Lipoproteins Are Major and Primary Mitogens and Growth Promoters for Human Arterial Smooth Muscle Cells and Lung Fibroblasts In Vitro

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Abstract Smooth muscle proliferation leading to excessive intimal thickening is of prime importance in atherosclerosis. Human arterial smooth muscle cells (SMCs) and human lung fibroblasts are rather insensitive to mitogens under plasma-free conditions in vitro. This prompted us to study the distribution and nature of the growth-promoting material in human plasma. SMCs were obtained from explants of human aortic media. More than 80% of the growth-promoting activity of plasma was present in the lipoprotein (LP) fraction. The growth-promoting capacity of the different LPs was determined on fractions isolated with density gradient ultracentrifugation. Cytotoxic effects appeared if low-density lipoprotein (LDL) was not protected from oxidation and were aggravated with platelet-derived growth factor (PDGF)-BB. Very-low-density lipoprotein, LDL, and high-density lipoprotein (HDL) stimulated DNA replication and cell growth by themselves. The stimulation was considerable and equaled that obtained with PDGF-BB only. It was strongly increased in the presence of PDGF-BB. The effect on SMCs was not uniform for subfractions of HDL. A light portion inhibited growth in the absence but strongly stimulated it in the presence of PDGF-BB. For fibroblasts, HDL subfractions had a uniform effect, suggesting a cell type–dependent difference. Addition of cholesterol or essential fatty acids did not induce a growth response similar to that of LPs. This speaks strongly against mere nutritional supplementation as responsible for the mitogenic and growth-promoting effect of LPs and suggests that the effect may be more specific. Disordered LP metabolism is strongly related to atherosclerosis, and certain LPs have a potential role for the deposition of lipids. In addition to this, the distinct mitogenic and growth-stimulating effect of LPs by themselves, as demonstrated in the present report, suggests a mechanism by which intimal thickening, which is a prerequisite for atherosclerosis, may be induced. The pronounced amplification of this effect with PDGF-BB, a substance that also has been implicated in atherogenesis, might promote growth leading to the excessive intimal thickening in the atherosclerotic plaque. (Arterioscler Thromb. 1994;14:288-298.)

Key Words • vascular smooth muscle • arteries • atherosclerosis • humans • cell division • growth substances • platelet-derived growth factor • lipoproteins • VLDL • LDL • HDL

Excessive increase of intimal smooth muscle is one of the most characteristic changes in atherosclerosis. Intimal proliferation has a key role in several other conditions leading to arterial insufficiency, e.g., restenosis after balloon dilatation, neointimal occlusion of grafts and coronary bypasses, and chronic vascular rejection after transplantation. Despite its importance, mechanisms responsible for the control of arterial intimal growth are as yet largely unknown.

The number of identified plasma constituents with influence on cellular growth is rapidly increasing, but the nature of plasma components necessary for sustained growth of untransformed cells in vitro is still unknown. In earlier experiments, we found that human arterial smooth muscle cells (SMCs) and human lung fibroblasts were rather insensitive to mitogens under plasma-free conditions in vitro, and this prompted us to investigate the material in plasma that permits multiplication and sustained growth of these cell types. The introductory experiments aimed at optimizing conditions for isolation and fractionation of lipoproteins and for cell incubation were, for convenience, guided by assays on fibroblast cultures, whereas the detailed effects under optimal conditions of effects of lipoproteins on replication and cell growth were done with both cell types.

Methods

Chemicals

Recombinant human platelet-derived growth factor, isoform BB (PDGF-BB) was purchased from Sigma except for the experiment shown in Fig 2, for which PDGF-BB was kindly provided by Professor C.-H. Heldin, Ludwig Institute for Cancer Research, Uppsala Branch, Uppsala, Sweden. Cholesterol (tissue-culture grade) was purchased from Sigma and purified by chromatography on aluminum oxide (see below). Recombinant human growth hormone, methionylated form (Somatonorm) and recombinant human insulin-like growth factor (IGF-I, native form) were obtained from KabiVitrum AB, Stockholm, Sweden. Epidermal growth factor was purchased from Collaborative Research (Waltham, Mass). Other chemicals are specified in the description of the separate experiments.

Cell Culture and Assay of Cell Growth

Human arterial tissue was obtained from abdominal aortas in conjunction with nephrectomy of young male kidney donors with fatal injuries. Isolation of the inner media by microdissection in buffered tissue-culture medium with elimination of
were passaged by trypsinization. For passaged cells, the culti-
vally"-like growth pattern and by immunohistochemical iden-
tification of α-smooth muscle actin with a specific monoclonal
antibody. Fibroblasts were plated (see "Methods") in multiwells to three parallel culture groups to be used for counting of cells, determination of culture size with the
MTT method (see "Methods"), and counting of nuclei. 

Each culture group received 5000, 10 000, 20 000, and 30 000 cells per well, with six parallel wells for each group for each cell number level. After cell attachment overnight, cells and nuclei were counted and the MTT values were determined. The coef-
ficient of variation was ≤10% for all three methods, and the y
error bars expressed as SEM are not visible because they do not
project outside the symbols.

the endothelium and connective tissue was described in detail
elsewhere. The isolated arterial media was raised into primary culture in a 1:1 mixture of Ham’s F12 and William’s
medium E (Gibco) containing 20 mmol/L N-hydroxethylpip-
egraine-N'-2-ethanesulfonic acid buffer, pH 7.3, 2.6 mmol/L
L-glutamate, 50 μg/mL neomycin, and 5% human whole-blood
serum, with incubation in 5% CO₂ in air at 37°C. The cells
were passaged by trypsinization. For passaged cells, the culti-
vating medium was MCDB 104 (Gibco) with 2.6 mmol/L
L-glutamate, 100 μg/mL streptomycin, 100 U/mL penicillin,
and 10% human whole-blood serum. SMCs were used between
the second and sixth passages. The smooth muscle nature of
the cultures was confirmed by their characteristic “hill-and-
valley”-like growth pattern and by immunohistochemical iden-
tification of α-smooth muscle actin with a specific monoclonal
antibody. Fibroblasts were human fetal lung fibroblasts (State
Bacteriological Laboratory, Stockholm, Sweden), and for the
present purpose they were used up to the 16th passage.

For growth measurements, cells were detached by
trypsinization; suspended in MCDB 104 with 2.6 mmol/L
L-glutamate, 100 μg/mL streptomycin, 100 U/mL penicillin,
and 3% platelet-poor plasma serum (PPPS); and plated into
multiwells (96-well plates for tissue culture; Falcon) that were
coated with 15 μg/cm² of fibronectin isolated from human
plasma. The number of cells plated was low enough to keep

the cultures well under confluence during the experiment. For SMCs, 4000 cells were plated per well. The fibroblasts were
smaller; therefore, 8000 cells were plated per well to give a cell
density similar to that of SMCs. After 24 hours, the PPPS-
containing medium was changed to plasma-free medium, and
the cells were incubated for at least 72 hours to obtain
quiescence. For this purpose, we used MCDB 104 with 2.6
mmol/L L-glutamate, 100 μg/mL streptomycin, 100 U/mL penicillin, 10
μg transferrin (Collaboratoy Research), and 2 mg ovalbumin
(grade V, Sigma) per milliliter.

This medium, with addition of 1 μmol/L insulin and 5
μg/mL cholesterol (see below), was used during the subse-
quent period of exposure to growth factors and samples in the
experiments in the first phase of the study. Later, MCDB 104
was changed to Iscove’s modified Dulbecco’s medium (to avoid
copper and iron components, with restriction of the ovalbumin
to 1.8 mg/mL and with inclusion of 0.2 mg/mL fatty acid-free
human serum albumin (Sigma) to serve as a peroxyl radical
trap. The other additions were unchanged or modified as
described in the figure legends.

Access to exogenous cholesterol is required for optimal
growth of certain cell types under cholesterol- and serum-free
conditions. Thirty thousand cells per dish were plated on fibronectin-
coated dishes as described in "Methods." After 3
days under serum- and plasma-free conditions, platelet-derived growth factor (PDGF)-BB, insulin-
like growth factor (IGF)-I, and human growth hor-
mones (hGH) (10, 2, and 40 ng/mL, respectively)
were added as indicated in the figure. Medium and
additions as above were renewed twice a week.

Triple or quadruplicate (days 12, 14) cultures of
each series were taken for cell counting at the time
intervals shown. Nineteen days later endothelial
growth factor (EGF) (5 ng/mL) and fibronectin (FN)
to a concentration as in 5% serum) or serum (5%)
were added as indicated to groups of triplicate
dishes of both previous series of cultures. The
experiment was terminated 1 week later.
various samples for which the effect on growth was to be tested. For studies on DNA replication, the cultures were incubated for 24 hours with the factors to be tested, after which 5 of

is given in the description of the respective experiment. For cultures in multiwells, the culture size was determined by measurement of mitochondrial activity as described in Reference 13 by the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to blue formazan product. In short, 25 µL of a solution of MTT in PBS (5 mg/mL) was added to each well culture, and the cultures were incubated in 5% CO₂ in air at 37°C for 5 hours. When MTT is reduced, it is converted to blue formazan crystals during incubation, and after incubation, these were dissolved by addition of 70 µL of 13% sodium dodecyl sulfate in 0.013N HCl during an additional period of 16 hours of incubation. The absorbance was read at 590 nm in an EIA multiwell photometer. Growth as measured by the MTT method was expressed as the net increase of absorbance after subtraction of the background absorbance of the growth factor–free control group for groups without PDGF-BB and of the PDGF-BB control group for cultures with added PDGF-BB and expressed as “MTT units.” The relation between MTT values and cell number, as estimated from the number of cells plated and counting of cells and nuclei, was studied in control experiments (Fig 1). There was a direct and linear relation between the number of cells and nuclei and MTT values from 5000 cells per multiwell.

Protein was measured by the method of Lowry et al10 as modified by Maxwell and coworkers11 with serum albumin as the standard.

Intracellular lipid was visualized by staining with fat red 7B (catalog No. F-1000, Sigma) as described in Reference 17.

Lipoprotein Isolation and Fractionation

Blood was collected from healthy male donors after 12 hours of fasting. During collection, the blood was gently mixed with the following additions and at the stated final concentrations: EDTA (sodium salt) 3.2 mmol/L, Na₂HPO₄ 0.02%, gentamicin sulfate 80 mg/L, soybean trypsin inhibitor 85 mg/L, Land proline 3 mg/L. The blood was immediately chilled on ice and plasma prepared by centrifugation at 3000g for 30 minutes at 4°C. The following inhibitors were added to the plasma to the stated final concentrations: benzamide 1 mmol/L, phenylmethylsulfonyl fluoride (PMSF) 2 mmol/L, pepstatin A 0.1 µmol/L, and butylated hydroxytoluene (BHT) 10 µmol/L. Platelet-poor whole plasma (WP) for isolation of lipoproteins was prepared by centrifugation of the plasma at 22 000g for 30 minutes at 4°C and collection of the supernatant. The lipoproteins were separated by density cushion ultracentrifugation of the WP essentially as described by Rudel et al18 with slight modifications. A "mock" plasma solution with the same background salt density as plasma (d = 1.0063) was used for the following composition was prepared: 0.02 mol/L phosphate buffer, pH 7.4, with 0.15 mol/L sodium chloride; 3 mmol/L EDTA; 10 µmol/L BHT; 1 mmol/L benzamide; 0.1 g/L Na₂HPO₄; and 80 mg/L gentamicin sulfate. The mock plasma was adjusted to a density of 1.225 by addition of solid KBr, and 7.0 mL was pipetted into a Quick-Seal centrifuge tube for an angle-head Ti 50.2 rotor for a Beckman L8M ultracentrifuge. WP was adjusted to density 1.31 by addition of solid KBr, and 32 mL was gently submerged below the density-adjusted mock plasma solution (d = 1.225). The tubes were centrifuged at 50 000 rpm (302 000g-max) for 22 to 26 hours at 4°C with slow acceleration and deceleration. The lipoproteins floated to the top of the tube and were collected by gentle suction into a syringe after puncturing of the tube wall. The heavy bottom zone of the tube was collected as lipoprotein-depleted plasma, and the intermediate zone was discarded.
The lipoproteins were further fractionated according to density with density gradient ultracentrifugation in a swinging-out 41 Ti rotor at 41,000 rpm (288,000g-max) for 24 hours and at 4°C. The solutions for the different density layers in the gradients were prepared by addition of KBr to the above-mentioned mock plasma. In some experiments, the gradients were shaped by layering density cushions as described by Kelly and Kruski to cover a density range of 1.006 to 1.31 and in some experiments as described by Pitas and Mahley to cover a density range of 1.006 to 1.21.

Fractionation of lipoproteins by gel filtration was done as described by Rudel et al on the agarose preparation BioGel A 5m, 200 to 400 mesh (Bio-Rad), and with conditions for elution as described.

**Results**

The inability for sustained growth in vitro of human arterial SMCs in the absence of plasma or serum is illustrated in Fig 2. Sustained growth was not obtained even with addition of PDGF-BB, PDGF-BB combined with IGF-I and human growth hormone, or PDGF-BB supplemented with epidermal growth factor, and, to promote attachment and spreading, fibronectin. This inability was not a result of cell senescence or other cellular changes, as indicated by the strong growth response elicited by addition of serum as late as 3 weeks after the start of the experiment. We did not find a capacity to promote sustained growth of human arterial SMCs and fetal lung fibroblasts under plasma- and serum-free conditions among a large number of known growth factors tested either alone or combined at different concentrations (not shown).

Preliminary studies on the plasma material necessary for sustained growth of SMCs had indicated that it was sensitive to proteolysis and oxidation. Protracted fractionation methods tend to increase the risk for artificial modification of the material. To obtain a rough estimate of the distribution of the activity among different major plasma fractions, we therefore decided initially to use fractionation by graded precipitation that could be done under conditions that minimized artificial change. Platelet-poor citrate plasma with additions to prevent proteolysis and oxidation (see the legend to the Table) was treated with BaCl₂ to remove prothrombin. Preliminary experiments had shown that the activity was not adsorbed to BaCl₂. The plasma was then fractionated by precipitation with ammonium sulfate followed by polyethylene glycol (PEG) at selected pH and solute concentrations to yield the major plasma fractions, immunoglobulins, albumin, and PEG-precipitated-minus albumin and -minus immunoglobulins (Table). More than 80% of the activity was found in the last-mentioned fraction (Table).

Gel filtration of an aliquot of the PEG-precipitated active material yielded four fractions (Fig 3), one (Fig 3, peak IV) minor, with elution properties identical to albumin, and the others (Fig 3, peaks I through III) with larger apparent molecular sizes. The elution properties of the latter fractions were similar to those reported for plasma lipoproteins. The activity was confined to the lipoprotein-like material as judged from direct counting of mitotic events in the cultures and from growth measurements with the MTT method.
To confirm its lipoprotein nature, the PEG-precipitated, active material was fractionated by density gradient ultracentrifugation as for plasma lipoproteins and the material separated into fractions corresponding to chylomicrons, very-low-density lipoproteins (VLDL), low-density lipoproteins (LDL), and high-density lipoproteins (HDL) (Fig 4).

The capacity of different lipoproteins to promote sustained growth was then studied directly. Plasma lipoprotein classes, as defined by particle size, were isolated by gel filtration (Fig 5, A) and, as defined by density, with ultracentrifugation in density gradients (Fig 6, A).

For lipoproteins as defined by particle size, HDL stimulated sustained growth of fetal lung fibroblasts...
with density gradient ultracentrifugation were used. Further studies, therefore, lipoprotein fractions isolated by gel filtration indicated that fractionation with the former method was not suitable for our purpose. For ultracentrifugation indicated that fractionation with the density gradient ultracentrifugation (see “Methods”) of material used for measurements of effects on growth in experiments shown in Figs 7 and 8. LDL indicates very-low-density lipoprotein; LDL, low-density lipoprotein; and HDL, high-density lipoprotein.

(Fig 5, B). LDL had a strong inhibitory effect paralleled by signs of cytotoxicity as judged from apparent cell injury and death in the cultures (not shown). These experiments were carried out in the absence of PDGF-BB.

For lipoproteins as defined by apparent density (Fig 6, A), all classes of lipoproteins strongly promoted the growth of fibroblasts both without and with PDGF-BB (Fig 6, B). Addition of PDGF-BB increased growth promotion by VLDL and HDL but decreased growth with LDL (Fig 6, B). Light-microscopic changes did not occur in the fibroblast cultures for any lipoprotein class in the absence of PDGF-BB or in cultures with PDGF-BB to which VLDL or HDL had also been added (not shown). For cultures that had received LDL and PDGF-BB, there was a varying degree of cellular swelling (Fig 6, C) and accumulation of lipids (Fig 6, D), and some cells had the appearance of foam cells (Fig 6, D). Cell death or injury was not apparent, however, which tallies with the considerable, although somewhat inhibited, growth even in these cultures (Fig 6, B, fractions 4 through 7).

The cytotoxic and inhibiting effects of LDL isolated according to size and density, respectively, indicated oxidative changes. Strict precautions had been taken to minimize changes of the material by oxidation and proteolysis during all manipulations of the material from the collection of the blood, during all steps of fractionation to the addition to the cultures (see “Methods”). These precautions and the starting lipoprotein material were identical for both types of lipoprotein isolations. The much more pronounced injurious effect of LDL isolated by gel filtration than by density gradient ultracentrifugation indicated that fractionation with the former method was not suitable for our purpose. For further studies, therefore, lipoprotein fractions isolated with density gradient ultracentrifugation were used.

Cell cultivation media generally contain copper and/or iron salts, which may promote changes by oxidative injury and death in the cultures (not shown). These precautions and the starting lipoprotein material were identical for both types of lipoprotein isolations. The much more pronounced injurious effect of LDL isolated by gel filtration than by density gradient ultracentrifugation indicated that fractionation with the former method was not suitable for our purpose. For further studies, therefore, lipoprotein fractions isolated with density gradient ultracentrifugation were used.

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Growth of human fetal lung fibroblasts without and with PDGF-BB and with addition of different lipoprotein fractions

**Addition to cell cultures**

![Graph showing effect of different density gradient lipoprotein fractions (see Fig 7) on growth of human fetal fibroblasts without and with platelet-derived growth factor (PDGF)-BB. With exception of the cell type and an incubation period of 3 days for the fibroblasts, the conditions were identical to those for the experiment shown in Fig 8, and the experiments shown in Figs 8 and 9 were performed strictly in parallel and with identical additions to both series. Growth values for growth factor-free and PDGF-BB controls were 665±8 and 702±6 MTT units, respectively. The values for platelet-poor plasma serum were 1122±57 and 1381±33 and for lipoprotein-depleted serum, 834±16 and 945±18 MTT units without and with PDGF-BB, respectively.

**LDL, and the heavier part of HDL (Fig 8). Thus, these lipoprotein fractions had, for SMCs, a direct growth-promoting effect in the absence of additional growth factor. In contrast, the lighter part of HDL inhibited growth in the absence of PDGF-BB, but for cultures to which PDGF-BB had been added, a strong stimulation was obtained. PDGF-BB stimulated growth by itself, and there was a general stimulating effect by added PDGF-BB over the whole lipoprotein density spectrum. All density classes of lipoproteins stimulated the growth of the fibroblasts even without PDGF-BB (Fig 9), and the stimulation increased when PDGF-BB was present. Essential inhibition did not occur. The stimulating effect of PDGF-BB as a single addition was weaker than that for SMCs.

The effects on SMC replication and growth of the pooled density gradient lipoprotein fractions are shown in Fig 10. All three lipoprotein classes were mitogenic by themselves, and the magnitude was considerable and equaled that of PDGF-BB (Fig 10, A). The incorporation of [3H]thymidine was strongly increased when the lipoproteins were combined with PDGF-BB, and for LDL and HDL, the incorporation equaled that for lipoprotein-depleted plasma serum and PPPS in combination with PDGF-BB. The lipoproteins also stimulated SMC growth by themselves to a degree that equaled that for PDGF-BB and lipoprotein-depleted plasma serum (Fig 10, B). As for thymidine incorporation illustrated above, SMC growth was strongly stimulated when the lipoproteins were combined with PDGF-BB.

Formation of lipid-loaded cells with the appearance of foam cells or inhibition of growth in the presence of PDGF-BB did not occur in the experiments shown in Figs 8 through 10, for which the culture medium did not contain copper and iron, in contrast to similar experi-
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Lipoproteins and Arterial Smooth Muscle Growth

Stimulation of cell growth by exposure to lipoproteins under certain conditions has been noted previously (see “Discussion”), and it has been assumed that this may be an effect of nutritional factors such as cholesterol and essential fatty acids provided by the lipoproteins. Several cell types, including the cells used in this study, require exogenous cholesterol for optimal growth under serum-free conditions. Exogenous cholesterol was provided in all growth experiments in the present work, and the experiment shown in Fig 11, A was undertaken to evaluate the effect of exogenous cholesterol and to ensure that the supply of cholesterol was not a limiting factor. The effect of cholesterol given initially or daily during the growth assay was compared for growth factor–free cultures, cultures with PDGF-BB, and/or cultures with total lipoproteins. There was no further stimulating effect with daily additions except for a marginal effect for the growth factor–free cultures, and the scheme with an initial addition of cholesterol was therefore adopted. The results indicate, furthermore, that the cholesterol component of lipoproteins is not responsible for the stimulation of growth.

The experiment shown in Fig 11, B through D, was undertaken to evaluate whether the strong growth-stimulating effect of lipoproteins might be due merely to supplement of essential fatty acids. The effects with increasing concentration of oleic and linoleic acids and of total lipoproteins without or with PDGF-BB were compared with strictly parallel culture groups in the same experiment. For oleic acid, there was a weak stimulation at the lower concentrations (Fig 11, B). For linoleic acid, there was also a weak stimulation but only at the lowest concentration (Fig 11, C). There was a pronounced cytotoxicity at higher concentrations for both fatty acids, and the toxic effect was considerably stronger for linoleic acid.

There was a strong direct concentration-dependent growth-stimulating response in the presence of lipoprotein
Distribution of Growth-Stimulating Activity After Fractionation of Plasma by Graded Precipitation With Ammonium Sulfate and Polyethylene Glycol at Different pH

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Net Increase of Growth, MTT Units, Mean±SD</th>
<th>Volume, mL</th>
<th>Total Activity</th>
<th>Growth-Stimulating Activity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precipitate ammonium sulfate, pH 6.5 (immunoglobulins)</td>
<td>88±16</td>
<td>17</td>
<td>1 496</td>
<td>7</td>
</tr>
<tr>
<td>Precipitate PEG, pH 4.5 (lipoproteins)</td>
<td>2544±824</td>
<td>7</td>
<td>17 808</td>
<td>82</td>
</tr>
<tr>
<td>Soluble in PEG, pH 4.5 (albumin)</td>
<td>55±9</td>
<td>45</td>
<td>2 475</td>
<td>11</td>
</tr>
</tbody>
</table>

PEG indicates polyethylene glycol.

Fresh-frozen platelet-poor citrate plasma was thawed and the following was added (final concentration): phenylmethylsulfonyl fluoride (PMSF) 2 mMol/L; butylated hydroxytoluene (BHT) 10 μmol/L; EDTA 2 mmol/L; aprotinin 3 μg/mL; pepstatin A 0.1 μmol/L; STI 85 μg/mL. The plasma was chilled on ice and gently stirred during all following steps, and centrifugations were done at 4°C. Barium chloride (1 mol/L) was added dropwise (80 mL/L plasma). After 1 hour, the suspension was centrifuged for 10 minutes at 5000g and the supernatant recovered for further fractionation. No activity had been found in the sediment in preceding experiments (not shown), and it was discarded. The pH of the supernatant was controlled, and benzamidine was added to 1 mmol/L and BHT to 10 μmol/L. Saturated ammonium sulfate solution in 50 mmol/L phosphate buffer, pH 6.5, with 2 mmol/L EDTA was added dropwise to a final concentration of 50% saturation of ammonium sulfate. After 15 minutes, the suspension was centrifuged at 4000g for 15 minutes. The sediment was saved for activity measurement. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) indicated immunoglobulins as major components (not shown). The pH of the supernatant was adjusted to 4.5 with hydrochloric acid, and after 15 minutes, the suspension was centrifuged as in the preceding step. The supernatant was saved for activity measurement, and the sediment was dissolved in a minimum volume of phosphate-buffered saline (PBS), pH 6.1, and the pH adjusted to 6.1 with sodium hydroxide. The solution was dialyzed (exclusion limit, 6000-8000, Spectrapore) against PBS, pH 6.1, with 1 mmol/L benzamidine and 10 μmol/L BHT. A small precipitate was removed by centrifugation as above. A 50% PEG 4000 solution, pH 6.1, was added to a final concentration of 12% PEG. After 15 minutes, the suspension was centrifuged as above. The pH of the supernatant, which had a light yellow color, was adjusted to 7.4. SDS-PAGE indicated that this fraction was composed mainly of albumin (not shown). The sediment was dissolved in a minimum volume of PBS, pH 7.4, with 1 mmol/L benzamidine and 10 μmol/L BHT. The color of the solution was red-brown. Aliquots of the fractions were dialyzed against PBS (Dulbecco), and their activity was measured with the MTT method on fibroblasts in the absence of platelet-derived growth factor-BB.

that leveled off at the highest concentration (Fig 11, D). Cytotoxicity was not noted. The responses observed for the fatty acids and for lipoprotein indicate that mere supplementation with essential fatty acids is not a likely mechanism for the stimulation of growth by lipoproteins.

Discussion

It is well known that untransformed human cells may require as yet unidentified plasma components for sustained growth in vitro even in the presence of competence factors such as PDGF and progression factors such as insulin and IGF-1.34 The human arterial SMC is no exception to this, and even the combination of growth factors like PDGF-BB, epidermal growth factor (EGF), human growth hormone, and IGF-1 supplemented with fibronectin to improve attachment and spreading does not support sustained growth (see Fig 2). The requirements for growth of this cell type are of great interest, since this growth plays a key role in the development of arterial stenosis due to intimal thickening especially apparent in atherosclerosis.1 This cell type also plays an important role in several other pathological processes.29 For example, foam cells appear in the cultures. This indicates that LDL promoted growth less than the same protective measures taken. However, in the absence of platelet-derived growth factor-BB.

lipoprotein-depleted serum26-30,31,34 or growth factors (PDGF, insulin, transferrin,25 EGF, fibroblast growth factor [FGF]30). Some groups have found that LDL prepared from hyperlipidemic31-33 or diabetic35 subjects promotes growth of serum-exposed cultures more strongly than LDL from normolipidemic subjects.

For HDL, the results are even more disparate. HDL was found to stimulate growth for cultures exposed to lipoprotein-depleted serum34 or to growth factors (insulin+transferrin+EGF or FGF34; insulin+PDGF29). In contrast, there are several studies in which HDL did not stimulate growth under similar conditions.26,31,32 Very little is known about the effects of HDL under strict serum- and growth factor-free conditions. Gospodarowicz et al37 noted stimulation of growth with HDL for SMC cultures seeded on dishes coated with endothelial extracellular matrix, but Libby and collaborators29 found that HDL by itself produced little or no growth in serum-free medium.

As to other lipoproteins, neither VLDL32 nor native lipoprotein(a)33 was found to stimulate SMC growth. These experiments were, however, performed in the presence of 5% calf serum, which might have concealed effects of the lipoproteins added.

Motivated by the earlier experiences of others and our own of the appearance of cytotoxic material in LDL fractions isolated with sequential flotation ultracentrifugation, a technique for which the risk of oxidative changes has also been pointed out in the literature,26 we tested less protracted fractionation methods. Cytotoxic material appeared in the LDL fraction after separation by gel filtration despite measures for optimal protection against oxidation and proteolysis. Cytotoxicity was not observed after fractionation with density gradients with the same protective measures taken. However, in the presence of PDGF-BB, LDL promoted growth less than when PDGF was absent, and lipid-loaded cells resembling foam cells appeared in the cultures. This indicates
that LDL was, despite these measures, still modified. These changes occurred only in the presence of PDGF-BB, which may be taken to suggest that modified LDL may have been taken up more effectively in the presence of PDGF-BB. It is known that PDGF activates scavenger receptor genes in vascular SMCs and fibroblasts, which suggests that induction of scavenger receptors might be responsible for the observed effects of modified LDL. Another possible mechanism might be that aggregation of the modified LDL had been promoted in the presence of PDGF-BB followed by an increased uptake by endocytosis.

For these experiments, the lipoproteins had been protected against oxidative changes throughout all manipulations except for the exposure to cells and culture medium during the growth assay. When factors that promote oxidation were eliminated in this step also by exclusion of copper and iron components from the cultivation medium and addition of serum albumin as a peroxyl radical trap, the above-mentioned indicators for oxidative changes of the LDL fraction disappeared.

Even with as different techniques for plasma fractionation as graded precipitation, gel filtration, and density gradient ultracentrifugation, we uniformly found the stimulating activity for sustained growth present in the lipoprotein fraction, and the present work demonstrates that VLDL, LDL, and HDL by themselves had a direct and primary growth-promoting effect on SMCs and fibroblasts and that these lipoproteins promoted sustained growth even in the absence of PDGF-BB.

With the lighter portion of the HDL fraction, a clear inhibition was obtained for SMCs without PDGF-BB. With PDGF-BB, in contrast, there was a strong stimulation. This PDGF-dependent transition from inhibition to strong stimulation was not seen for fibroblasts that were stimulated by HDL even in the absence of PDGF-BB. It is possible, therefore, that the PDGF-dependent transition from inhibition to strong stimulation is SMC-specific. The distribution of this effect in the density gradient may indicate that the effect is connected to a subfraction of HDL, HDL2. It may also point to lipoprotein(a). The latter alternative is of special interest from an atherogenic point of view, since lipoprotein(a) has an atherogenic potential. We are currently engaged in studies designed to clarify this point.

The response of the fibroblasts was different from that of SMCs in other respects as well. Detailed analysis of the effect of the separate lipoprotein classes showed stimulation of growth by material from the whole density spectrum, and the effect was enhanced in the presence of PDGF-BB. The inhibition noted by some fractions for SMCs was not observed for the fibroblasts. In addition, for the fibroblasts, LDL had a much stronger stimulating effect than for SMCs. This difference was very pronounced in the presence of PDGF-BB.

The different responses of SMCs and fibroblasts; the distinct and primary growth-promoting effects of VLDL, LDL, and HDL if properly protected against oxidation; and the different effects on growth of the various lipoproteins and subfractions are of great interest and have not, as far as we know, been demonstrated earlier. The different responses to lipoproteins of the two cell types might be a manifestation of differences between a potentially contractile cell type, such as the SMC, and fibroblasts. Alternatively, the different lipoprotein responses may reflect differences between adult and fetal cells, keeping in mind that the SMCs of the present work were derived from adult aortic media and the fibroblasts from fetal tissue. From an atherogenic point of view, the latter explanation may be as interesting as the former. It has been proposed that the intimal SMC, i.e., the cell that is responsible for building up the intimal thickening, would be derived from immature stem cells that are activated by arterial injury and possibly other mechanisms with relation to atherosclerosis. It is thought that such cells might retain certain fetal properties. From this aspect, it is interesting to note the prompt induction of apparently vital, lipid-loaded cells, some of which appeared as foam cells, from the fetal fibroblasts when they were incubated with slightly changed LDL (in the presence of PDGF-BB) and the fact that the fetal lung fibroblasts showed the strongest stimulation for growth with LDL, considered as an atherogenic lipoprotein, in contrast to the adult medial SMCs, which were less sensitive. A further, more detailed comparative analysis of the effects of LDL on young and old SMCs and fibroblasts was not carried out because it was clearly beyond the aims of the present study.

It has been assumed that the growth-promoting effect of lipoproteins noted for mitogen-stimulated cells may be due to mere supplementation with nutrients. Addition of cholesterol or essential fatty acids did not induce a growth response similar to that of lipoproteins, as noted in the present study. This speaks strongly against mere nutritional supplementation as responsible for the mitogenic and growth-promoting effect of lipoproteins and suggests that the effect may be more specific.

Disordered serum lipoprotein metabolism is strongly related to the development of atherosclerosis, and certain lipoproteins have a potential role for deposition of lipids and formation of foam cells in the intima. In addition to this role, the distinct mitogenic and growth-stimulating effects of VLDL, LDL, and HDL by themselves, as demonstrated in the present report, may point to a mechanism by which the intimal thickening, which is a prerequisite for atherosclerosis, may be induced. The pronounced amplification of this effect in the presence of PDGF-BB, a substance that has also been implicated in atherogenesis, might be a mechanism for the promotion of SMC growth to the excessive intimal thickening in the atherosclerotic plaque.

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