Metabolic Cooperation between Vascular Endothelial Cells and Smooth Muscle Cells in Co-culture: Changes in Low Density Lipoprotein Metabolism

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ABSTRACT A microcarrier co-culture system for aortic endothelial cells and smooth muscle cells (SMCs) was developed as a model for metabolic interactions between cells of the vessel wall. Low density lipoprotein (LDL) metabolism in SMCs was significantly influenced by co-culture with endothelium. The numbers of high affinity receptors for LDL was increased more than twofold (range, 2.1–5.6), with concomitant increases in LDL receptor-mediated endocytosis and degradation. These effects reached a plateau at an endothelial cell/SMC ratio of 1. Kinetic analysis of the endocytic pathway for LDL in SMCs indicated that, in co-culture with endothelium, there was no alteration in the binding affinity of LDL to its receptors but that the internalization rate constant declined and the rate constant for degradation increased. This analysis suggested that the formation and migration of endocytic vesicles was the rate-limiting step of enhanced LDL metabolism under co-culture conditions.

Two mechanisms by which endothelial cells influenced smooth muscle LDL metabolism were identified. First, mitogen(s) derived from endothelial cells stimulated entry of SMCs into the growth cycle, and the changes in LDL metabolism occurred as a consequence of G1-S transition. Second, SMC lipoprotein metabolism was stimulated in the absence of mitogens by a low molecular weight (<3,500) factor or factors. Co-culture was a required condition for the latter effect, suggesting that the mediator(s) may be unstable or that cell–cell communication was necessary for expression. These results (*a*) demonstrate that vascular cell interactions can modify LDL metabolism in SMCs, (*b*) provide some insights into the mechanisms responsible, and (*c*) identify co-culture as an experimental approach appropriate to certain aspects of vascular cell biology.

Vascular endothelial cells and smooth muscle cells (SMCs)[1] co-exist in close apposition to each other in all blood vessels except capillaries. Investigations of the metabolic interactions that may occur between these cells are essential to an understanding of vascular homeostasis and the pathogenesis of vascular diseases such as atherosclerosis. A number of recent studies have drawn attention to the importance of vascular cell interactions. Pharmacological studies of endothelial cell–SMC interactions by Furchgott and Zawadzki have shown that endothelial cells are essential for the relaxation of arterial SMCs by acetylcholine in isolated blood vessel preparations (1). In tissue culture, Hajjar et al. (2) have measured changes in the activities of intracellular enzymes of cholesterol metabolism in SMCs when they were overlaid with a monolayer of endothelial cells. In the area of cellular growth control, vascular cell interactions have been investigated primarily by the use of cell-conditioned media (3–5) or by co-cultures in which endothelium and SMCs were plated together (3, 6, 7).

In attempts to dissect such interactions, it is necessary to separate effects that may be mediated via cell–cell contact

[1] *Abbreviations used in this paper:* ECCM, endothelial cell-conditioned medium; EDGF, endothelial cell-derived growth factor; PDS, plasma-derived serum; SMC, smooth muscle cell.

from those that may occur by soluble mediators. Although cell-conditioned medium may contain soluble mediators, these could become ineffective if metabolically or structurally unstable. Transfer of cell-conditioned media is of necessity unidirectional, thereby preventing feedback signaling between cells. In developing a model for vascular cell co-culture, we have used microcarrier technology (8) to provide a highly flexible arrangement for bringing different cell populations close together without their actually touching (7).

The importance of low-density lipoprotein (LDL) as a source of exogenous cholesterol for normal eukaryotic cells and in the accumulation of free and esterified cholesterol in intimal cells during early atherogenesis is well documented (9, 10). In the studies reported here, LDL metabolism was measured in SMCs co-cultured with endothelial cells. The pathway of high-affinity receptor-mediated endocytosis of LDL in SMCs was investigated by kinetic analysis. We document significant endothelial-specific changes in LDL metabolism of SMCs and identify two major mechanisms of such interactions.

MATERIALS AND METHODS

Microcarrier Cultures of Endothelial Cells: Endothelial cells were isolated from bovine aorta by collagenase digestion (11) and grown to confluence (Fig. 1 *a*) on the surface of solid plastic microcarrier beads (Biosilon, Nunc; Distributor, Vangard International Inc., Neptune, NJ) as previously described, except that after plating, the microcarriers were transferred to 1-L stirring flasks (Techne Microcarrier System MCS-104, Techne, Inc., Princeton, NJ) and maintained in suspension by gentle elliptical agitation (30 rpm). Each flask contained 5 g dry wt of microcarriers in 500 ml culture medium, corresponding to a surface area for cell growth of 2,000 cm^2. Approximately 2 $\times$ 10^8 endothelial cells per stirrer flask were generated. Culture medium (Dulbecco's modified Eagle's medium containing 10% calf serum, 2 μmol/ml glutamine, and 100 U penicillin and 100 μg streptomycin per ml) was replaced each week. Calf serum was obtained from M.A. Bioproducts (Walkersville, MD). At confluence, there were ~150 endothelial cells per bead. Cells were readily trypsinized from the plastic microcarriers (trypsin/EDTA mixture, M.A. Bioproducts) for determination of cell numbers by a Coulter Counter (Coulter Electronics Inc., Hialeah, FL) or hemocytometry. For determination of cell protein, microcarrier-bound cells were dissolved in a solution of 0.1% SDS (Bio-Rad Laboratories, Richmond, CA) and the microcarriers were vigorously pipetted. The beads settled within 30 s and the supernatant was removed for protein determination by the Lowry procedure (12).

Culture of Arterial SMCs: Bovine aortic SMCs were obtained as outgrowth from arterial explants by the method of Ross (13). After reaching confluence, they were subcultured for use in passage 1, 2, or 3. SMCs were not used beyond the third passage. In preparation for co-culture, 5 $\times$ 10^3 cells/cm^2 were plated in Costar dishes (35-mm diam.; Costar, Cambridge, MA) in Dulbecco's modified Eagle's medium containing 10% calf serum. After cell attachment and spreading, the medium was replaced with Dulbecco's modified Eagle's medium containing 5% plasma-derived serum (PDS; see below), a medium deficient in growth factors for SMCs.

Other Cells on Microcarriers: In some experiments, other cells were plated on microcarriers to serve as nonspecific controls for endothelial cells. SMCs (prepared as described above), Swiss 3T3 cells (ATCC CCL92), or normal human skin fibroblasts (ATCC CCL109) obtained from the American Type Culture Collection were grown to confluent density on microcarriers as described for endothelium.

Co-culture System: A microcarrier co-culture system was devised as detailed in reference 7. In brief, a shallow plastic cylinder was reversibly attached to the underside of a petri dish lid (Fig. 1 *b*). The lower end was closed by a silicon-treated nylon net (1-μm pore; Spectramesh Medical, Los Angeles, CA). Microcarrier cells were loaded into the cylindrical chamber. When the lid was placed onto a culture dish, the lower part of the chamber was submerged in culture medium. A second cell population (SMCs in these studies) was grown conventionally in the culture dish. Thus, the culture medium was shared by both cell populations, thereby allowing humoral interchange between them without direct cell contact. This arrangement permitted great flexibility in the numbers of microcarrier-bound cells in co-culture. The cell types were separated by removing the culture dish lid. Structural, functional, and viability characteristics of endothelial cells in the system were recently reported (8).

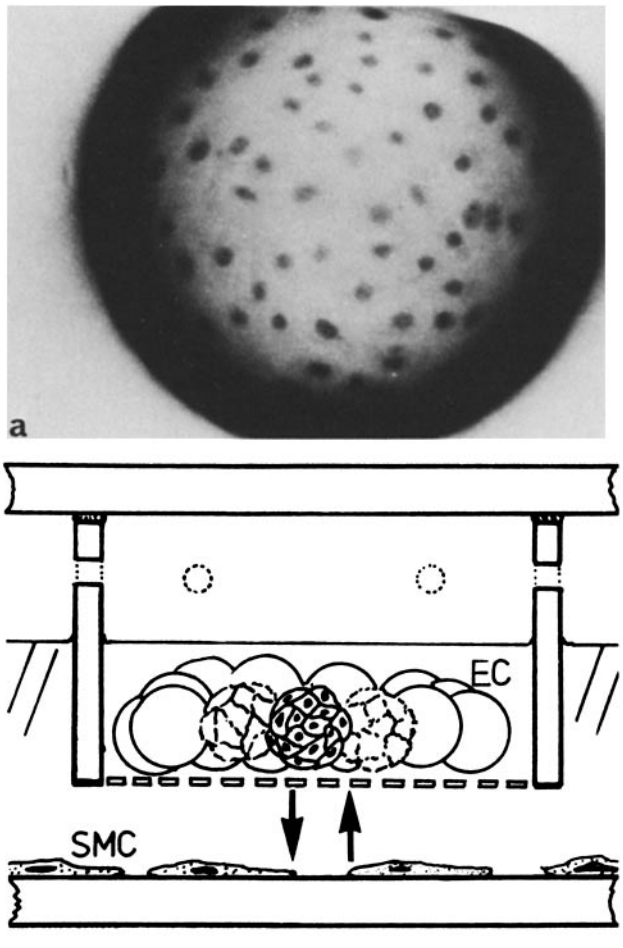

FIGURE 1 Microcarrier co-culture of bovine arterial endothelial cells and SMCs. (*a*) Solid polystyrene microcarrier with confluent monolayer of endothelium (~200 cells). Cell nuclei were stained with hematoxylin. $\times$ 375. (*b*) Diagram of co-culture arrangement. *EC*, endothelial cells. Cylinder containing microcarriers is attached to the underside of culture dish (35-mm diam) lid by a thin layer of sterile silicon grease. Distance between cylinder and SMC monolayers is 0.5–1.0 mm. Arrows indicate the potential for bidirectional humoral interactions.

In this paper, the term single culture refers to SMCs in a monolayer in the absence of any other cell type.

Dialysis Membrane Barrier between Cells: In some experiments, larger molecules (e.g., growth factors) were retained in the co-culture cylinder and smaller molecules were permitted to diffuse into the lower chamber. A dialysis membrane (cut-off of 3,500 mol wt) was secured across the opening of the cylinder by a tight-fitting plastic ring. Its effectiveness was tested using ^{125}I-labeled lysozyme, soybean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin, and insulin. The molecular weight range of these tracers was from 6,000 (insulin) to 66,200 (albumin). More than 95% of trichloroacetic acid (TCA)-precipitable ^{125}I-counts was retained in the upper cylinder in the presence of each of these tracers.

Plasma-derived and Lipoprotein-deficient Media: Platelet-poor plasma was isolated and recalcified by the methods of Vogel et al. (14). The resulting PDS was passed over a carboxymethyl-Sephadex (Pharmacia Fine Chemicals, Piscataway, NJ) chromatography column to remove residual growth factors, dialyzed against phosphate-buffered saline (pH 7.4), and stored frozen.

Lipoprotein-deficient medium was prepared by a modification of the method of Weinstein et al. (15). Human plasma obtained from the American Red Cross was dialyzed for 24 h against three changes of 0.15 M NaCl (pH 7.4) at 4°C. The density was adjusted to 1.25 g/cm^3 by the addition of NaBr. The solution was centrifuged for 48 h at 60,000 rpm in a Beckman Ti60 rotor (Beckman Instruments Inc., Palo Alto, CA). The bottom fraction was isolated and dialyzed

for 24 h against two changes of 0.1 M Tris (pH 7.4) at 4°C. Cationic growth factors were removed by carboxymethyl-Sephadex chromatography, and the solution was dialyzed then sterilized by microporous filtration (0.45 μm pore; Schleicher & Schuell, Keene, NH). This procedure resulted in a plasma fraction deficient in both lipoproteins and growth factors. The ability of culture medium containing 5% of this fraction or 5% PDS to maintain SMCs at G_0/G_1 phase of the cell cycle was tested by [^{3}H]thymidine autoradiography. Less than 10% of the cells synthesized DNA in these media.

^{125}I-LDLs: Human LDLs were isolated from fresh plasma by a modification (16) of conventional precipitation techniques (17). 2 U plasma was dialyzed against three changes of 0.15 M NaCl containing 10^{-3} M disodium EDTA, pH 8.6, at 4°C overnight. 35-ml aliquots were pipetted into centrifuge tubes to which were added 1.4 ml sodium heparin (5,000 U/ml) and 1.75 ml 1 M manganese chloride. The contents of tubes were mixed well and stood on ice for 30 min, then centrifuged at 2,400 rpm for 30 min at 4°C. The supernatant was aspirated from each tube, and the precipitate containing LDL was dissolved by the addition of 3.5 ml 2 M NaCl containing 10^{-3} disodium EDTA (pH 8.6) to each tube. After prolonged mixing, any undissolved residue was discarded. The total volume was adjusted to 50 ml with 2 M NaCl/EDTA solution, pH 8.6. 4 ml of 0.15 M NaCl at room temperature was layered on top of 8 ml of the solution, and the tubes were centrifuged at 41,000 rpm in a Beckman SW41 rotor at 15°C for 20 h. The LDL band was removed by tube puncture, pooled, and dialyzed for 24 h against three changes of 0.15 M NaCl at 4°C. Purity was assessed by double radial immunodiffusion, immunoelectrophoresis, and paper strip electrophoresis (18).

LDL was iodinated with Na^{125}I (New England Nuclear, Boston, MA) by the method of McFarlane (19) as modified by Bilheimer et al. (20). Protein concentration was measured by Lowry assay. Non-protein bound radioactivity was removed by exhaustive dialysis against 0.15 M NaCl/EDTA, pH 8.6. The integrity and quality of ^{125}I-LDL were verified by precipitation (>96%) with 10% TCA, chloroform-methanol extraction (21), immunodiffusion, and immunoelectrophoresis. LDL isolated and labeled as described above was compared with LDL isolated by sequential ultracentrifugal flotation (22). LDL prepared in either fashion gave identical results when incubated with SMCs. Specific activity of ^{125}I-LDL ranged between 100 and 400 cpm/ng protein.

Lipoprotein Metabolism: Binding, internalization, and degradation of LDL were determined using standard procedures (23). Single cultures or co-cultures of vascular cells were incubated with lipoprotein-deficient, growth factor-depleted medium for 48 h. Cells were incubated with ^{125}I-LDL at 37°C for periods appropriate to each experiment. At the end of incubation, the dishes were rapidly chilled to 4°C, and the medium was removed for determination of LDL degradation. The cells were washed six times with ice-cold Hanks' balanced salt solution containing 2 mg/ml bovine albumin, then rinsed once with Hanks' balanced salt solution alone. The cells were incubated at 4°C with Hanks' balanced salt solution containing 10 mg/ml heparin (Sigma Chemical Co., St. Louis, MO) as described by Goldstein et al. (24). The heparin-containing medium was removed and counted to determine the total amount of high affinity LDL binding. The cells were rinsed with Hanks' balanced salt solution and dissolved in 0.1% SDS for determination of internalized ^{125}I-counts and total cellular protein. LDL degradation was determined as TCA-soluble ^{125}I-monoiodotyrosine activity in the culture medium. Free ^{125}I was removed by oxidation to iodine and partition into chloroform (25).

In all experiments, nonspecific LDL metabolism was determined by inclusion of excess unlabeled LDL and was subtracted to determine receptor-mediated metabolism. Empty dish or empty microcarrier controls were incubated in parallel and subtracted. For incubations of co-cultured cells, the contribution to LDL degradation of cells on microcarriers alone was determined and subtracted from the total degradation by both populations of cells in co-culture.

Thymidine Autoradiography: Cultures were incubated with 0.1 μCi/ml [^{3}H]thymidine (6–7 Ci/mmol; New England Nuclear) for 20 h at times indicated in the figure legends, and processed for autoradiography of labeled DNA as previously described (26).

Kinetic Analysis of Receptor-mediated LDL Metabolism: The model was derived from the known characteristics of ^{125}I-apoprotein-labeled LDL metabolism (23). After reversible binding to cell surface receptors, LDL is internalized by the formation of endocytic vesicles. Within the cell, LDL dissociates from the receptor which is recycled to the cell surface. The vesicle fuses with a lysosome in which the apoprotein is degraded into its constituent amino acids which diffuse from the cell. Schematically, the process can be described as:

$$\text{LDL} + \text{Rec} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{LDL-Rec} \xrightarrow{k_2} \text{LDL}_{\text{int}} \xrightarrow{k_3} \text{LDL}_{\text{d}},$$

where LDL is the free concentration of LDL (micrograms per milliliters), and Rec, LDL-Rec, LDL_{int}, and LDL_{d} represent the free receptor, LDL-receptor complex, internalized LDL, and degraded LDL, respectively (nanograms per milligram). Ligand binding is a reversible, bimolecular reaction with association constant k_1 (milliliters per microgram per minute) and dissociation constant k_{-1} (minutes^{-1}). The rate of internalization is first order in cell surface-bound LDL with rate constant k_2 (minutes^{-1}), and the rate of degradation is first order for intracellular LDL with rate constant k_3 (minutes^{-1}).

Material balances on bound, internalized, and degraded LDL yield:

$$\frac{d[\text{LDL} - \text{Rec}]}{dt} = k_1[\text{LDL}_o][\text{Rec}] - (k_{-1} + k_2)[\text{LDL} - \text{Rec}], \quad (1)$$

$$\frac{d[\text{LDL}_{\text{int}}]}{dt} = k_2[\text{LDL} - \text{Rec}] - k_3[\text{LDL}_{\text{int}}], \text{ and} \quad (2)$$

$$\frac{d[\text{LDL}_{\text{d}}]}{dt} = k_3[\text{LDL}_{\text{int}}]. \quad (3)$$

$[\text{LDL}_o]$ represents the concentration of labeled LDL added at the beginning of the experiment and is constant. The total number of receptors is constant (27) and is equal to the sum of free and ligand-bound receptors:

$$[R_T] = [\text{LDL} - \text{Rec}] + [\text{Rec}]. \quad (4)$$

A constant of interest is K_{int}:

$$K_{\text{int}} = \frac{k_{-1} + k_2}{k_1}. \quad (5)$$

K_{int} is the ratio of the rate constants for LDL − Rec dissociation and internalization to the rate constant for binding. It is analogous to the half-saturation constant defined in Michaelis-Menten kinetics (28). k_{-1} is negligible relative to k_2 (27).

Equations 1–4 are a set of linear first order ordinary differential equations and were solved by standard methods (29). The rate constants k_1, k_2, k_3, R_T, and K_{int} were determined by simultaneous, nonlinear regression of data for bound, internalized, and degraded LDL using a Bayesian parameter estimation of multiresponse data (30) as reported previously (31).

RESULTS

Endothelial Cell–specific Stimulation of LDL Metabolism in SMCs

In experiments to measure LDL metabolism in co-culture, confluent endothelial cells on microcarriers and quiescent, subconfluent SMCs were separately pre-incubated with 5% lipoprotein-deficient growth factor–depleted medium for 48 h. The cell populations were then brought together in co-culture. ^{125}I-LDL was added at various intervals for 4 h of incubation with the cells at the end of which LDL metabolism was measured. As shown in Fig. 2, there was a significantly increased expression of LDL receptors on SMCs in the presence of endothelial cells. This effect was endothelial cell–specific because substitution of endothelial cells by more SMCs (Fig. 2), empty microcarriers, 3T3 cells, or human skin fibroblasts (data not shown) did not significantly increase LDL metabolism above single culture levels. Throughout a 54-h period of co-culture with endothelium, elevated LDL binding to SMCs was maintained or increased.

The mass of LDL bound, internalized by receptor-mediated endocytosis, and degraded in the lysosomal system of SMCs was significantly stimulated over a range of LDL concentrations (Fig. 3*a*). The effects of increasing numbers of endothelial cells upon SMC lipoprotein metabolism after co-culture for 48 h are shown in Fig. 3*b* at a fixed concentration of LDL (20 μg/ml). Enhanced LDL binding, internalization, and degradation saturated when endothelial cell protein was increased

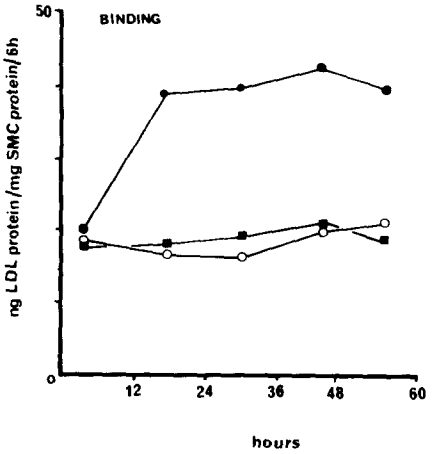

FIGURE 2 Stimulation of LDL binding to SMCs during 60 h of co-culture with endothelial cells. 2×10^5 SMC were preincubated in 2% PDS for 24 h, then in 5% lipoprotein-deficient, growth factor-depleted medium for 48 h. Microcarrier-bound endothelial cells (or SMCs for co-culture controls) were treated similarly. Co-cultures (either endothelium/SMC 1:1 [●] or SMC/SMC 0.7:1 [■] were established at time 0. ^{125}I-LDL (10 μg/ml) was added to replicate dishes for separate 6-h periods at 0, 15, 30, 44, and 54 h. At the end of each incubation with LDL, specific high affinity binding of ^{125}I-LDL to SMC receptors at 37°C was measured. Nonspecific binding and empty dish control values were subtracted in the determination of specific binding. Each point is the mean of four dishes. Standard deviations were <20% of the mean. O, SMC alone.

to 0.15 mg. This corresponds to ~2×10^5 endothelial cells. The number of SMCs in the dish was routinely $2–3 \times 10^5$; therefore in co-culture an endothelial cell/SMC ratio of ≤1 was sufficient to invoke maximum effects upon smooth muscle LDL metabolism. Bound and internalized LDL saturated at a level threefold higher than controls (0 endothelial cell protein), whereas increased LDL degradation saturated five times higher than in single culture, reflecting intracellular compartmental differences in the kinetics of LDL metabolism. To investigate these differences, we performed a kinetic analysis of LDL receptor-mediated endocytosis.

Kinetic Analysis of Stimulated LDL Metabolism

After SMCs were incubated with endothelium in co-culture or with medium alone for various intervals, ^{125}I-LDL was added for 4 h. The resulting data for smooth muscle LDL metabolism were fitted using a Bayesian parameter estimation approach with simultaneous regression of bound, internalized, and degraded LDL. This approach allowed assessment of the effects of endothelial cells upon LDL metabolism in SMCs at 37 instead of at 4°C (where measurements are restricted to determination of binding alone) and provides excellent agreement with conventional Scatchard analysis (31). The rate constants obtained are shown in Table I. The major effect of co-culture was to more than double the total number of LDL receptors expressed by SMCs (an average 3.2-fold increase; range, 2.1–5.6). In addition there were changes in the major rate constants for LDL metabolism. The association rate constant (k_1) for LDL-receptor binding decreased in co-culture, indicating that binding occurred 33% more slowly than in single culture (range, 15–38%). The rate constant for receptor-mediated endocytosis of LDL (k_2) decreased 42% in co-culture (range, 21–49%). The overall affinity of LDL for its receptor, K_{int}, however, was unchanged because K_{int} is derived from a ratio of k_2/k_1. In contrast, the rate of degradation of LDL (k_3) was increased 51% in co-culture (range, 33–74%).

To visualize how these changes in the rate constants influenced LDL metabolism, model-generated curves are presented in Fig. 4 in which differences in the number of LDL receptors have been normalized. The kinetic equations were divided by the total number of LDL receptors. This eliminated differences in receptor number between control and co-cultures. The curves for bound and degraded LDL were virtually identical for control and co-culture (Fig. 4, *a* and *c*, respectively), reflecting an unchanged affinity of LDL for the receptor, K_{int}, and an unaltered rate of degradation of the internalized compartment of LDL. The curves for LDL internalization from the surface, however, were significantly different (Fig. 4*b*), which indicates that, on a receptor-independent basis, the steady-state internalized concentration in co-culture was decreased relative to control. This occurred because of a decrease in the internalization rate constant and an increase in the degradation rate constant. In co-culture therefore, LDL was internalized less efficiently.

Mechanisms of Endothelial-specific Stimulation of LDL Metabolism in SMCs

CELL CYCLE-ASSOCIATED EFFECTS: SMCs and fibroblasts bind and degrade more LDL after exposure to purified mitogens (32–34). The changes of LDL metabolism coincide with G_1-S phases of the cell cycle. To determine whether cell cycle played a role in stimulating SMCs lipoprotein metabolism during co-culture with endothelium, we determined by [^{3}H]thymidine autoradiography the proportion of SMC nuclei that synthesized DNA during a 24-h period. The results of various treatments are shown in Table II. In lipoprotein-deficient, growth factor–depleted medium, only 2.5% of SMCs in single culture entered the growth cycle. Co-culture with endothelium, however, increased this fraction to 63%, whereas co-culture with other cells on microcarriers (control for nonspecific effects) did not stimulate DNA synthesis. Although DNA synthesis was stimulated, the SMCs did not proliferate significantly (i.e., complete the cell cycle) in lipoprotein-deficient medium, presumably because of a limited supply of exogenous sterols, which prevented completion of mitosis (35), but if sufficient lipoprotein was present (2 or 5% PDS), SMC proliferation occurred in the presence of endothelial cells (Fig. 5, *a* and *b*). In 2% PDS, the SMC proliferative response to increasing numbers of endothelial cells was approximately linear over a range of endothelial cell protein concentrations of up to 0.3 mg (Fig. 5*c*). The mitogenic effects upon quiescent SMCs in PDS or lipoprotein-deficient medium were therefore endothelial specific. Consequently, stimulation of LDL metabolism in SMCs during co-culture with endothelium was consistent with the effects of endothelial-derived mitogens, such as that described by Gajdusek et al. (3) and characterized recently by DiCorleto et al. (36).

CELL CYCLE–INDEPENDENT STIMULATION OF LDL METABOLISM IN SMCS: To separate the effects of mitogen upon LDL metabolism from any influence of smaller nonmitogenic agents, we modified the co-culture system. A dialysis membrane of molecular weight cut-off 3,500 was inserted between the endothelial cells and the SMCs as described

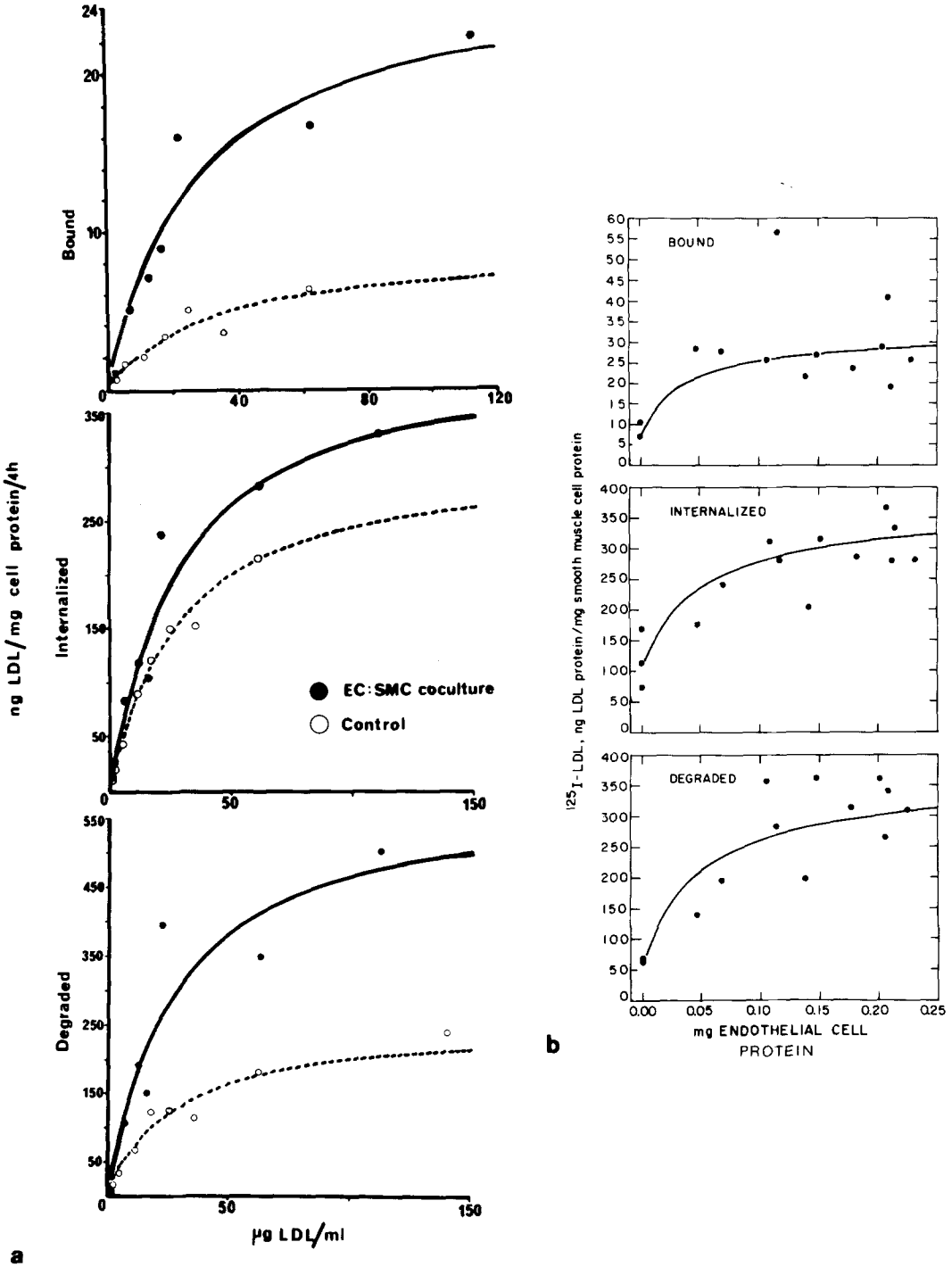

FIGURE 3 (*a*) Relation of high affinity binding, internalization, and degradation of ^{125}I-LDL in SMCs to the concentration of LDL in single cultures of SMCs and co-cultures of endothelial cells with SMCs. After preincubation with lipoprotein-deficient, mitogen-depleted medium for 48 h, 3×10^5 SMCs were incubated for 48 h more either in single culture (○) or in co-culture (●) with 2×10^5 confluent endothelial cells. After the addition of ^{125}I-LDL at various concentrations for 4 h, high affinity binding, internalization, and degradation in SMCs were determined. Each point is the mean of four dishes. Range of nonspecific LDL metabolism was 20–40% total and varied with LDL concentration. (*b*) Influence of increasing numbers of co-cultured endothelial cells on SMC high-affinity binding, internalization, and degradation of ^{125}I-LDL. 2×10^5 SMCs were incubated in co-culture with increasing numbers of confluent, microcarrier-bound endothelial cells. After 48 h, 10 μg ^{125}I-LDL was added for 4 h, and LDL metabolism was then determined in the SMCs. Each point is a single experiment. 0.1 mg endothelial cell protein is equivalent to $\sim 2 \times 10^5$ cells.

in Materials and Methods. The effects upon LDL metabolism in SMCs with the membrane in place are shown in Fig. 6*a*. More than one-third of the increase in LDL receptor expression measured in unmodified co-culture was retained with the membrane present. Furthermore, LDL internalization and degradation were stimulated to 35 and 50%, respectively, of the levels measured in unmodified co-culture. Confirmation that this effect was cell cycle–independent was obtained

TABLE I. *Kinetic Analysis* of LDL Metabolism in Cultured Arterial SMCs at 37°C*

Kinetic parameter	SMCs, single culture	Endothelial cells-SMCs, co-culture‡	% Change	
			Mean	Range
Total numbers of LDL receptors/cell, R_T	8,323 (±5,066)	27,234 (±4,824)	+220§	112–457
Binding association constant, k_1 ($ml/\mu g$ per min)	1.79×10^{-2} ($\pm 5.4 \times 10^{-3}$)	1.20×10^{-2} ($\pm 2.6 \times 10^{-3}$)	−33‖	15–38
Internalization rate constant, k_2 (min^{-1})	0.506 (±0.128)	0.294 (±0.036)	−42‖	21–49
Degradation rate constant, k_3 (min^{-1})	5.1×10^{-3} ($\pm 9.4 \times 10^{-4}$)	7.7×10^{-3} ($\pm 2.8 \times 10^{-3}$)	+51‖	33–74
Affinity constant for LDL receptor, K_{int} ($\mu g \cdot ml^{-1}$)	27.1 (±7.8)	28.5 (±6.6)	Unchanged	

* Calculated at 37°C using a Bayesian parameter estimation with simultaneous regression of bound, internalized, and degraded LDL. See Materials and Methods.
‡ Endothelial cell/SMC ratio, 1.0.
§ Significantly different from single culture, $P < 0.001$.
‖ Significantly different from single culture, $P < 0.01$.

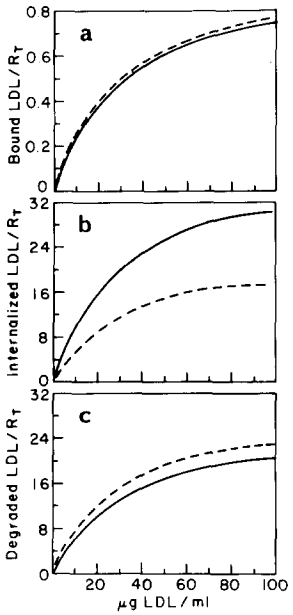

FIGURE 4 High affinity LDL metabolism in SMCs in co-culture with endothelial cells after normalization for receptor number. Data for binding (*a*), internalization (*b*), and degradation (c) of ^{125}I-LDL in SMCs in single culture and in co-culture with endothelial cells were normalized for receptor numbers. Superimposition of the curves for single culture (——) and co-culture (– – –) demonstrates unchanged affinity of LDL for the receptor (a) and similar degradation rates for the LDL already internalized and available for lysosomal proteolysis (c). The disparity in *b* demonstrates that in co-culture with endothelium, LDL was internalized less efficiently by SMCs than in single culture.

TABLE II. *[^{3}H]Thymidine Autoradiography of Cultured Arterial Smooth Muscle Cells**

Culture medium and treatment	% Labeled cell nuclei‡
5% Lipoprotein-free, growth factor–deficient serum (LPDM)	2.5 ± 0.6
2% PDS	8.5 ± 2.5
10% Calf serum	47.0 ± 14.3
0.4% Calf serum	5.4 ± 2.8
Endothelial cell–derived medium§	54.7 ± 7.5
2% PDS, co-culture with:	
Confluent endothelial cells	55.0 ± 17.1
SMCs	8.8 ± 0.7
Human skin fibroblasts	9.5 ± 1.3
5% LPDM, co-culture with:	
Confluent endothelial cells	63.0 ± 12.3
SMCs	4.6 ± 4.1

* SMCs were preincubated in 5% PDS for 48 h in all cases before switching to the media indicated after a brief wash in balanced salt solution. [^{3}H] Thymidine was added for 24 h, and the cells were washed, fixed, and processed for light autoradiography.
‡ Percentage of total cell nuclei counted (at least 1,000 per sector of dish) that were labeled by [^{3}H]thymidine.
§ 2% LPDM conditioned for 48-h with confluent endothelial cells; 1 ml/10^6 cells.

from SMC autoradiography. As shown in Fig. 6*b*, the growth fraction of SMCs remained low (4%) in co-culture in the presence of the membrane, whereas co-culture without membrane resulted in stimulation of DNA synthesis (42% labeled cells) as a result of endothelial cell–derived mitogen. Endothelial cell–conditioned medium (ECCM), concentrated sevenfold and containing potent quantities of smooth muscle mitogen, was ineffective in stimulating SMC growth (4.4% labeling) when contained within the dialysis membrane. In contrast, unconcentrated ECCM applied directly to SMCs elicited a potent mitogen effect (44% labeling).

We conclude from these studies that in co-culture endothelial cells can mediate LDL metabolism in SMCs by a mechanism independent of the SMC cycle.

CELL CYCLE–INDEPENDENT STIMULATION OF LDL METABOLISM IN SMCS REQUIRES CO-CULTURE: To determine whether conditioned medium from endothelial cells also contained a factor or factors that stimulate LDL metabolism in SMCs in the absence of cell growth, the following experiments were performed. ECCM was prepared by incubating lipoprotein-deficient, growth factor–depleted medium with confluent endothelial cells for 72 h. Part of the ECCM was concentrated fivefold and the remainder was diluted 1:1 with unconditioned medium. 1 ml of either concentrated or diluted ECCM was placed into the co-culture cylinder separated from SMCs by a dialysis membrane. As shown in Fig. 7, there was no significant stimulation of SMC LDL metabolism. In the same experiment, unconcentrated ECCM added directly to SMCs stimulated LDL binding, internalization, and degradation by 238, 360, and 560%, respectively (mitogenic response; 65.2% labeled cells). Thus, the ability of co-culture to stimulate LDL metabolism in SMCs in the absence of a mitogenic response contrasts sharply with the failure of ECCM to do so even when concentrated to fivefold potency.

These results suggest that the sharing of culture medium by both cell types is necessary for enhanced LDL metabolism by SMCs in the absence of cell proliferation and imply that either an unstable product of endothelial cells may influence LDL metabolism, or that reciprocal communication between endothelial cells and SMCs is required to activate the mechanism.

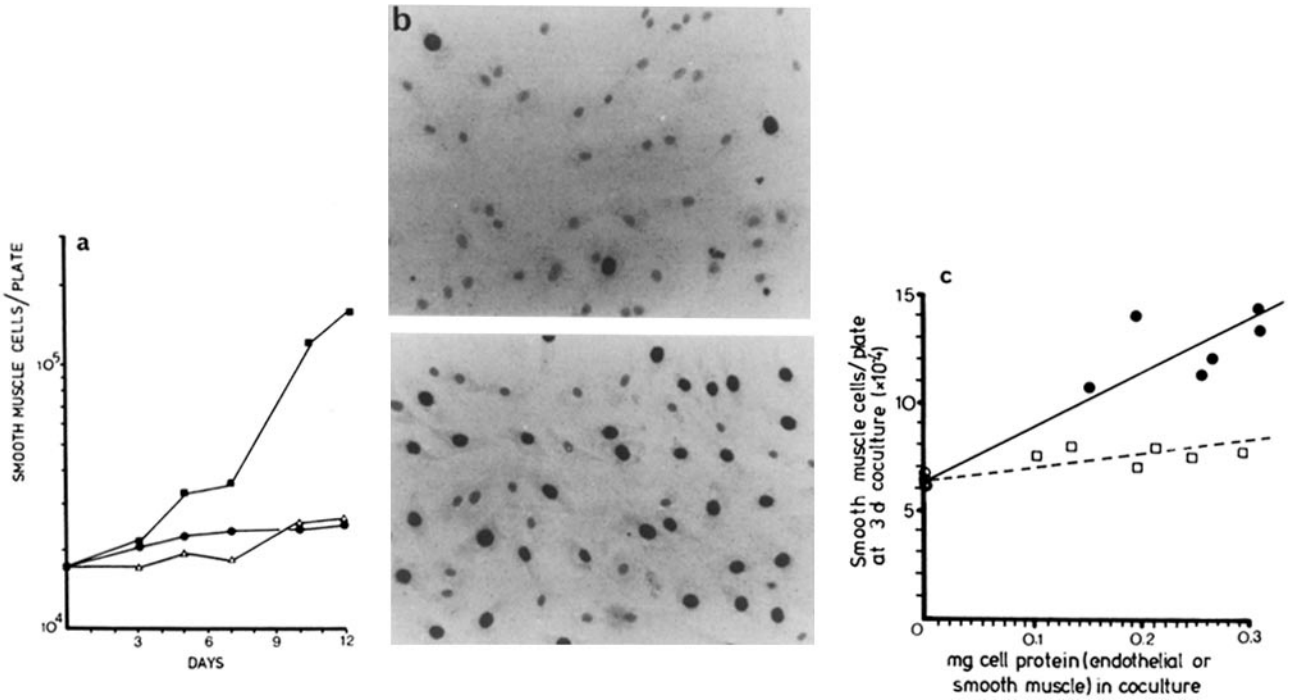

FIGURE 5 Mitogenic effects upon SMCs by co-culture with endothelial cells. 1.8×10^4 SMCs were incubated with 2% PDS for 48 h to arrest their growth. (*a*) At day 0, all dishes received fresh 2% PDS, and the SMCs were then co-cultured with equal numbers of microcarrier-bound confluent endothelial cells (■), SMCs (●), or empty microcarriers (△). Smooth muscle cell numbers were determined by a Coulter Counter on the days indicated. Each point is the mean of four observations. 95% confidence limits were within 20% of the mean. (*b*) [^{3}H]Thymidine autoradiography of SMCs co-cultured with microcarrier bound SMCs (*top*) or endothelial cells (*bottom*) for 48 h in 2% PDS. Endothelial SMC ratio, 1.0. × 230. (*c*) Effects of increasing numbers of endothelial cells upon SMC growth. Increasing numbers of confluent microcarrier-bound endothelial cells (●) or microcarrier-bound SMCs (□) were co-cultured with 5×10^4 SMCs for 3 d in 2% PDS, at which time SMC numbers were counted. 0.1 mg cell protein is equivalent to $\sim 2 \times 10^5$ endothelial cells and 1.5×10^5 SMCs.

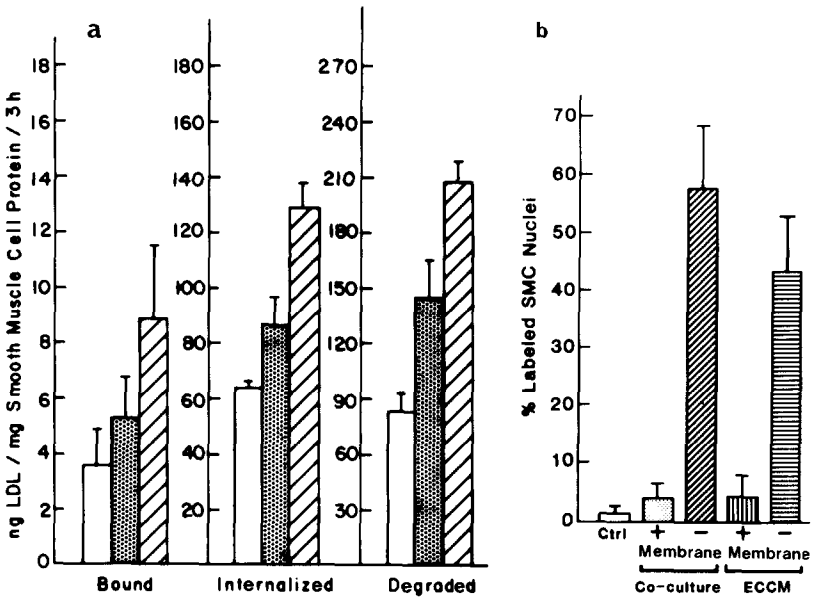

FIGURE 6 Endothelial cell-derived mitogen-dependent and independent stimulation of SMC LDL metabolism in co-culture. (*a*) Specific high affinity LDL binding, internalization, and degradation in SMCs in single culture (□), co-culture with endothelium while a semipermeable membrane was interposed between the cell populations (□); or co-culture with endothelium without interference (▨). After the various conditions had been in effect for 24 h, 5 μg ^{125}I-LDL was added for 3 h, and specific LDL metabolism in SMCs was determined. Endothelial/SMC ratio was 1.0 (0.15 mg endothelial cell protein). Each bar is the mean ± SD of four determinations. (*b*) Effectiveness of membrane interposed between endothelium and SMCs in preventing EDGF(s) from reaching SMCs. SMCs were incubated with [^{3}H]thymidine for 24 h. □, single culture of SMCs; □, co-culture with confluent endothelial cells with dialysis membrane between the cell populations; ▨ co-culture with endothelial cells, membrane absent; ▥, seven fold concentrated ECCM, membrane present; ▤, unconcentrated ECCM, membrane absent.

DISCUSSION

The principal reason for the development of in vitro models of cell–cell interactions such as the co-culture system used in these studies is to reconstruct some of the complexities of the cellular environment that exist in vivo while retaining the experimental advantages associated with tissue culture. The microcarrier co-culture system was designed to study humoral interactions between cells and represents an improvement of other available co-culture techniques because it combines an increased surface area for cell adhesion with easy transfer of the microcarriers.

The presence of endothelial cells increased the mass of LDL that was bound, internalized, and degraded by arterial SMCs. The effects were endothelial cell-specific and were not induced by fibroblasts, 3T3 cells, or a second population of

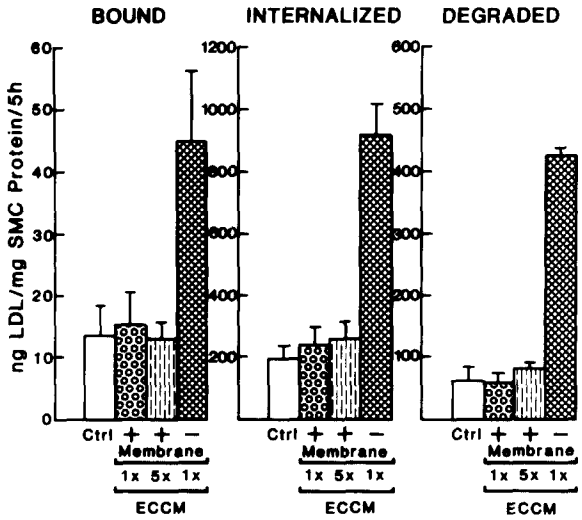

FIGURE 7 Absence of mitogen-independent stimulation of LDL metabolism in SMCs incubated with ECCM. ECCM was prepared by incubation of 2% lipoprotein-deficient, growth factor-depleted medium with confluent endothelial cells at a ratio of 1 ml medium/10^6 cells. After 48 h, the medium was collected and centrifuged to remove particulate cell debris, and part was concentrated fivefold by ultrafiltration. SMCs were preincubated with lipoprotein-deficient, growth factor-depleted medium for 48 h after, and then fresh medium was added. □, SMC cultured alone; ▩, SMCs cultured with unconcentrated ECCM or with 5-fold concentrated ECCM (▥) placed in the upper chamber of the co-culture apparatus and separated by a dialysis membrane (3.5 k D). A fourth group of SMCs was directly exposed to unconcentrated ECCM (▩). 24 h later 50 μg ^{125}I-LDL/ml was added for 5-h, and then specific LDL metabolism in the SMCs was determined. Each bar is the mean ± 1 SD of 9–12 determinations in three separate experiments.

SMCs. Within 12 h of co-culture, receptors that bound LDL with high affinity increased in number. As a consequence, the mass of LDL that was internalized and degraded was increased. Kinetic analysis of LDL binding and endocytosis provided a number of insights into the altered dynamics that occur in SMCs in co-culture with endothelial cells. First, it was possible to measure LDL binding affinity and the association rate constant at 37 rather than at 4°C, where changes in cell surface membrane fluidity may influence binding to the receptor. Second, kinetic analysis provided a better understanding of the changing rates of LDL metabolism in SMCs when experimental conditions were altered by co-culture with endothelial cells. We determined that although there was an increase in the number of LDL receptors expressed at the cell surface, which in turn was associated with increases in the mass of ligand internalized and degraded, the rate of binding to the receptors declined slightly. Furthermore, the internalization rate constant decreased by 42% and that for degradation increased by 51%. The affinity constant for the LDL receptor, however, was unchanged. Thus, in co-culture, LDL bound to more receptors at a slightly slower rate than it did in controls, but with the same affinity. Internalization of the LDL-receptor complex by the SMCs then occurred at a slower rate than normal. Upon reaching the lysosomal compartment, however, LDL was degraded more efficiently than in controls. The mechanism for a rate-limiting internalization process is unclear. A number of possibilities are (*a*) a limitation in the number of LDL-receptor complexes that can be accommodated in a coated pit-vesicle unit; (*b*) a limited rate of membrane recycling after internalization of an LDL-receptor loaded vesicle; and (*c*) limitations in the amounts and activities of elements that may direct the formation and translocation of vesicles, such as clathrin and cytoskeleton. These possibilities require further investigation.

Two general mechanisms by which endothelial cells influenced LDL metabolism in SMCs were identified in co-culture—stimulation of the SMC cell cycle, and a cell cycle-independent mechanism.

A number of investigators have demonstrated that endothelial cells can produce growth factors for SMCs, fibroblasts, and 3T3 cells (3, 4, 36). The mitogenic activity is collectively referred to as endothelial cell-derived growth factor (EDGF), at least part of which appears to be identical to platelet-derived growth factor by criteria of biochemical properties (36) and competition studies for the platelet-derived growth factor receptor on cultured fibroblasts (37). A close relationship has been reported between LDL metabolism and cell cycle in cultured fibroblasts and SMCs after exposure to purified platelet-derived growth factor (32, 33) and fibroblast growth factor (34). Platelet-derived growth factor and fibroblast growth factor stimulated the binding, internalization, and degradation of LDL by increasing the number of high-affinity receptors per cell, whereas receptor affinity remained unchanged (32). Endothelial cell-specific stimulation of LDL metabolism and growth of SMCs in the co-culture system are therefore consistent with the activity of EDGF, since the effects upon LDL metabolism are via the cell cycle.

Aspects of EDGF-dependent alterations of smooth muscle LDL binding and internalization in co-culture agree with data generated by others using ECCM as a source of EDGF (4), and fibroblasts as the target cells. In contrast, however, LDL degradation was markedly different in co-culture experiments than in those that employed ECCM. Cell-conditioned medium significantly inhibited degradation in the presence of enhanced receptor-mediated binding and internalization (4, 38). In contrast, we measured significant stimulation of the degradation rate constant (k_3) in co-culture. Cornicelli et al. (38) showed that the inhibition was attributable to NH_4^+ produced by porcine endothelial cells. We therefore analyzed our bovine cell system for the presence of NH_4^+. The concentrations of NH_4^+ were <1 mM, well below that required to significantly inhibit LDL degradation (39). Thus, we have not found any evidence for interference of the endocytic pathway of LDL in SMCs by endothelial cell-derived NH_4^+ in the bovine culture system. Rather, the co-culture studies we describe here demonstrate that smooth muscle LDL degradation was significantly stimulated in the presence of endothelial cells, a pattern consistent with the effects of purified growth factors.

A significant conclusion from the co-culture experiments is that LDL metabolism in SMCs can be influenced by endothelial cells in circumstances when EDGF is prevented from acting on the SMCs. The dialysis membrane allowed only smaller molecular weight endothelial products to diffuse to the SMCs and it excluded mitogens. As determined by [^{3}H]-thymidine autoradiography, the cells remained quiescent, yet LDL binding, internalization, and degradation were significantly increased. In contrast, when the microcarriers were replaced with ECCM within the dialysis membrane, there was no change in smooth muscle LDL metabolism. These experiments indicate that interactions between the two cell types in co-culture were different than when conditioned medium

was used and suggest that either an unstable low molecular weight product of endothelial cells was able to modify smooth muscle LDL metabolism or that cell–cell communication was necessary. Whichever mechanism prevails, the results demonstrate that co-culture was required for the detection of these kinds of interactions between cells. Clearly, a co-culture arrangement increases the probability of detecting the effects of a short-lived agent produced by endothelial cells. It was not possible to determine the contribution of such an agent to EDGF-mediated enhancement of LDL metabolism in these experiments, or to know whether its effects were overridden by EDGF. We speculate, however, that under circumstances in which EDGF production is suppressed or its effects are antagonized by inhibitors (40), LDL metabolism in SMCs can be mediated by endothelial cells via an unstable product that lacks mitogenic activity. Recently, Hajjar et al. (41) have reported that prostacyclin influences cholesterol metabolism in cultured SMCs. Prostacyclin has a half-life of several minutes and can be produced by both endothelial cells and SMCs (42, 43). Physiological levels of prostacyclin significantly increased both acid (lysosomal) cholesteryl ester hydrolase and neutral (cytosolic) cholesteryl ester hydrolase activities, resulting in accelerated cholesterol loss from the cells. Whether these changes in the activities of enzymes involved in cellular cholesterol metabolism are related to our measurements of ^{125}I-apoprotein-labeled LDL in SMCs is being studied.

In summary, we have investigated metabolic cooperation between endothelial cells and SMCs in co-culture and have demonstrated that endothelial cells influence SMC lipoprotein metabolism by cell cycle–dependent and mitogen-independent mechanisms. Kinetic analysis of the LDL pathway indicated that the internalization step was rate-limiting for mitogen stimulation of LDL metabolism. The studies focus on the interaction between two vascular cell populations in tissue culture and suggest that important local humoral interactions exist between them. Such interactions may be relevant to normal blood vessel maintenance as well as to pathological changes associated with atherogenesis.

We are most grateful to Drs. M. A. Gimbrone and R. S. Cotran for helpful discussions, and to Dr. G. King of the Joslin Diabetes Center, Boston, for a gift of ^{125}I-insulin.

The work was supported by National Heart, Lung and Blood Institutes grants HL24612, HL22602, and HL21429. George Truskey was the recipient of a Whitaker Health Sciences Fund Predoctoral Fellowship. Dr. Henry Warren was supported by National Research Service Award 2T32 RR07000-09 from the National Institutes of Health.

Received for publication 7 November 1984, and in revised form 22 January 1985.

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