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Modified low density lipoproteins suppress production of a platelet-derived growth factor-like protein by cultured endothelial cells

(acetylated low density lipoprotein/atherosclerosis/lipid accumulation)

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ABSTRACT **Cultured endothelial cells (EC) produce a platelet-derived growth factor-like protein (PDGF-c) that stimulates the growth of cultured cells of mesenchymal origin. We have examined the effect of native plasma low density lipoprotein (LDL) and chemically modified LDL on production of PDGF-c by EC. Acetyl-LDL, but not native LDL, suppressed the production of PDGF-c by bovine aortic EC. Half-maximal inhibition was observed at a concentration of 25–75 μg of cholesterol per ml, and maximal inhibition (0–25% of control) at 150 μg of cholesterol per ml. EC treated with acetyl-LDL showed no morphological damage, there was no change in cell number, and the effect on production of PDGF-c was substantially reversed upon removal of the acetyl-LDL. The observed inhibition of PDGF-c production was specific, since total cellular and secreted protein synthesis were unaffected by acetyl-LDL. Acetyl-LDL suppressed PDGF-c production in both bovine aortic and human umbilical vein EC, but not in rat heart EC. This cell specificity correlated with the presence of scavenger receptors as measured by degradation of ^{125}I-labeled acetyl-LDL and uptake of fluorescently labeled acetyl-LDL. Dimethylpropanediamine-LDL, a cationic modified lipoprotein, also inhibited PDGF-c production. The inhibition by both types of modified LDL was accompanied by significant intracellular cholesterol accumulation, suggesting a role for EC lipid composition in the regulation of production of PDGF-c.**

Platelet-derived growth factor (PDGF) is a potent mitogen for cultured cells of mesenchymal origin (see ref. 1 for review). The principal source of PDGF in human serum is the platelet, but PDGF-like proteins are also secreted by cells in culture, including several transformed cell lines (2, 3) and several classes of cells relevant to vascular pathology, including macrophages (4), arterial smooth muscle cells under certain conditions (5, 6), and endothelial cells (EC) (7–9). The latter cells secrete multiple mitogens, including a PDGF-like protein (designated PDGF-competitor protein or PDGF-c), which has been identified as such by biochemical and immunological criteria (10) and by its ability to competitively block binding of ^{125}I-labeled PDGF to its specific cell-surface receptor (11). In addition, Collins *et al.* (12) recently demonstrated an mRNA from cultured EC with complete homology to the larger (the B chain) of the two heterodimeric chains of PDGF.

Conditions affecting EC production of PDGF-c or expression of its gene have recently been reported. Bovine aortic EC constitutively secreted PDGF-c (as well as non-PDGF-like mitogens) into serum-free medium at a constant rate for at least 3 weeks; certain agents that caused EC injury, such as endotoxin and phorbol esters, stimulated production up to 4-fold (13). Jaye *et al.* (14) showed that the reversible organization of human umbilical vein EC into three-dimensional tubular structures was accompanied by decreased levels of mRNA for the B chain of PDGF. Evidence for production of PDGF-c by endothelium *in vivo* was recently demonstrated by Barrett *et al.* (15), who reported detectable levels of mRNA for PDGF B in freshly isolated EC from human umbilical vein and bovine aorta. The levels of PDGF-c message per cell were, however, at least an order of magnitude less than the level expressed by cultured EC.

Although there are no reports of lipid-loading by endothelium *in vivo*, the accessibility of these cells to serum lipoproteins makes cellular lipid composition a strong candidate for regulation of EC function. EC, like macrophages, have an active scavenger receptor and preferentially bind, internalize, and degrade acetylated low density lipoprotein (LDL) (16) or malondialdehyde-altered LDL (17) relative to native LDL. Several recent reports described alterations in EC and macrophage function due to the uptake of modified lipoproteins via the scavenger receptor (18–20). In this study, the effects of LDL and chemically modified LDLs on production of PDGF-c by EC are reported.

MATERIALS AND METHODS

Reagents, Cell Cultures, and Lipoproteins. All tissue culture materials were obtained from GIBCO with the exception of bovine serum from HyClone (Logan, UT). Homogeneous human PDGF was purified by the method of Raines and Ross (21) and ^{125}I-labeled PDGF (^{125}I-PDGF) was prepared as described (22). L-[4,5-^{3}H]leucine (130 Ci/mmol; 1 Ci = 37 GBq) was obtained from Amersham and [^{14}C]oleic acid (50 mCi/mmol) was from ICN. Bovine serum albumin used in binding assays was from Armour (CRG-7; Kankakee, IL). Crystalline bovine serum albumin (Sigma), sterilized by passage through a 0.22-μm filter (Millipore), was used in long-term incubations. Soybean trypsin inhibitor and all reagents for analysis of cell cholesterol and DNA were obtained from Sigma.

Human LDL was isolated from fresh plasma by differential ultracentrifugation as the $1.019 < d < 1.060$ g/ml fraction as described (23). LDL prepared by this method has been shown to be free of protein contamination by immunoelectrophoresis (24). Homogeneity of the LDL was confirmed by the appearance of a single band after agarose gel electrophoresis (23). LDL was acetylated (acetyl-LDL) by reaction with acetic anhydride according to the method of Fraenkel-Conrat

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Abbreviations: DMPA, dimethylpropanediamine; EC, endothelial cell(s); LDL, low density lipoprotein; PDGF, platelet-derived growth factor; ^{125}I-PDGF, ^{125}I-labeled PDGF; PDGF-c, PDGF-like protein that competes for binding to the PDGF receptor; ^{125}I-acetyl-LDL, ^{125}I-labeled acetylated LDL; diI-acetyl-LDL, acetylated LDL labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate.

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(25). The extent of acetylation was measured using trinitrobenzenesulfonic acid according to the method of Habeeb (26); 50–75% of the lysine residues were modified in all acetyl-LDL preparations. Homogeneity and altered electrophoretic mobility of acetyl-LDL were confirmed by agarose gel electrophoresis. Dimethylpropanediamine LDL (DMPA-LDL) was prepared according to the method of Basu *et al.* (27). ^{125}I-labeled acetyl-LDL (^{125}I-acetyl-LDL) was prepared by acetylation of LDL iodinated by the method of Pittman *et al.* (28). Native LDL, acetyl-LDL, DMPA-LDL, and ^{125}I-acetyl-LDL were dialyzed exhaustively against 0.9% NaCl/0.3 mM EDTA, pH 7.0, and sterilized by filtration. Acetyl-LDL labeled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (dil-acetyl-LDL) was purchased from Biomedical Technologies (Cambridge, MA).

Primary cultures of bovine aortic EC, rat heart EC, human umbilical vein EC, and human foreskin fibroblasts were isolated and maintained in medium containing 5% calf serum as described (29). The medium used was a mixture (1:1) of Dulbecco–Vogt modified Eagle's medium and Ham's F-12 medium supplemented as described (29). In the experiments reported here, the bovine EC were used between passages 15 and 25, and rat heart EC were used between passages 12 and 15. Identical results were obtained with bovine EC between passages 6 and 40. The human umbilical vein EC were used as primary cultures only.

Collection of Conditioned Medium and Cells. Confluent cultures of EC in 4-cm^2 wells were rinsed with serum-free medium and the test lipoprotein was added in 0.5 ml of serum-free medium containing bovine serum albumin (2 mg/ml). Unless otherwise noted, the cells were incubated for 3 days at 37°C in a humidified atmosphere containing 5% CO_2/95% air. The EC-conditioned medium was centrifuged at 8000 × *g* for 10 min to remove loosened cells and debris. To measure cellular cholesterol and DNA, the cells were released from dishes by treatment with trypsin and were quantitatively transferred to a microfuge tube containing trypsin inhibitor. Cells were collected by centrifugation and dispersed in phosphate-buffered saline in a Branson sonifier (Heat Systems, Plainview, NY) with a standard microtip for 10–15 sec.

Analytical Procedures. PDGF-c in EC-conditioned medium was quantitated by a specific radioreceptor assay, sensitive to 0.05 ng of PDGF, measuring competition with ^{125}I-PDGF for binding to PDGF receptors, and was carried out essentially as described (13) utilizing subconfluent cultures of human foreskin fibroblasts as target cells. This assay has been validated for use with bovine aortic and human umbilical vein EC in that the protein measured by the radioreceptor assay was biochemically and immunologically indistinguishable from PDGF (10, 11). EC protein synthesis was determined by incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable material as described (13); newly synthesized cellular protein and secreted protein were determined separately. The activity of the acetyl-LDL (scavenger) receptor was measured by degradation of ^{125}I-acetyl-LDL to form trichloroacetic acid-soluble noniodide material as described by Goldstein and Brown (30). The presence of scavenger receptors on EC was also examined qualitatively by fluorescence microscopy of cells incubated with dil-acetyl-LDL as described by Voyta *et al.* (31). Esterification of cholesterol was determined by incubating cells with an albumin complex containing [^{14}C]oleic acid (0.27 mM, final concentration) and measuring formation of [^{14}C]cholesteryl ester by hexane/ethanol extraction of the cells and thin-layer chromatography as described (32). Cell DNA was measured by the method of Labarca and Paigen (33) with calf thymus DNA as standard. Lipoprotein cholesterol was measured by a colorimetric procedure (Boehringer Mannheim) and cellular cholesterol was measured by the method of Gamble *et al.* (34) with calibrated human serum as standard.

All results shown are representative of at least two experiments. Unless otherwise indicated, all measurements were made on triplicate wells and the results are expressed as the mean ± SEM.

RESULTS

Incubation of confluent bovine aortic EC with acetyl-LDL resulted in concentration-dependent suppression of production of PDGF-c (Fig. 1). The half-maximal inhibition in this experiment was at ≈60 μg of cholesterol per ml. This number ranged from 25 to 75 μg of cholesterol per ml with several acetyl-LDL preparations. In some experiments, the amount of PDGF-c in the medium, after incubation of cells with maximal doses of acetyl-LDL, was below the level of detection of the radioreceptor assay (≈0.05 ng). The inhibition was specific for acetyl-LDL since native LDL, at a cholesterol concentration at which acetyl-LDL showed maximal inhibition, did not affect PDGF-c production (Fig. 1). Bovine serum albumin acetylated to the same extent as LDL (69%) and added at similar protein concentration (100 μg/ml) also did not alter PDGF-c production (data not shown). In a cell-free control experiment, the concentration of PDGF-c in EC-conditioned medium was not affected by incubation with acetyl-LDL, thus showing that the decreased production of PDGF-c was not due to modification or binding of the protein by acetyl-LDL and subsequent inability to be recognized by the PDGF receptor. Furthermore, addition of acetyl-LDL during both phases of the radioreceptor assay—i.e., the initial competition with unlabeled PDGF and the subsequent incubation with ^{125}I-PDGF—did not affect the results of the assay. To further test whether acetyl-LDL inhibited the appearance of PDGF-c by binding to the protein, acetyl-LDL was added to medium after a conditioning interval that allowed accumulation of PDGF-c in the medium. Acetyl-LDL did not decrease the amount of PDGF-c in preconditioned medium in the presence of cells (Table 1). This experiment further showed that the decreased appearance of PDGF-c was not due to increased degradation of secreted PDGF-c but was likely due to decreased production (or possibly increased degradation of a precursor).

Three lines of evidence suggested that the suppression of PDGF-c production was a specific effect rather than the result of acetyl-LDL-mediated injury to the EC. First, no significant morphological changes were discerned by phase-

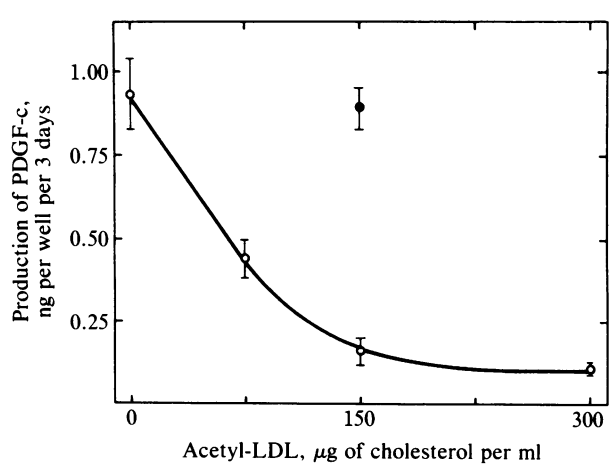

FIG. 1. Effect of acetyl-LDL on production of PDGF-c by bovine aortic EC. Bovine aortic EC were incubated for 3 days with acetyl-LDL (○) or native LDL (●) at the concentrations shown. The amount of PDGF-c in the conditioned medium was determined by radioreceptor assay.

Table 1. Effect of acetyl-LDL on the level of PDGF-c in previously conditioned medium

Addition to wells		PDGF-c in conditioned medium, ng per well
0–72 hr	72–96 hr	
Medium	—	0.88 ± 0.02
Acetyl-LDL	—	0.04 ± 0.02
Medium	Medium	1.23 ± 0.08
Medium	Acetyl-LDL	0.91 ± 0.11

Bovine aortic EC were incubated for 72 hr with or without acetyl-LDL (150 μg of cholesterol per ml). In separate but identically treated wells, EC were incubated in medium without acetyl-LDL for 72 hr. Incubation was then continued for 24 hr with or without acetyl-LDL (150 μg of cholesterol per ml). The conditioned medium was removed after 72 or 96 hr as indicated, and the amount of PDGF-c was determined by radioreceptor assay.

contrast microscopy after incubating cells for 3 days with acetyl-LDL (up to 400 μg of cholesterol per ml). Second, the number of attached cells, as measured by DNA content, was unaffected by incubation with acetyl-LDL. Finally, the effect of acetyl-LDL on PDGF-c production was reversible. After bovine aortic EC were incubated with acetyl-LDL for 3 days, replacement of medium with medium without acetyl-LDL resulted in substantial restoration (65–100%) of the production of PDGF-c compared to cells not incubated with acetyl-LDL. The specificity of acetyl-LDL with respect to the proteins affected was also examined. Neither EC secreted protein synthesis nor cell layer protein synthesis was affected by incubation of cells with acetyl-LDL (Fig. 2). This argues for specificity of the effect and also provides further evidence for the viability of the cells.

A time course of the effect of acetyl-LDL on production of PDGF-c is shown in Fig. 3. The decrease in production of PDGF-c was detectable after incubation of cells with acetyl-LDL for 8 hr. Shorter incubation periods were not examined because of the sensitivity limitation of the radioreceptor assay. No production of PDGF-c in the presence of acetyl-LDL was observable after 8 hr.

All EC cultures examined in our laboratory, including cells isolated from several species, produce significant amounts of PDGF-c. The effect of acetyl-LDL on PDGF-c production was examined in EC from bovine, human, and rat sources.

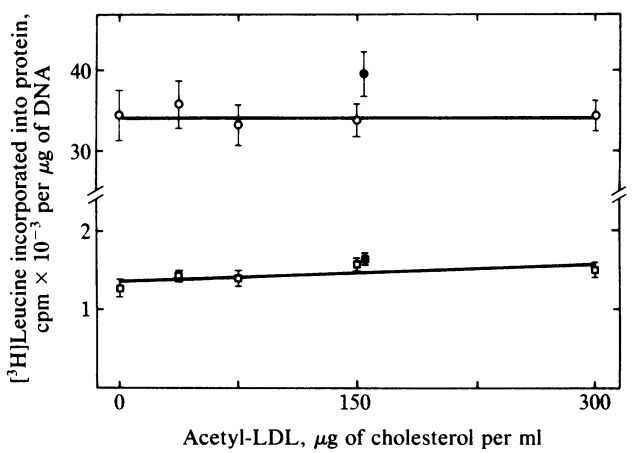

FIG. 2. Effect of acetyl-LDL on protein synthesis by bovine aortic EC. Bovine aortic EC were incubated for 64 hr with acetyl-LDL or native LDL at cholesterol concentrations shown. [3H]Leucine (3 μCi per well) was then added and the cells were further incubated for 8 hr in the presence of the added lipoprotein. Protein synthesis was determined as described. Cell-layer protein synthesis (○, acetyl-LDL; ●, native LDL) and secreted protein synthesis (□, acetyl-LDL; ■, native LDL) were determined separately.

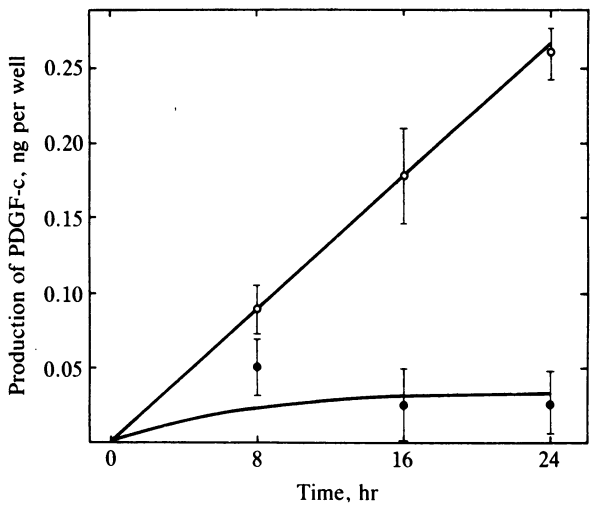

FIG. 3. Time course of production of PDGF-c. Bovine aortic EC were incubated in the absence (○) or presence (●) of acetyl-LDL (300 μg of cholesterol per ml). Conditioned medium was collected from triplicate wells for each time point at 8-hr intervals. The amount of PDGF-c in the conditioned medium was determined by radioreceptor assay.

Acetyl-LDL suppressed production in both bovine aortic and human umbilical vein EC but not in rat heart EC (Table 2). To test whether the species differences reflected differences in acetyl-LDL uptake, cells were compared with respect to their ability to degrade ^{125}I-acetyl-LDL. Degradation of ^{125}I-acetyl-LDL was measured rather than binding because degradation measures entry of the lipoprotein into a metabolically active pool and is not susceptible to problems of nonspecific binding. The bovine and human EC degraded ^{125}I-acetyl-LDL to a much greater extent than the rat heart EC and human foreskin fibroblasts, indicating that only the bovine and human EC have scavenger receptors (Fig. 4); the total absence of the scavenger receptor on human fibroblasts has been demonstrated (35). These results were confirmed by studies in which cultures were incubated with dil-acetyl-LDL, a fluorescent marker used for identification of EC; both bovine aorta and human umbilical vein EC showed extensive fluorescence following incubation (4 hr at 37°C) with dil-acetyl-LDL, whereas the rat heart EC showed background levels of fluorescence (data not shown).

The possibility that PDGF-c production is correlated with EC cholesterol content was investigated. The effect of acetyl-LDL on cellular cholesterol (free plus esterified) is shown in Fig. 5. In seven separate experiments, the cholesterol content of bovine aortic EC was increased by 20–50%

Table 2. Effect of acetyl-LDL on production of PDGF-c by EC from several species

EC source	Acetyl-LDL, μg of cholesterol per ml	Production of PDGF-c: ng per well per 3 days	Production of PDGF-c: % inhibition
Bovine aorta	0	1.04 ± 0.03	
	150	0.14 ± 0.08	87
	300	0.03 ± 0.03	97
Human umbilical vein	0	2.34 ± 0.16	
	150	0.03 ± 0.02	99
	300	0.06 ± 0.04	97
Rat heart	0	1.91 ± 0.07	
	150	2.04 ± 0.05	0
	300	1.92 ± 0.04	0

EC were incubated with acetyl-LDL for 3 days. PDGF-c in the conditioned medium was determined by radioreceptor assay.

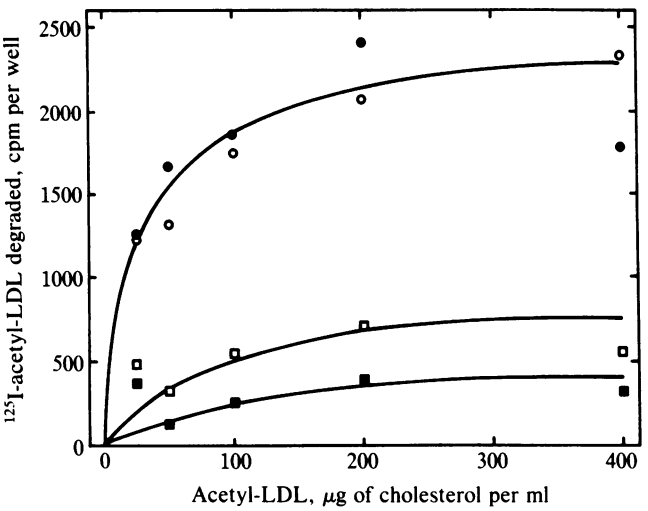

FIG. 4. Degradation of ^{125}I-acetyl LDL by EC isolated from several species. ^{125}I-acetyl-LDL (3122 cpm per μg of cholesterol) was incubated for 18 hr with confluent cultures of bovine aortic EC (●), primary human umbilical vein EC (○), rat heart EC (□), and human foreskin fibroblasts (■) in 0.5 ml of serum-free medium containing 2 mg of bovine serum albumin per ml. The cell-mediated degradation was calculated by subtracting the trichloroacetic acid-soluble, noniodide radioactivity in cell-free wells from that in wells with cells. The radioactivity in the cell-free wells, due to degradation of ^{125}I-acetyl-LDL during both storage and incubation at 37°C, was 0.25% of the total radioactivity added and, at nonsaturating concentrations of ^{125}I-acetyl-LDL, it was ≈30% of that in the wells with bovine or human EC. Standard errors in duplicate wells were generally <5% of the mean.

by prior incubation with a saturating concentration of acetyl-LDL. An alternative method for loading cells with cholesterol was examined to test the necessity for cholesterol entry by the scavenger pathway. LDL made positively charged by covalent linkage to DMPA presumably binds nonspecifically to electronegative regions of the cell surface and is subsequently internalized by adsorptive endocytosis (18). Incubation of bovine aortic EC with DMPA-LDL resulted in a large increase in cellular cholesterol and diminished production of PDGF-c (Table 3). The effect of DMPA-LDL was specific since there was no inhibition of total protein synthesis. To demonstrate that cholesterol from acetyl-LDL and DMPA-LDL entered a metabolically active pool and was not simply adsorbed to the cell surface, esterification of cholesterol with

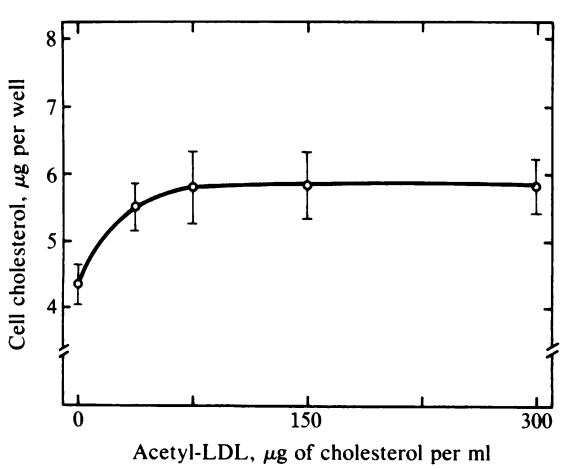

FIG. 5. Effect of acetyl-LDL on cell cholesterol. Bovine aortic EC were incubated for 3 days with acetyl-LDL at the concentrations indicated. Total cholesterol (free plus esterified) was determined as described.

Table 3. Effect of DMPA-LDL on production of PDGF-c by bovine aortic EC

	PDGF-c, ng per well per 3 days	Cell cholesterol, μg per well	Total protein synthesis, cpm per well
Medium alone	0.88 ± 0.02	2.53 ± 0.89	24,000 ± 100
DMPA-LDL	0.17 ± 0.09	7.35 ± 1.47	26,100 ± 800

Bovine aortic EC were incubated for 3 days with DMPA-LDL (20 μg of cholesterol per ml) or with medium alone. The amount of PDGF-c in the conditioned medium was determined by radioreceptor assay. Protein synthesis by the cell layer and cell cholesterol was determined as described.

[^{14}C]oleic acid to form [^{14}C]cholesteryl oleate was measured. Acetyl-LDL and DMPA-LDL stimulated cholesterol esterification 2.2-fold and 4.5-fold, respectively, compared to lipoprotein-free controls in a 24-hr incubation, indicating entry of the cholesterol into an active cellular pool; LDL did not significantly stimulate esterification.

DISCUSSION

In this report, we have shown that acetyl-LDL dramatically and specifically suppresses production of PDGF-c by EC. The inhibition is not due to injury or cell death, because the cells are unchanged by morphological criteria, there is no change in cell number, and the inhibition is reversed upon removal of acetyl-LDL. Furthermore, acetyl-LDL does not inhibit synthesis of total cellular or secreted protein, suggesting not only viability of the EC cultures but also specificity of the acetyl-LDL with respect to the proteins affected. The production of the non-PDGF-like mitogens by bovine aortic EC was also unaffected by acetyl-LDL (data not shown). Although the mechanism by which acetyl-LDL exerts its effect has not been fully resolved, it is likely that the scavenger receptor is required since (*i*) the amounts required for half-maximal degradation of ^{125}I-acetyl-LDL and half-maximal inhibition of PDGF-c production are similar and (*ii*) PDGF-c production is inhibited by acetyl-LDL only in cells possessing an active scavenger receptor. However, since it is unlikely that the cationic DMPA-LDL is recognized by the scavenger receptor, it is probable that modified LDLs entering the cell by several pathways can suppress production of PDGF-c.

The decreased appearance of PDGF-c may be due to inhibition of one of the biosynthetic steps—e.g., gene transcription, mRNA translation, intracellular processing, or secretion. The last alternative, defective secretion, is unlikely since lysis of acetyl-LDL-treated EC did not release a measurable quantity of PDGF-c (data not shown). In view of the report that binding of modified proteins by the scavenger receptor triggers neutral protease secretion by murine macrophages (36), the effect of acetyl-LDL on degradation of PDGF-c was examined. Addition of acetyl-LDL to preincubated EC-conditioned medium did not decrease the amount of PDGF-c. It remains possible, however, that the modified lipoproteins stimulate degradation of intracellular PDGF-c or a precursor. Evidence for high molecular weight precursors to PDGF has, in fact, been obtained by immunoprecipitation of cell extracts of human osteosarcoma cells that produce PDGF (37).

The molecular species responsible for suppression of PDGF-c production has not been identified. Components of modified LDLs that are candidates include apoprotein B, cholesterol, cholesteryl esters, neutral glycerides, phospholipids, and possibly oxidized lipids. A role for cholesterol in this regulatory process is possible since suppression of PDGF-c production by modified LDLs is always accompanied by significant cholesterol accumulation. Increased cel-

lular cholesterol content can alter the level of expression of several proteins including the LDL receptor and hydroxymethylglutaryl (HMG)-CoA reductase (38). Cholesterol decreases the level of HMG-CoA reductase in UT-1 cells, a varient of Chinese hamster ovary cells, by decreasing the rate of transcription of the gene coding for HMG-CoA reductase and increasing the rate of degradation of the enzyme (39). The significance of cholesterol in the suppression of PDGF-c production by acetyl-LDL has not been proven; it is possible that other acetyl-LDL-derived lipids may accumulate in the EC and be responsible for this effect.

The inhibition by modified lipoproteins of PDGF-c production may help to resolve an apparent inconsistency that has recently become evident. The amount of PDGF (c-*sis*) mRNA in endothelium freshly scraped from bovine aorta is at least an order of magnitude less than the level expressed by cultured EC (15). The increased expression of PDGF mRNA by EC *in vitro* may be explained by a difference in lipid composition of cultured cells versus cells *in vivo*. We speculate that EC *in vivo*, continuously exposed to physiological concentrations of plasma lipoproteins, maintain a sufficiently high level of a regulatory component (e.g., cholesterol) to suppress production of PDGF-c. Cultured EC, however, may be deficient in this regulatory component, resulting in constitutive PDGF-c production. Modified lipoproteins might supply the deficient component to cultured cells and restore physiological control to PDGF-c production. Whether this model is proven to be true or not, the ability to reversibly alter this function of EC should prove useful in understanding the role of EC-derived growth factors in vascular pathology, and more generally, in understanding the regulation of growth factor production by normal and transformed cells.

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