultures exposed to 15 μg/ml LDL. Exposure of cells to oxidized LDL led to an increase of more than 100% in the amount of PDGF A–competing activity released into the medium compared with control and LDL-treated cultures (p<0.005; Table 1). No PDGF B–competing material was found in any of the cultures. Essentially similar results were obtained with a radioimmunossay for total PDGF (Table 1).

The mitogenic activity of the conditioned medium was assayed by determining the ability to stimulate DNA synthesis in cultured rat arterial SMCs. There was no significant increase in mitogenic activity in media from cultures given oxidized LDL compared with media from control cells or cultures exposed to native LDL (Table 1).

The cells were then investigated for the presence of PDGF receptors. The binding specificity of PDGF AA and BB was analyzed by using SMCs isolated from the renal artery. The binding of [125I]PDGF AA was effectively removed by addition of excess unlabeled PDGF AA. Although higher concentrations were required, unlabeled PDGF BB also had the ability to compete with [125I]PDGF AA binding. Contrarily, the binding of [125I]PDGF BB was removed only by unlabeled PDGF BB and not by PDGF AA (Figure 3). These findings are in accordance with studies indicating that PDGF BB may bind to both the α- and β-receptor, whereas PDGF AA only binds to the α-receptor.16 The specific binding of PDGF AA (binding that could not be displaced with a 20-fold excess of unlabeled PDGF AA) to control cells was 32.3±0.5 pg/well. Exposure of the cells to 10 μg/ml native or oxidized LDL resulted in a small increase in PDGF AA binding (37.7±1.6 and 37.3±3.8 pg/well, respectively; Figure 3). The binding of PDGF BB to the cells was higher than for PDGF AA (42.5±16.6 pg/well).

This may be due to the presence of a higher number of β-receptors or by the fact that PDGF BB binds not only to the β-receptor but also to the α-receptor. Preincubation of cells with native LDL gave rise to a marked increase in PDGF BB binding (104.4±8.7 pg/well, p<0.005), whereas the effect of oxidized LDL was less prominent in this respect (80.2±11.0 pg/well, p<0.01; Figure 3).

To further analyze the effect of lipoproteins on PDGF receptor expression, saturation binding studies were performed. In these experiments cells isolated from a mesenteric artery were used. The results indicated an increased saturation binding of PDGF AA after exposure to native and oxidized LDL (Figure 4). In these experiments no effect on PDGF BB binding was observed. Thus, there may exist regional differences in respect to regulation of PDGF receptor expression between renal and mesenteric artery cells. On the other hand, mesenteric artery SMCs demonstrated both an increased expression of the PDGF β-receptor gene and an increased response to PDGF BB stimulation after lipoprotein exposure (data not shown).

Finally, we analyzed the ability of the cells to respond with an increased rate of DNA synthesis when stimulated with PDGF AA and BB. The cells were serum starved for 48 hours, exposed to native or oxidized LDL for 1 hour, transferred to serum- and lipoprotein-free media for another 16 hours, and then incubated with [3H]thymidine and various concentrations of PDGF AA and BB for another 24 hours. In cultures neither exposed to lipoproteins nor stimulated with PDGF, only 2.6±0.7% of the cells were labeled. Preincubating the cells with native or oxidized LDL led to a fivefold increase in the rate of DNA synthesis compared with control cultures (14.7±4.5% and 15.8±1.2%, respectively, p<0.005; Figure 5). In control cultures addition of 4 ng/ml PDGF AA resulted in a labeling index of 7.2±2.7% (Figure 5). Although this rate of DNA synthesis was significantly higher than that for cells given no PDGF AA (p<0.05), the AA homodimer of PDGF appears to be a poor mitogen for human arterial SMCs.

PDGF BB was found to be a much more potent mitogen for human SMCs than was PDGF AA, giving rise to a sixfold increase in DNA synthesis at the concentration of 4 ng/ml. In cells preincubated with native LDL, DNA synthesis in response to 4 ng/ml PDGF BB was 75% higher than in control cells given the same concentration of PDGF BB (p<0.01). Furthermore, in cells preincubated with oxidized LDL, stimulation with 4 ng/ml PDGF BB resulted in a
A threefold higher rate of DNA synthesis than in control cells ($p<0.001$; Figure 5).

Dose–response experiments demonstrated that the maximal stimulatory effect of oxidized LDL occurred at a concentration of 3 µg/ml, whereas the maximal effect of native LDL occurred at a concentration of 10 µg/ml (Figure 6). To study the influence of superoxides in mediating the effect of native and oxidized LDL, SMCs were preincubated with 10 µg/ml superoxide dismutase for 2 hours. This treatment was found to completely block the stimulatory effect of both native and oxidized LDL (Figure 7).

The influence of the length of the lipoprotein exposure period on initiation of DNA synthesis was studied by incubating SMCs with 10 µg/ml native and oxidized LDL for different time periods. They were then rinsed, transferred to serum-free F-12 medium for 16 hours, and subsequently stimulated with 4 ng/ml PDGF AA or BB for 24 hours. A stimulatory effect of both native and oxidized LDL was observed for exposure times of up to 4 hours for PDGF BB and AA, whereas no stimulatory effect could be identified after longer exposures (Figure 8). The lag phase required for lipoprotein-dependent stimulation of PDGF-induced DNA synthesis was analyzed by exposing SMCs to native and oxidized LDL for 1 hour, followed by incubating the cells in serum-free medium for different times, and finally by stimulating the cells with PDGF AA or BB for 24 hours. The results indicate that the lag phase is 2–6 hours (Figure 9).

![Figure 4](image1.png)

**FIGURE 4.** Line plots showing binding of platelet-derived growth factor (PDGF) AA and BB to human mesenteric artery smooth muscle cells (SMCs) exposed to native and oxidized low density lipoprotein (LDL). SMCs were grown to confluence in 24-well culture plates and serum starved by transfer to Ham's medium F-12 for 48 hours. The cells were then exposed to medium F-12 ($\circ\circ\circ$), 10 µg/ml native LDL ($\bullet\bullet\bullet$), or 10 µg/ml oxidized LDL ($\Delta\Delta\Delta$) for 1 hour; rinsed; and kept in Ham's medium F-12 at 37°C for another 16 hours. They were then cooled to 4°C for 2 hours, rinsed with binding buffer, and incubated with increasing concentrations of 125I-PDGF AA (panel A) or 125I-PDGF BB (panel B) without or with addition of a 20-fold excess of unlabeled ligand in 0.5 ml binding buffer for 2 hours at 4°C. Specific binding was calculated by subtracting binding in the presence of excess unlabeled PDGF from binding without addition of unlabeled PDGF ($SD<20\%$).

![Figure 5](image2.png)

**FIGURE 5.** Line plots for autoradiographic analysis of initiation of DNA synthesis in human renal artery smooth muscle cells (SMCs). Subconfluent SMCs grown on glass coverslips in 24-well culture plates were growth arrested by transfer to Ham's medium F-12 for 48 hours. The cells were then exposed to medium F-12 ($\circ\circ\circ$), 10 µg/ml native low density lipoprotein (LDL) ($\bullet\bullet\bullet$), or oxidized LDL ($\Delta\Delta\Delta$) for 1 hour at 37°C; rinsed; and kept in medium F-12 for 16 hours. They were then incubated with increasing concentrations of platelet-derived growth factor (PDGF) AA (panel A) or PDGF BB (panel B), 0.1% bovine serum albumin, and 2 µCi/ml [3H]thymidine for 24 hours, and the fraction of labeled nuclei was then determined by autoradiography. Each value represents mean ± SD of triplicate cultures.
FIGURE 6. Line plot of the dose–response effect of native and oxidized low density lipoprotein (LDL) on serum-induced DNA synthesis. Subconfluent human peripheral vein smooth muscle cells grown on glass coverslips in 24-well culture plates were growth arrested by transfer to Ham's medium F-12 for 48 hours. The cells were then exposed to native (■—■) or oxidized (●—●) LDL for 2 hours, rinsed, and kept in medium F-12 for 16 hours. They were then incubated with 5% fetal calf serum and 2 μCi/ml [3H]thymidine for 24 hours, and the fraction of labeled nuclei was then determined by autoradiography. Each value represents mean±SD of triplicate cultures.

Discussion

Our results confirm earlier studies demonstrating that LDL may promote growth of SMCs and that this effect is partly due to an increased sensitivity to exogenous mitogens. They also suggest that the mechanism responsible for this effect involves both an increased endogenous synthesis of PDGF as well as an increased expression of PDGF receptors. A similar effect on SMC growth activation has been described for interleukin-1, which has been demonstrated to enhance DNA synthesis in SMCs by activating the endogenous production of PDGF and fibroblast growth factor as well as the surface expression of fibroblast growth factor receptors.

Oxidation of LDL did not remove the stimulatory effects on SMC growth. Contrarily, dose–response experiments indicated that oxidized LDL was slightly more effective than native LDL, suggesting that at least some of the stimulatory activity was formed as a result of lipid oxidation. This hypothesis was further supported by the finding that the stimulatory effects of both native and oxidized LDL were effectively inhibited by addition of superoxide dismutase, suggesting that the effects of both types of lipoprotein preparations were mediated by formation of superoxides. Experiments analyzing gene expression, PDGF production, and PDGF receptor expression were performed only with one concentration of LDL (10 μg/ml for both the native and oxidized preparation). Thus, it is not possible to determine whether oxidized LDL was more potent than native LDL in these respects also.

The stimulatory effect of the lipoproteins began to disappear at concentrations above 10 μg/ml and at exposures longer than 4 hours in serum-free medium. These findings are in accordance with studies performed on lipoprotein-exposed mononuclear leukocytes, demonstrating that both the stimulatory effect of low concentrations of oxidized LDL as well as the cytotoxic effects of higher concentrations of oxidized LDL are inhibited by superoxide dismutase. One possible explanation for these findings is that the lipid peroxides present in oxidized LDL initiate formation of superoxide anions in the cells and that these activate or potentiate the mitogenic signal system, but that the cytotoxic effects become predominant if their concentration increases beyond a certain level. Accordingly, studies performed on hamster fibroblasts have shown a growth-stimulatory effect of low concentrations of superoxides and hydrogen peroxides, whereas these molecules become growth inhibiting and cytotoxic at higher concentrations. It has recently also been demonstrated that the antioxidant α-tocopherol inhibits growth of PDGF-stimulated SMCs, adding further support to the hypothesis that free radicals may play a role in the stimulation of SMC replication.

Our knowledge of the mechanisms involved in regulation of the PDGF genes in SMCs is still very limited. Raines et al have reported that interleukin-1 stimulates PDGF A gene transcription in human SMCs, and it has also been demonstrated that transforming growth factor–β has a similar effect. More is known about the regulation of the PDGF genes in cultured endothelial cells. By using nuclear runoff analysis, Kavanough and coworkers have demonstrated that transforming growth factor–β stimulates transcription of both the A- and B-chains, whereas thrombin and phorbol esters stimulate the expression of B-chain transcripts only. These authors were also able to show that forskolin, a potent activator of adenyl cyclase, completely blocked thrombin-induced transcription of the B-chain.

Studies analyzing the effect of preparations of chemically modified and oxidized LDL on gene expression in cultured vascular cells have been equivocal. In contrast
FIGURE 8. Line plots showing effect of lipoprotein exposure time on stimulation of platelet-derived growth factor (PDGF)–induced DNA synthesis. Subconfluent human iliac artery smooth muscle cells grown on glass coverslips in 24-well culture plates were growth arrested by transfer to Ham's medium F-12 for 48 hours. The cells were then exposed to 10 μg/ml native (■—■) or oxidized (▲—▲) low density lipoprotein for the indicated times, rinsed, and kept in medium F-12 for 16 hours. They were then incubated with 4 ng/ml PDGF AA (panel A) or PDGF BB (panel B), 0.1% bovine serum albumin, and 2 μCi/ml [3H]thymidine for 24 hours, and the fraction of labeled nuclei was then determined by autoradiography. Each value represents mean±SD of triplicate cultures.

to our present results, Fox and DiCorleto37,38 have in an elegant series of studies been able to demonstrate that acetylated LDL inhibits production of PDGF in endothelial cells. Oxidized LDL has been found to inhibit the release of PDGF39 and tumor necrosis factor40 from macrophages. The mechanism responsible for this difference is presently unclear, but differences in the concentrations used and addition of antioxidants, as well as differences in the chemical composition of the lipoprotein preparations, may play a role.

In the concentrations used here, PDGF AA is a less potent mitogen for human SMC than is PDGF BB. This may at least partly be due to the higher number of PDGF β-receptors on the cells. This may also explain why conditioned media from cells exposed to oxidized LDL, which should contain approximately 1.5 ng/ml PDGF AA, do not give rise to a significant increase in DNA synthesis in rat arterial SMCs (Table 1). However, in cells stimulated to produce PDGF AA by exposure to oxidized LDL, intracellular interaction with PDGF α-receptors may be more efficient and result in stimulation of DNA synthesis. A small increase in DNA synthesis was also observed when native LDL–exposed cells were stimulated with PDGF AA, whereas no response was seen in cells preincubated with oxidized LDL (Figure 5). The lack of effect of PDGF AA in the latter cells may be due to the fact that PDGF α-receptors are occupied by endogenously produced PDGF A or to downregulation of PDGF α-receptors.

Analysis of conditioned medium from cells exposed to native and oxidized LDL by a radioreceptor assay indicated that oxidized LDL stimulated secretion of PDGF AA from the cells. The interpretation of this assay is complicated by the fact that any PDGF BB in the medium would displace the binding of both 125I–PDGF AA and BB, whereas any PDGF AA in the medium would displace only 125I–PDGF AA. However, in the present experiments no increase in 125I–PDGF BB

FIGURE 9. Line plots showing lag phase required for lipoprotein-dependent stimulation of platelet-derived growth factor (PDGF)–induced DNA synthesis. Subconfluent iliac artery smooth muscle cells grown on glass coverslips in 24-well culture plates were growth arrested by transfer to Ham's medium F-12 for 48 hours. The cells were then exposed to 10 μg/ml native (■—■) or oxidized (▲—▲) low density lipoprotein for 1 hour, rinsed, and kept in medium F-12 for the indicated times. They were then incubated with 4 ng/ml PDGF AA (panel A) or PDGF BB (panel B), 0.1% bovine serum albumin, and 2 μCi/ml [3H]thymidine for 24 hours, and the fraction of labeled nuclei was then determined by autoradiography. Each value represents mean±SD of triplicate cultures.
competing activity occurred, indicating that the increase in \(^{125}\text{I}-\text{PDGF AA}\) competing activity is explained solely by increased amounts of PDGF AA in the conditioned medium.

Both native and oxidized LDL stimulated DNA synthesis in the absence of exogenous mitogens. The mechanism responsible for this increase is not clear, but in cells exposed to oxidized LDL may at least partly be accounted for by endogenous production of PDGF AA and autocrine stimulation of cell growth. Alternatively, lipoproteins may have direct mitogenic effects on SMCs. Several studies have reported an increased rate of proliferation of arterial SMCs grown in hypercholesterolemic serum.\(^1^8\) However, it has not been possible to identify the mechanism responsible for the growth-stimulatory effect. Libby and coworkers\(^1^9\) have demonstrated that lipoproteins are not growth factors in themselves but rather function by potentiating growth of mitogen-stimulated cells. The present finding that LDL increases the number of PDGF \(\beta\)-receptors on SMCs may provide an explanation for these results.

It is important to keep in mind that the present results may be valid only for cultured cells. Furthermore, many experiments were performed on cells between passages eight and 16, raising the possibility that they may have obtained transformed properties, but the finding that these cells were unable to form colonies in soft agar argues against this possibility. Another confounding factor that should be taken into account is the possibility that the observed effects are due to endotoxin contamination of the lipoprotein preparations. However, this explanation is less likely because the endotoxin levels were very low, the effects of both native and oxidized LDL were inhibited by preincubation with superoxide dismutase, and isolated endotoxins added in an identical manner were without growth-stimulatory effect. The present findings may have interesting implications for the connection between hypercholesterolemia and the intimal proliferation of SMCs characteristic of atherosclerosis. A high serum level of cholesterol enhances the penetration of LDL particles into the arterial intima\(^1^4\) where they may become oxidatively modified by endothelial cells and SMCs.\(^1^2\) This LDL could then initiate SMC proliferation by activating an endogenous production of PDGF AA in the cells, leading to an autocrine stimulation of cell growth, and by increasing the number of PDGF receptors, making the cells more responsive to stimulation with PDGF released from surrounding macrophages and endothelial cells. There are several lines of evidence supporting the hypothesis that regulation of PDGF AA secretion and PDGF receptor expression are important for the development of atherosclerosis. SMCs in human atherosclerotic plaques express PDGF AA,\(^1^5\) and SMCs isolated from such plaques produce a PDGF-like mitogen.\(^1^6\) In contrast to SMCs in the normal media, SMCs in human atherosclerotic plaques express PDGF \(\beta\)-receptor mRNA\(^1^7\) and PDGF \(\beta\) surface receptors.\(^1^7\) The present results suggest that the expression of PDGF AA and PDGF \(\beta\)-receptors found in SMCs in human atherosclerotic plaques may be partly due to the interaction of SMCs with LDL.

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References


Native and oxidized LDL enhances production of PDGF AA and the surface expression of PDGF receptors in cultured human smooth muscle cells.

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