Interaction of arterial cells. I. Endothelial cells alter cholesterol metabolism in co-cultured smooth muscle cells

David P. Hajjar,[1] Domenick J. Falcone, James B. Amberson, and John M. Hefton

Departments of Pathology, Biochemistry, and Cell Biology and Anatomy, The New York Hospital-Cornell Medical Center, New York, NY 10021

Abstract Results of previous in vivo experiments indicated that the presence of arterial endothelium modifies cholesteryl ester (CE) metabolism and the retention of low density lipoproteins (LDL) in injured arteries. We describe herein the effects of bovine arterial endothelial cells (ENDO) on the CE cycle, fluid phase endocytosis, and cell proliferation in co-cultured bovine arterial smooth muscle cells (SMC). Following several days of cultivation on confluent SMC, ENDO were removed from SMC by treatment of the co-cultures with 1.0% collagenase (type II). Removal of only ENDO from the co-culture dishes was confirmed by immunofluorescent staining for Factor VIII antigen, hemotoxylin-eosin staining, and biochemical analyses. We observed that ENDO grown to 75% confluency on confluent SMC induced: *1*) a reduction of CE hydrolysis as a result of decreased lysosomal CE hydrolytic activity in SMC as compared to SMC cultured alone; and *2*) an increase in the rate of incorporation of labeled oleate into CE as a result of increased acyl CoA:cholesterol O-acyltransferase activity in SMC as compared to SMC cultured alone. Neither endothelial cell-derived culture media (ECDM) nor fibroblasts modulated CE metabolism in co-cultured SMC. Additional experiments showed that the presence of endothelial cells or ECDM decreased the proliferation of co-cultured SMC by 50%, but enhanced the endocytotic rate of labeled sucrose into SMC threefold. Results of experiments described herein demonstrate that, in addition to providing a thromboresistant surface and regulating permeability, endothelial cells may also serve to modulate cholesteryl ester metabolism in smooth muscle cells derived from the arterial wall. —**Hajjar, D. P., D. J. Falcone, J. B. Amberson, and J. M. Hefton.** Interaction of arterial cells. I. Endothelial cells alter cholesterol metabolism in co-cultured smooth muscle cells. *J. Lipid Res.* 1985. **26:** 1212–1223.

Supplementary key words atherosclerosis • cell proliferation • cholesteryl ester metabolism • co-culture of arterial cells • endocytosis

Current interest in the culture of arterial endothelial cells (ENDO) and smooth muscle cells (SMC) results partly from the important role of these cells in arterial disease. A striking feature of the healthy arterial wall is that ENDO and the underlying SMC are in a quiescent growth state with little cholesterol and cholesteryl ester (CE) accumulation. If the endothelium is damaged, however, SMC in the injured vessel are exposed to blood cells and proteins, including lipoproteins, which contribute to the intracellular and extracellular pools of arterial lipid (1). The mechanisms controlling these processes remain undefined.

It has been a major objective of this laboratory to study the role of the endothelium in the control of the metabolism of the underlying SMC in order to elucidate those factors responsible for arterial lipid accumulation following injury. Such factors have included altered cholesterol metabolism (2) and the contributions of glycosaminoglycans (3) and lipoproteins (4). Results demonstrated that re-endothelialized neointima contained increased glycosaminoglycan concentration, increased retention of low density lipoproteins (LDL), and altered CE metabolism which favored cholesterol and CE deposition (2–5). These findings, supported by other laboratories (6–8), suggest that the endothelium may directly affect SMC metabolism.

To focus attention on the specific role of the endothelium in the control of SMC metabolism and/or the interaction of these cell types, a variety of in vitro systems have been utilized. Jones (9) cultured cloned ENDO over arterial SMC, Merrilees and Scott (10) cultured arterial ENDO side-by-side with aortic SMC, and Warren et al. (11) and Davies et al. (12, 13) have developed a co-culture model which utilizes a microcarrier system. Results of these various approaches have demonstrated that ENDO can modulate SMC metabolism.

Abbreviations: ACAT, acyl CoA:cholesterol O-acyltransferase; ACEH, acid cholesteryl ester hydrolase; CE, cholesteryl esters; ECDM, endothelial cell-derived culture medium; ECM, endothelial cell culture medium; ENDO, endothelial cells; FCS, fetal calf serum; IF, immunofluorescence; LDL, low density lipoprotein; NAGase, N-acetyl-β-glucosaminidase; Neut. Gluc., neutral-α-glucosidase; NCEH, neutral cholesteryl ester hydrolase; NCS, newborn calf serum; P-lipid, phospholipid; PBS, phosphate-buffered saline; PC, phosphatidylcholine; SMC, smooth muscle cells; TG, triacylglycerols.

[1] Address all reprint requests and correspondence to: Dr. David P. Hajjar, Department of Pathology, Cornell University Medical College, 1300 York Avenue, New York, NY 10021.

Since we previously demonstrated that re-endothelialization of the injured artery alters its metabolism (2, 3), we have used a co-culture system designed primarily to further investigate the metabolic effects of ENDO on SMC. Specifically, we assessed the CE cycle as well as endocytotic and proliferative activity in SMC co-cultured with ENDO.

MATERIALS AND METHODS

Materials

Cholesteryl [1-^{14}C]oleate (sp act 55.6 mCi/mmol), [9,10-^{3}H]oleic acid (sp act 13.3 Ci/mmol), [1-^{14}C]oleic acid (sp act 59.0 mCi/mmol), [1,2-^{3}H]cholesterol (sp act 54.8 mCi/mmol), [1-^{14}C]oleoyl CoA (sp act 55.0 mCi/mmol), [^{14}C-U]leucine (sp act 320.0 mCi/mmol), [^{14}C(U)]-sucrose (sp act 673.0 mCi/mmol), and Aquasol-2 liquid scintillation fluid were purchased from New England Nuclear Corp., Boston, MA. Unlabeled oleic acid, cholesterol, cholesteryl oleate, and triolein were obtained from Applied Science Laboratories, Inc., State College, PA. Phospholipids and bile salts were purchased from Supelco, Inc., Bellefonte, PA. Collagenase (CLS, type II; 157 units/mg) was obtained from Worthington Biochemical Corp., Freehold, NJ and neutral protease (dispase; grade II; 0.5 units/mg) was obtained from Boehringer-Mannheim, Indianapolis, IN. The following were obtained from Sigma Chemical Co., St. Louis, MO: bovine serum albumin (Fraction V, essentially fatty acid free), calf thymus DNA, 2-mercaptoethanol, 4-methylumbelliferyl-2-acetamido-2-deoxy-β-D-glucopyranoside, and 4-methylumbelliferyl-α-D-glucopyranoside. All other chemicals were reagent grade. Tissue culture media, antibiotics, and amino acids were purchased from Flow Laboratories, Inc., McLean, VA. Screened fetal and newborn calf serum (heat-inactivated) were obtained from M.A. Bioproducts, Walkersville, MD; and, tissue culture supplies were purchased from Becton-Dickinson Labware, Oxnard, CA.

Rabbit anti-human Factor VIII was purchased from Calbiochem-Behring, La Jolla, CA, and goat anti-rabbit IgG-FITC conjugated F(ab')$_2$ fragments were obtained from Cappel Inc., Cochranville, PA.

Cell culture

Culture medium used to propagate bovine arterial SMC consisted of Eagle's Minimum Essential Medium (MEM) supplemented with 20% fetal calf serum, 1% glutamine, 1% penicillin–streptomycin, and 1% Fungizone. Cells were cultured in Falconware in a 37°C incubator containing 5% CO_2–95% air.

Smooth muscle cells were derived from bovine arteries collected within 2 hr postmortem. Arterial segments were washed several times with phosphate-buffered saline (PBS, pH 7.4) to remove traces of grossly visible blood. The adventitial and ENDO layers were removed by scraping. The preparation of arterial explants, the propagation, maintenance, and harvesting of the arterial SMC that grow out from the intima-media explants, and the identification of these cultured cells by transmission electron microscopy have been described previously (14–16). Arterial SMC were subpassaged twice before they were used in the biochemical studies.

Fibroblasts were cultured according to established methods (17). These cells were subpassaged four times before they were used in the biochemical studies.

Endothelial cells were cultured from bovine arteries previously cut into 2″ × 2″ pieces. The luminal side of the artery was placed down in a petri dish containing 1.0% dispase prepared in MEM alone, sufficient to cover the luminal surface. After incubation for 30 min at 37°C, this dispase solution was discarded, ENDO were removed from the luminal surface with sterile cotton applicators, and ENDO were collected in sterile RPMI-1640 plus 10% newborn calf serum (NCS). The cells were collected by centrifugation at 1000 rpm for 5 min. The ENDO pellet was resuspended in Endothelial Culture Medium (ECM: RPMI-1640 supplemented with 10% NCS, 0.01% gentamycin, and 2 mM L-glutamine). The ENDO were seeded at a density of 5 × 10^5 cells/cm^2 on plastic surfaces precoated with either 0.1% gelatin or 0.1% plasma-derived fibronectin. Cultures were incubated at 37°C in an atmosphere of 5% CO_2–95% air with 100% humidity. After 4–5 days, cells reached confluency and were detached with 0.1% collagenase (type II) for 3–5 min at 37°C. Collagenase activity was then inhibited by the addition of NCS. Detached cells were washed with ECM, collected by centrifugation, and resuspended in ECM in preparation for co-culture experiments. Endothelial cells were previously subpassaged three to five times.

Co-culture of ENDO and SMC

Smooth muscle cells were trypsinized, resuspended in MEM, and seeded into Falcon 60 × 15 mm^2 dishes at a final density of 5 × 10^5 cells/cm^2. Standard incubations were carried out until SMC reached confluency. ENDO (8.0 × 10^5) were then added to confluent SMC exposed previously to 0.1% fibronectin for 5 min to provide maximal proliferation of ENDO on co-cultured SMC. Cells were allowed to remain in co-culture for several days depending on the experiment. Endothelial cells grew to 75% confluency on confluent, fibronectin-coated SMC. Preliminary studies demonstrated that no significant differences were observed if ENDO were sparse or 75% confluent on SMC with regard to the metabolic parameters studied in vitro. Control SMC were similarly exposed to 0.1% fibronectin.

Separation of ENDO from SMC

Endothelial cells were selectively removed from the underlying co-cultured SMC by treating with 2 ml of 1.0% collagenase (type II) for 3–5 min at 37°C. The ENDO were discarded; SMC cultured alone or in the presence of ECDM were treated in a similar manner with 1.0% collagenase.

ENDO-conditioned media and fibroblasts

To determine whether the potential effects of ENDO on SMC proliferation, endocytosis, or CE metabolism resulted from the obligatory presence of ENDO or ENDO-derived soluble stimuli, we cultured SMC in the presence of ENDO-conditioned media (ECDM). This medium was obtained from confluent ENDO cultured after 48 hr. Following 48 hr, ECDM was collected, passed through a 0.45-μm Millipore filter, and diluted 1:1 (v/v) with fresh ECM to give a final serum concentration of 10%. Preliminary experiments revealed that similar metabolic, proliferative, and endocytotic effects were observed whether ECDM was filtered, unfiltered, or mixed 2:1 (v/v) with fresh culture media. The SMC were cultured with ECDM for the designated period of time for each experiment. At the conclusion of the experiment, media were decanted and SMC were detached as previously outlined. The parameters used to assess CE metabolism, endocytosis, and proliferation are outlined below.

To determine whether the widespread contact or potential for contact of ENDO with underlying SMC was an important factor in these experiments, we also utilized a co-culture system in which widespread contact would be unlikely. For this reason, ENDO were cultured on DEAE Sephadex (Cytodex-3) beads at a ratio of approximately 150 cells/bead (18). Fibroblasts were also cultured on beads at a final ratio of approximately 70 cells/bead since they can serve as a control for cell specificity with regard to potential alterations in SMC metabolism. Neither ENDO nor fibroblasts grew to a confluent state on the beads.

Approximately 8×10^5 ENDO on microcarriers or 6×10^5 fibroblasts on microcarriers were added separately to some of the confluent SMC in the 60×15 mm^2 dishes using 2 ml of RPMI-1640 plus 10% NCS. In experiments with fibroblasts, cultures were prepared on microcarriers since they could not be completely separated from the confluent SMC if the fibroblasts were plated on the cultured SMC.

Cells previously grown on microcarrier beads and co-cultured with SMC were removed from the underlying SMC by aspirating the microcarriers off the dish, treating the co-cultures with 1.0% collagenase as described previously, and washing the attached SMC twice with PBS.

Morphological characteristics

Co-cultured ENDO with SMC and cultured SMC following removal of co-cultured ENDO with collagenase were examined after hematoxylin-eosin staining by light microscopy.

Factor VIII/von Willebrand factor immunofluorescence

The presence or absence of ENDO, as well as ENDO distribution on co-cultured SMC, was monitored by indirect immunofluorescence. Incubation of cell cultures with rabbit anti-human Factor VIII/vWF, followed by detection with FITC-conjugated goat anti-rabbit IgG (19), was used to confirm the presence or absence of ENDO on the co-cultured SMC.

Isolation of plasma proteins

Fibronectin was isolated from human plasma (20). Human low density lipoprotein (LDL) (d 1.019–1.063 g/ml) was isolated from fresh human plasma by ultracentrifugation (21). LDL was determined to be free of other lipoproteins or proteins by electrophoresis in agar-agarose gels (5).

Enzyme activities

Following the removal of ENDO, ECDM, or the various cells grown on microcarrier beads from SMC, SMC were recovered from the plastic dish at 4°C with a rubber policeman with 2.0 ml of ice-cold isotonic sucrose buffer (250 mM sucrose–1 mM EDTA–0.1 M Tris-HCl, pH 7.2). Cells were homogenized in an ice bath (0–4°C) for 2 min. Aliquots were taken for the assessment of CE metabolizing enzymes activities, marker enzymes, DNA, and protein.

Cholesteryl ester metabolizing enzymes

Acid cholesteryl ester hydrolase (ACEH). Activity of ACEH was assayed in SMC by the method of Haley, Fowler, and de Duve (22) with the use of 13 μM cholesteryl [1-^{14}C]-oleate as substrate suspended in an egg phosphatidylcholine–digitonin dispersion. Assay conditions were optimized as previously reported (2, 15). After the incubation with 150 μg of cell protein for 60 min, released oleic acid was separated by organic solvent extraction, and radioactivity was assayed. Efficiency of fatty acid extraction was 90% or greater. For preliminary studies, different methods of substrate preparation were examined. The substrate composition and the incubation conditions used were similar to those previously described (2).

Neutral cholesteryl ester hydrolase (NCEH). Cholesteryl [1-^{14}C]oleate served as substrate for the optimal assay of NCEH activity in arterial SMC (23). It was prepared as

a mixed micelle consisting of cholesteryl oleate, phosphatidylcholine, and sodium taurocholate (23, 24). Constituents of the mixed micelle were at a final 1:4:2 molar ratio. This substrate provided the highest enzyme activity in arterial SMC as compared to other substrates tested (23).

For the assay of NCEH activity, an incubation mixture was prepared by the addition of 50 μl of micellar cholesteryl [1-^{14}C]oleate to 800 μl of 100 mM potassium phosphate buffer (pH 7.0) containing 0.05% bovine serum albumin. The reaction was begun by addition of 150 μl of SMC homogenate diluted in homogenization medium (ca. 150 μg protein). Arterial cell homogenates were prepared in 250 mM sucrose–1 mM EDTA–100 mM Tris-HCl (pH 7.2) (2, 15). Incubations were carried out in stoppered tubes at 37°C for 60 min. Substrate blanks were run under identical conditions with isotonic sucrose buffer added in place of the enzyme. The principal components of the final reaction mixture were enzyme, 6.0 μM cholesteryl oleate, 23.7 μM phosphatidylcholine, 12.5 μM sodium taurocholate, 0.04% bovine serum albumin, and 85 mM potassium phosphate buffer, pH 7.0. The reaction was stopped and the unhydrolyzed substrate was removed as described previously (25). The amount of liberated [1-^{14}C]oleate in the upper aqueous phase was determined by the addition of a 1.0-ml aliquot to 15 ml of Aquasol-2 liquid scintillation cocktail for counting. Extraction of a fatty acid standard by the same procedure routinely gave 85–90% of the [1-^{14}C]oleate in the upper phase.

Acyl CoA:cholesterol acyltransferase (ACAT). Activity of ACAT in SMC was assayed by measuring the synthesis of CE from radioactive fatty acyl CoA employing exogenous free cholesterol incorporated into phospholipid liposomes (26). Unilamellar liposomes consisting of 15 μCi [1-^{14}C]-oleoyl CoA, egg PC, and exogenous cholesterol were prepared as described (2).

The ACAT assay was initiated by the addition of 250 μl diluted SMC homogenate (ca. 250 μg of protein) to a solution containing 250 μl of the liposomal preparation and 500 μl of 150 mM Tris-HCl buffer (pH 7.4) containing 10 mM mercaptoethanol and 0.05% defatted bovine serum albumin. The final reaction mixture (1.0 ml) contained enzyme, 12.5 μM oleoyl CoA, 10.0 μM exogenous cholesterol, and 1.0 mM egg PC in 75 mM Tris-HCl buffer (pH 7.4). This buffer also contained 5 mM mercaptoethanol and 0.025% bovine serum albumin. The incubation was carried out at 37°C for 60 min. Incubation medium with substrate but without homogenate served as a control. All assay conditions were optimized as previously described (2, 26). After incubation the reaction was stopped with 5 ml of chloroform–methanol 2:1 (v/v) containing 50 μg of unlabeled cholesteryl oleate as carrier, and the lipid was extracted by the procedure of Folch, Lees, and Sloane Stanley (27). Extracted lipids from the organic phase were separated by thin-layer chromatography (TLC) (28). Individual spots were identified and areas of the plate corresponding to the position of CE were scraped into vials containing 15 ml of Aquasol-2 and counted.

Cellular marker enzymes

N-acetyl-β-glucosaminidase and neutral-α-glucosidase activities were assayed in arterial SMC according to procedures described by Peters, Muller, and de Duve (29).

Units of activity

For all hydrolases, 1 unit of activity corresponds to 1 nmol of substrate hydrolyzed per min. One unit of ACAT corresponds to 1 nmol of oleoyl CoA esterified to cholesterol per min. Activity is expressed as milliunits per mg DNA.

Cellular cholesteryl [1-^{14}C]oleate hydrolysis

Cholesteryl [1-^{14}C]oleate (10 μCi) was incorporated into LDL using 10% DMSO according to the method of Brown, Dana, and Goldstein (30). Labeled cholesteryl oleate, mixed with buffer containing DMSO, was incubated 15 min at 37°C. LDL was then added and the incubation was continued for 3 hr at 37°C. The solution was dialyzed overnight against a total of 4 l of buffer containing 0.15 M NaCl–0.3 mM EDTA (pH 7.0) at 4°C. Radiolabeled LDL was stored at 4°C and used in the assays within 10 days.

Smooth muscle cells (5×10^5) grown in 60×15 mm^2 dishes were fed 2.0 ml of RPMI-1640 plus 45 μg of LDL-cholesteryl [1-^{14}C]oleate (13,000 cpm) for 36 hr. At time 0, SMC received unlabeled 100 nmol of oleate–20 nmol of albumin. Some dishes were plated with 7×10^5 ENDO on top of the confluent SMC or with 2.0 ml of ECDM. After incubation at 37°C for the indicated time, the supernatants were removed and discarded. All dishes were washed with PBS and then treated with 1.0% collagenase. The remaining SMC monolayers were washed at 4°C with 3.0 ml of 0.15 M NaCl–50 mM Tris-HCl (pH 7.2). Lipid extraction from the cells was then performed in situ by the procedure of Hara and Radin (31). Each dish was treated with 2 ml of hexane–isopropyl alcohol 3:2 (v/v) for 30 min, an internal standard ([^{3}H]cholesterol, 7,000 cpm/dish), and carrier lipids. The organic solvent was removed after 30 min and replaced with 1 ml of fresh solvent for a second 15-min extraction. The combined extracts were evaporated under N_2 and dissolved in 250 μl of dichloromethane–methanol 2:1 (v/v) for lipid fractionation by TLC (32). Cholesteryl esters and free cholesterol were separated on TLC plates (28), and lipid spots were scraped and counted. The remaining cell protein precipitate in the dishes was dissolved in 0.2 N NaOH for 1 hr and aliquots were then used for protein determinations (33).

Average recoveries of the radiolabeled lipids were approximately 75%. Results are expressed as pmol of cholesteryl [^{14}C]oleate remaining from the hydrolysis of cholesteryl [1-^{14}C]oleate/mg protein.

Incorporation of [^{14}C]oleic acid into esterified lipid

To each dish of 5 × 10^5 SMC cultured alone or in the presence of 7 × 10^5 ENDO or 2.0 ml ECDM, a [^{14}C]oleic acid–albumin mixture (34) was added to give a final concentration of 100 μM oleate–20 μM albumin (6200 cpm/5 × 10^5 SMC per dish). As a source of free cholesterol, 50 μg of unlabeled LDL was added. Following a 24- or 48-hr incubation, ENDO, ECDM, or SMC media were removed. SMC were washed twice with PBS. Lipid extraction, lipid fractionation, and protein assays were then carried out. An internal standard ([^{3}H]cholesterol, 6200 cpm/dish) and carrier lipids were also added to the dishes. Lipid classes were separated by TLC, and spots corresponding to phospholipids, TG, and CE were scraped and counted. Esterification was expressed as nmol of [^{14}C]oleic acid incorporated/mg of protein.

Total protein synthesis

Protein synthesis in SMC was determined by measuring [^{14}C]leucine incorporation into material precipitable by trichloroacetic acid (TCA) (35). Cells grown in RPMI-1640 plus 10% NCS were labeled for 24 hr in medium containing 3.2 μCi of [^{14}C-U]leucine and 50 μg/ml sodium ascorbate. The incorporation was linear during this time period. After labeling, the medium was withdrawn and pooled with one serum-free medium wash of the cell layer. TCA was added to a final concentration of 5% and samples were allowed to precipitate at 4°C with albumin as a protein carrier. The cell layer was scraped with a rubber policeman in serum-free medium, freeze-thawed twice to disrupt the cells, then TCA-precipitated at a final concentration of 5% TCA at 4°C. Both medium and cell layer precipitates were pelleted by centrifugation at 1000 rpm. The pellets were resuspended in 5% TCA and re-precipitated once again to remove unincorporated [^{14}C]leucine. The final pellets were neutralized with 0.1 N NaOH and aliquots were taken for protein determinations by the method of Lowry et al (33) and for scintillation counting to determine radioactivity.

Nonspecific binding of [^{14}C]leucine to cells or dishes was determined by the method of Burke and Ross (35). Results of these experiments were then subtracted from those from the experimental groups to correct for nonspecifically bound [^{14}C]leucine.

Rate of fluid endocytosis

Fluid-phase endocytosis was measured in SMC cultured alone or in the presence of ENDO or ECDM. Endocytotic rates were measured by monitoring SMC uptake of [U-^{14}C]sucrose from the medium by the method of Davies and Ross (36). Radiolabeled sucrose was added in 10 μl of PBS to a final activity of 15 μCi/ml of culture media per 5 × 10^4 cells. Following incubation at 4°C (control to inhibit endocytosis) or at 37°C, ENDO or ECDM were removed and the remaining SMC were washed twice with ice-cold PBS containing 0.1% BSA. Each culture dish was then washed twice with PBS alone. Cells were dissolved (36), aliquots were taken for DNA analysis, and the remaining cell preparation was counted with Aquasol-2 in a liquid scintillation counter. Rates of fluid-phase endocytosis were expressed as dpm [^{14}C]sucrose/mg DNA over time.

DNA and protein assays

DNA was measured in cell preparations by the method of Kissane and Robins (37). Calf thymus DNA served as a standard. Protein was assessed by the method of Lowry et al. (33) with bovine serum albumin as a standard.

Statistical methods

The hydrolytic rates of CE and the synthetic rates of esterified lipid over time in the SMC cultured alone or in the presence of ENDO or ECDM were compared with two-way analyses of variance following log transformation of the data (38).

Enzyme activity analyses and cell counts in each group were compared separately by a single factor analysis of variance (38).

RESULTS

Endothelial cells were cultured on confluent SMC. Our preliminary electron microscopy studies demonstrate that ENDO did not consistently rest directly on the SMC but were separated either by spaces or by extracellular matrix. These observations are similar to those reported by Jones (9) and Merrilees and Scott (10). The area occupied by ENDO on the co-cultured SMC was enhanced by pretreating SMC with fibronectin. In our experiments, neither type I or type II collagen nor gelatin increased spreading of arterial ENDO on arterial SMC.

To confirm that ENDO were completely removed from the underlying co-cultured SMC with 1.0% collagenase, we quantitated DNA and protein in the dishes containing SMC alone, co-cultured cells, and co-cultured SMC following treatment with collagenase (**Table 1**). Enzymic treatment of the co-cultures reduced the DNA and protein content to levels similar to those found in SMC culture alone (also treated with collagenase). In addition, dishes were stained with hematoxylin-eosin and for the presence of Factor VIII. These procedures were carried out to confirm the presence and absence of co-cultured

TABLE 1. DNA and protein content of smooth muscle cells co-cultured with endothelial cells before and after collagenase treatment

Culture System	DNA Content (μg)	Protein Content (μg)
SMC cultured alone	52 ± 4[a]	365 ± 40[c]
SMC co-cultured with ENDO	120 ± 7[a,b]	712 ± 43[c,d]
SMC + collagenase treatment to remove ENDO	48 ± 5[b]	352 ± 31[d]

Smooth muscle cells (5–6 × 10^5 cells/dish) were grown in 60 × 15 mm dishes in RPMI-1640 plus 10% NCS. Endothelial cells (ENDO) were added to the 4-day-old cultures at a density of 7–8 × 10^5 cells to some of the dishes using the same media. After 72 hr, SMC cultured alone and SMC co-cultured with ENDO were treated separately with 1.0% collagenase (type II) for 3 min at 37°C. This treatment removed only the ENDO; the SMC remained attached to the dish. DNA and protein contents were subsequently determined on the SMC remaining on the dishes. Data represent the mean ± SE for six individual dishes.

[a]Values with the same corresponding letters are significantly different ($P < 0.05$).

ENDO with SMC following collagenase treatment (**Fig. 1**). The immunofluorescence and biochemical data indicated that ENDO were completely removed and that the SMC remained attached to the dish.

Biochemical studies indicated that a small but significant reduction ($P < 0.05$) in CE hydrolysis occurred in intact, slowly proliferating SMC co-cultured with ENDO but not by ECDM alone (**Fig. 2**). This was attributable to reduced lysosomal (acid) CE hydrolytic activity (**Table 2**). This decreased hydrolysis was observed when ENDO were either sparse or semi-confluent on the co-cultured SMC. In addition, ENDO grown on microcarriers that were subsequently plated on SMC produced a similar effect. These findings suggest that widespread contact between ENDO and SMC may not be necessary to modulate these metabolic effects in co-cultured SMC. In addition, no differences in ACEH activity were observed in SMC co-cultured in the presence of fibroblasts cultivated on microcarrier beads. In the case of cytoplasmic (neutral) CE hydrolysis, no differences in CE hydrolytic activity could be demonstrated in any of the cells studied (Table 2).

Similar to CE hydrolysis, CE synthesis in SMC was also influenced by ENDO. We observed increased synthesis of CE from labeled oleic acid in SMC in the presence of ENDO 24 and 48 hr after initiating the experiment (**Table 3**). A 2.5-fold increase in ACAT activity in co-cultured SMC appears to account for the observed increase in CE synthesis (Table 2). Similar to our findings for CE hydrolysis, ENDO, cultured on SMC as a semi-confluent monolayer or on microcarrier beads, induced a modulatory effect on cholesterol esterifying activity in co-cultured SMC. Addition of ECDM alone to SMC did not produce an effect. Also, no differences in ACAT activity in SMC were observed in the presence of fibroblasts (Table 2). With regard to the synthesis of other esterified lipids, we also observed a significant ($P < 0.05$) increase in the incorporation of [^{14}C]oleic acid into phospholipids and TG in SMC co-cultured with ENDO at 24 hr following the initiation of the experiment (Table 3). The addition of ECDM to SMC did not produce a significant effect on the synthesis of these esterified lipids.

Similar to lysosomal CE hydrolysis, there was a twofold decrease in NAGase activity (a lysosomal marker) in SMC co-cultured with ENDO as compared to SMC cultured alone. However, we were unable to demonstrate this effect in SMC when ENDO were grown on microcarriers and then co-cultured with SMC. Likewise, ECDM or fibroblasts had no effect on NAGase activity in co-cultured SMC (Table 2). No differences in neutral-α-glucosidase activity could be demonstrated in any of the experimental groups. These results paralleled those obtained with cytoplasmic NCEH activity (Table 2).

To ascertain whether the interaction of ENDO with SMC in co-culture would produce other cellular effects in addition to alterations in lipid metabolism, we measured protein synthesis by studying the incorporation of [^{14}C]-leucine into protein by SMC. We found no differences in the amount of labeled TCA-precipitable protein synthesized by SMC cultured alone or co-cultured with ENDO or ECDM (SMC alone: 28.1 ± 3 × 10^3 cpm/mg prot.; ENDO/SMC: 33.3 ± 5 × 10^3 cpm/mg prot.; ECDM/SMC: 28.5 ± 4 × 10^3 cpm/mg prot.; X ± SE, n = 5).

Cell proliferation studies indicated that ENDO or ECDM significantly ($P < 0.05$) inhibited the proliferation of cultured SMC after 60 hr of co-incubation (**Table 4**). The inhibitory effect of ECDM on SMC growth in vitro was striking and in contrast to our findings regarding CE metabolism. Fibroblasts had no effect on the proliferative rate of co-cultured SMC during the 60-hr period (data not shown).

The endocytotic rate of labeled sucrose by SMC was influenced by ENDO and ECDM during the 60-hr incubation (**Fig. 3**). There was a threefold increase in the uptake of labeled precursor in SMC co-cultured with ENDO or ECDM as compared to SMC cultured alone. Fibroblasts did not alter endocytotic activity in SMC over the experimental period (data not shown).

DISCUSSION

Arterial ENDO appear to modulate the phenotypic expression of co-cultured SMC such that their metabolism differs from SMC cultured alone. Recently we showed that the vascular wall exhibits profound alterations in metabolism after arterial injury (2, 3). In fact, the regenerated endothelium can modulate these observed metabolic alterations in the injured blood vessel (2, 3). As reported herein, we have now extended these findings by demonstrating that arterial ENDO can induce a metabolic response in co-cultured arterial SMC similar to those observed in re-endothelialized arteries.

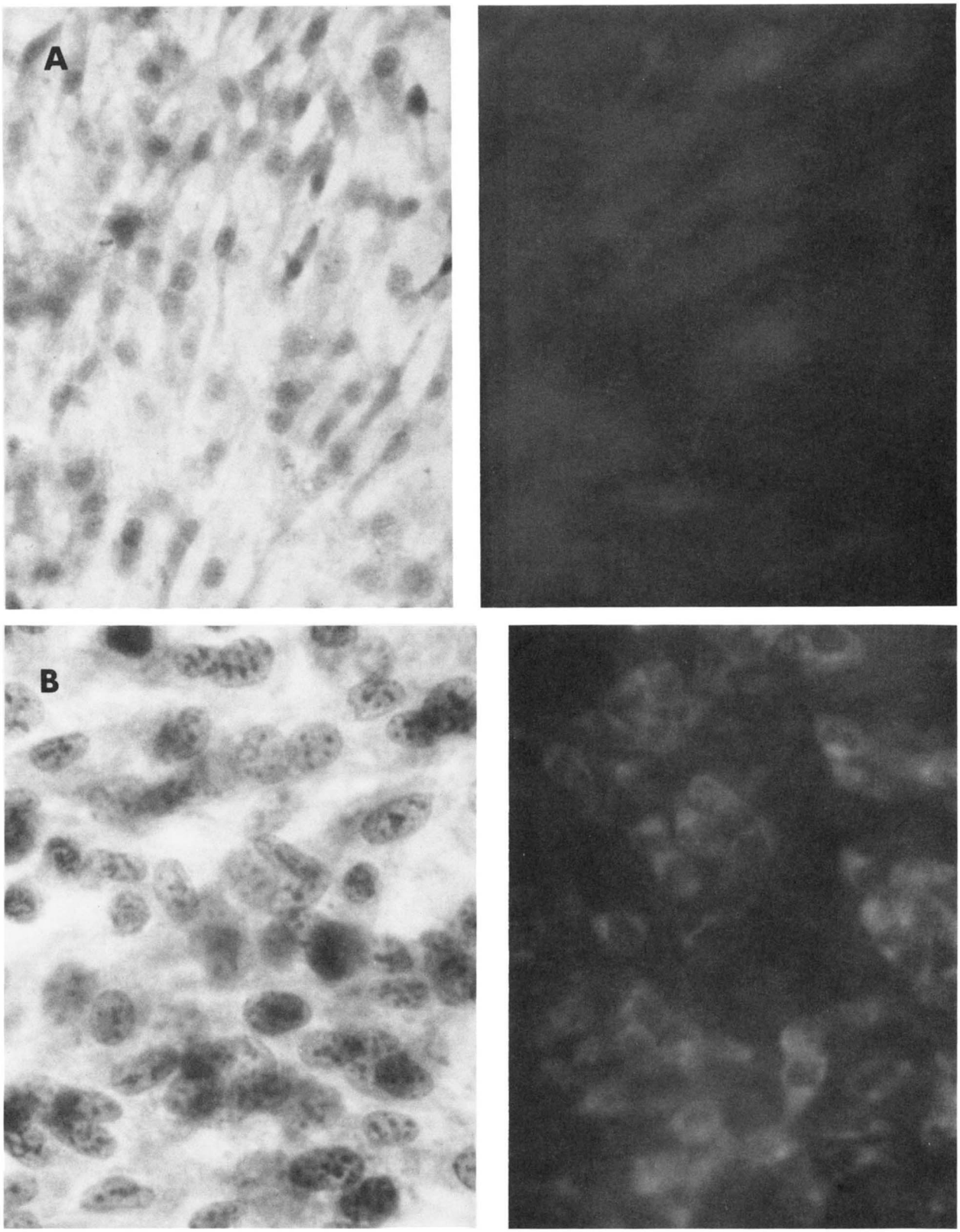

Fig. 1. Light and immunofluorescence microscopy of arterial ENDO co-cultured with arterial SMC. Photomicrographs of equivalent areas from duplicate coverslips on which bovine arterial SMC or a 48-hr co-culture of bovine arterial ENDO on SMC were grown. Left half of each figure represents cells stained with hemotoxylin and eosin. Right half of each figure represents cells stained with anti-factor VIII rabbit serum and FITC-conjugated anti-rabbit serum. (A) Bovine arterial SMC following a 1.0% collagenase treatment to remove co-cultured ENDO (H + E: × 250; IF: × 320). Note the lack of ENDO following enzymatic treatment. (B) Co-cultured bovine arterial ENDO on fibronectin-coated SMC for 48 hr (H + E: × 400; IF: × 320). It is noteworthy that the ENDO do not grow as a continuous monolayer on confluent SMC. ENDO do not grow as a continuous monolayer in our co-culture system even after 21 days in culture.

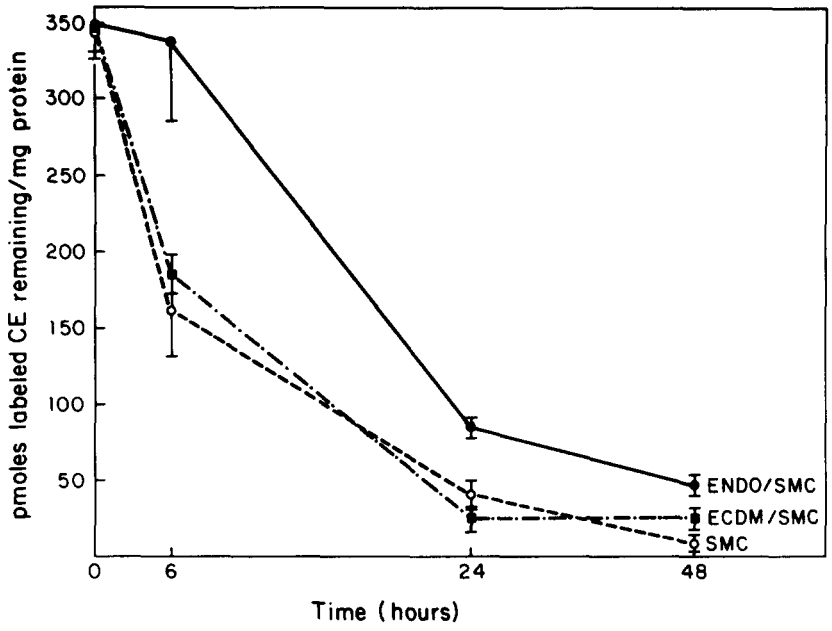

Fig. 2. Time course of hydrolysis of cholesteryl [1-^{14}C]oleate by SMC cultured alone, SMC co-cultured with ENDO, or SMC cultured with ECDM. Smooth muscle cells (5×10^5 cells) were fed 2.0 ml RPMI-1640, and 45 μg of LDL containing [1-^{14}C]cholesteryl oleate (13,000 cpm) for 36 hr. ENDO (7×10^5 cells) or 2.0 ml ECDM were added separately to some of the SMC for the designated period in the presence of RPMI-1640 plus unlabeled 100 nmol of oleate–20 nmol of albumin to prevent re-esterification of the released radiolabeled oleic acid from the hydrolyzed CE. Cells were not fed during the experimental period. At the indicated time, ENDO or ECDM were removed as described in the Methods section, and the SMC were washed twice with PBS. Lipid was extracted in situ, and the amount of radiolabeled CE remaining in SMC was determined as described in the Methods section. Each point is the mean (± SE) for four separate determinations. ECDM had no effect on CE hydrolysis in intact SMC cultured alone as compared to SMC co-cultured with ENDO ($P < 0.05$).

TABLE 2. Enzyme activities* in arterial smooth muscle cells cultured alone or in the presence of arterial endothelial cells or fibroblasts

	CE Metabolism			Cell Marker Enzymes	
Culture System	ACEH	NCEH	ACAT	NAGase	Neutral Gluc.
SMC alone	18.3 ± 0.8[a]	10.3 ± 0.7	2.3 ± 0.4[e]	10.0 ± 2.7[i]	1.8 ± 0.3
ENDO/SMC	9.5 ± 0.3[a,b]	8.8 ± 0.6	5.2 ± 0.7[e,f]	4.2 ± 0.8[i,j]	2.1 ± 0.2
ECDM/SMC	21.4 ± 3.1[b]	9.9 ± 1.9	3.0 ± 0.7[f]	11.9 ± 3.1[j]	2.4 ± 1.0
SMC + microcarriers alone	19.5 ± 1.1[c]	11.2 ± 1.2	2.5 ± 0.1[g]	10.8 ± 1.9	1.1 ± 0.7
ENDO-microcarriers/SMC	10.1 ± 0.7[c,d]	8.9 ± 1.2	6.1 ± 0.7[g,h]	13.7 ± 2.1	2.8 ± 1.0
Fibroblasts-microcarriers/SMC	17.9 ± 2.1[d]	12.7 ± 2.1	2.6 ± 0.9[h]	10.0 ± 1.7	1.5 ± 0.5

Smooth muscle cells were grown to confluency in 60 × 15 mm petri dishes (5.0×10^5 cells) in RPMI-1640 plus 10% NCS. At time 0, SMC were washed with PBS and either 2.0 ml of culture media containing 7.0×10^5 ENDO, 2.0 ml ECDM (see Methods section), 2.0 ml solution of 5×10^5 DEAE Sephadex (Cytodex-3) beads, 2.0 ml of these microcarriers containing 8.0×10^5 ENDO, or 2.0 ml of these microcarriers containing 6.0×10^5 fibroblasts were added separately to the attached SMC. Cells were incubated at 37°C for 48 hr. Cells were not fed during the experimental period. ENDO, ECDM, or microcarriers were then removed from the SMC as described in the Methods section. SMC were washed twice with PBS, and were harvested for the assessment of CE metabolic activities, cell marker enzyme activities, and DNA. Data represent the mean ± SE for eight individual dishes.

*mU/mg DNA ± SE.

[a]Values with the same corresponding letters are significantly different ($P < 0.05$).

TABLE 3. Synthesis of esterified lipid in arterial smooth muscle cells cultured alone or in the presence of arterial endothelial cells or endothelial cell-conditioned media*

	Esterified Lipid					
	Cholesteryl Esters		Phospholipid		Triglycerides	
	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
SMC alone	1.3 ± 0.1[a]	2.4 ± 0.6[c]	24.0 ± 1.5[e]	19.3 ± 4.1[g]	101.7 ± 8.5[i]	177.3 ± 51.5[j]
ENDO/SMC	4.7 ± 1.7[a,b]	18.9 ± 1.6[c,d]	51.3 ± 2.9[e,f]	33.3 ± 4.7[g,h]	200.4 ± 14.9[i]	285.7 ± 28.2[j,k]
ECDM/SMC	1.7 ± 0.2[b]	2.3 ± 0.8[d]	30.4 ± 1.8[f]	20.7 ± 0.5[h]	134.2 ± 10.6[i]	132.2 ± 22.4[k]

Smooth muscle cells were grown to confluency in 60 × 15 mm petri dishes (5.0×10^5 cells) in RPMI-1640 plus 10% NCS. At time 0, SMC were washed once with PBS, then pulsed with 2.0 ml RPMI-1640 plus 100 nmol of [1-^{14}C]oleate (6200 cpm)–20 nmol of albumin and 50 μg of unlabeled LDL (as a source of cholesterol). One group of intact SMC also contained 7.0×10^5 ENDO while another group of SMC was fed 2.0 ml ECDM. Final volume in all three groups was 2.0 ml. Cells were not fed during the experimental period. At the designated times, ENDO and ECDM were removed (see Methods section). Intact SMC were washed twice with PBS, and the amount of radiolabeled esterified lipid was assessed in all groups of SMC. Data represent the mean ± SE for four individual dishes.

* nmol of labeled oleate incorporated/mg of protein.

[a] Values with the same corresponding letters are significantly different ($P < 0.05$).

Our results indicate that ENDO co-cultured with SMC alter the CE cycle in SMC (Tables 2 and 3; Fig. 2). With respect to CE synthesis, ENDO enhanced the incorporation of labeled oleic acid into esterified lipid by co-cultured SMC. This was particularly evident in CE and was due to a stimulatory effect on ACAT activity. No differences in cytosolic CE hydrolysis (NCEH activity, Table 2) were observed between co-cultured SMC and SMC cultured alone. With respect to lysosomal CE hydrolysis, we observed a small reduction in CE hydrolysis in SMC co-cultured with ENDO as compared to SMC cultured alone (Fig. 2), due to decreased ACEH activity (Table 2). These results are supported, in part, by the findings of Davies et al. (12) who studied the catabolism of labeled LDL in SMC co-cultured with ENDO grown on microcarrier spheres. Their results suggest that the presence of ENDO enhances the uptake of LDL by co-cultured SMC but inhibits LDL degradation. Paradoxically, they also reported that ECDM had an inhibitory effect on LDL binding, internalization, and degradation in co-cultured SMC (12). In our experiments, we were unable to demonstrate an effect with ECDM on cholesterol esterification or CE hydrolysis (Tables 2 and 3; Fig. 2).

Phenotypic modulation of cultured SMC does not appear to be influenced by serum components (39), but may be regulated by other factors. In our co-culture system, specific component(s) released into the nutrient medium, derived from co-cultured ENDO, may alter SMC metabolism. For example, ENDO but not ECDM, influenced the CE cycle in our system, suggesting a requirement for the obligatory presence of ENDO in order to induce an effect on CE metabolism in SMC. In addition, we have also observed that both sparse and semi-confluent ENDO exert similar metabolic effects on co-cultured SMC, indicating that close apposition of large numbers of ENDO with SMC is unnecessary in order to elict these responses in CE metabolism. This conclusion is supported by our observation that ENDO grown on microcarriers produce similar modulation in co-cultured SMC. In contrast to these findings, both ENDO and ECDM enhanced fluid endocytosis of labeled sucrose into confluent SMC (Fig. 3) and significantly decreased SMC proliferation in vitro (Table 4). It has been suggested that both ENDO and ECDM may inhibit SMC growth in culture through the ability of ENDO to release heparin or heparin-like materials into the media (40–42). In contrast to these findings, however, others (43, 44) have demonstrated that ENDO stimulate SMC growth in vitro by releasing mitogens. These conflicting reports may reflect the variable factors in experimental design, such as cell density and the subpassage of cells, utilized in these studies.

Our observations that ENDO stimulate CE synthesis and inhibit CE hydrolysis in co-cultured SMC are consistent with our previous observations that metabolic function in the injured artery is altered by the presence of regenerated endothelium. However, these results are

TABLE 4. Proliferation of arterial smooth muscle cells in culture in the presence or absence of arterial endothelial cells or endothelial cell-conditioned media

	Cell Number*	
	24 hr	60 hr
SMC	27 ± 3 × 10^3/dish	58 ± 7 × 10^3/dish[a]
ENDO/SMC	28 ± 4 × 10^3/dish	39 ± 4 × 10^3/dish[a]
ECDM/SMC	21 ± 3 × 10^3/dish	27 ± 3 × 10^3/dish[a]

Smooth muscle cells were plated in 60 × 15 mm dishes at a sparse concentration of 5×10^4. At time 0, ENDO (5×10^4 cells/dish) in RPMI-1640 plus 10% NCS or 2.0 ml ECDM (see Methods section) were added. Cells were not refed during the experimental period. At the designated time periods, ENDO or ECDM were removed (see Methods section), and SMC were counted using a hemocytometer. The SMC remained 99 + % viable during the experimental period. Data represent the mean for five separate analyses.

* Number of SMC/60 × 15 mm Primaria dish ± SE.

[a] Values with the same corresponding letters are significantly different ($P < 0.05$).

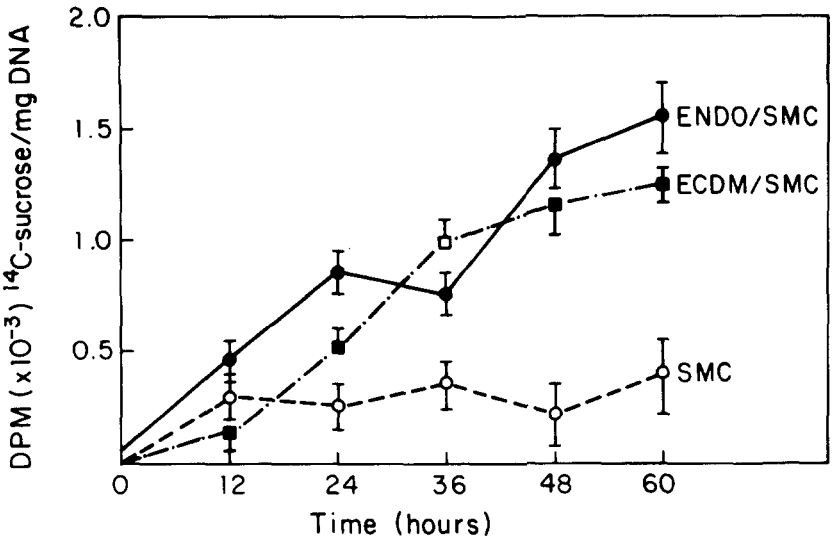

Fig. 3. Time course of fluid-endocytotic rates of labeled sucrose into arterial SMC cultured alone, SMC co-cultured with arterial ENDO or SMC cultured with ECDM. Semi-confluent SMC (5×10^4 cells) were fed RPMI-1640 plus 10% NCS. Endothelial cells were plated on SMC at a density of 7×10^4 cells in RPMI-1640 plus 10% NCS. Two ml of ECDM was also added separately to SMC. Total volume in all groups was 2.0 ml. Cells were not fed during the experimental period. At designated time periods, ECDM and ENDO were removed from the SMC (see Methods section), and endocytotic activity and DNA analysis were evaluated in treated and untreated SMC. Each point is the mean (± SE) for six separate determinations. There was a threefold increase in the uptake of labeled sucrose in SMC co-cultured with ENDO or ECDM as compared to SMC cultured alone ($P < 0.05$).

difficult to extrapolate to the uninjured artery since SMC in culture are modulated cells (45, 46). Cultured SMC exhibit profound alterations in metabolism similar to those found in injured arteries (2, 3). The presence of a feeder layer of ENDO may help to maintain a more differentiated state in the co-cultured SMC.

In conclusion, our data suggest for the first time that there are specific interactions between SMC and the overlying ENDO with respect to the modulation of CE metabolism. This effect appears to require the continued presence of ENDO because ECDM did not elicit a similar response. Contrary to these findings, both ENDO and ECDM can alter other cellular processes, including proliferation and endocytosis in co-cultured SMC. These additional results suggest that SMC may respond differently to ENDO-derived humoral factors such as heparinoid compounds or the more labile prostaglandins. In previous experiments we showed that prostaglandin I_2 can enhance lysosomal and cytosolic CE hydrolytic activities (15, 26), while prostaglandin E_2 inhibited CE synthetic (ACAT) activity in SMC cultured alone (26). Since ENDO (as well as SMC) synthesize several eicosanoids including PGI_2 and PGE_2 (47, 48), we were surprised to find the opposite effects in our co-culture system, viz., that ENDO caused an inhibition of acid CE hydrolase activity and an increase in CE synthetic (ACAT) activity in co-cultured SMC. We speculate that ENDO may alter the normal response of SMC to eicosanoids, thus modulating the phenotypic expression of CE metabolism in SMC. The mechanism(s) by which this occurs has not been elucidated. We are presently attempting to define those ENDO-derived factors that may be involved in the modulation of CE metabolic function in SMC derived from the arterial wall.

Preliminary reports of this work were presented at meetings of the Federation of American Societies for Experimental Biology in April 1983 and April 1985. These studies were supported in part by Research Grants HL-18828 from the National Heart, Lung, and Blood Institute of the National Institutes of Health. Dr. Falcone is currently a recipient of an Andrew Mellon Foundation Teacher-Scientist Award and an NIH New Investigator Research Award. Dr. Hajjar is an Established Investigator of the New York Heart Association.

Manuscript received 2 March 1985.

REFERENCES

1. St. Clair, R. W. 1976. Metabolism of the arterial wall and atherosclerosis. *Atheroscler. Rev.* **1:** 61–117.
2. Hajjar, D. P., D. J. Falcone, S. Fowler, and C. R. Minick. 1981. Endothelium modifies the altered metabolism of the injured aortic wall. *Am. J. Pathol.* **102:** 28–39.
3. Salisbury, B. G. J., D. P. Hajjar, and C. R. Minick. 1985. Altered glycosaminoglycan metabolism in injured arterial wall. *Exp. Molec. Pathol.* **42:** 306–319.
4. Falcone, D. J., D. P. Hajjar, and C. R. Minick. 1984. Lipoprotein and albumin accumulation in re-endothelialized and de-endothelialized aorta. *Am. J. Pathol.* **114:** 112–120.

5. Falcone, D. J., D. P. Hajjar, and C. R. Minick. 1980. Enhancement of cholesterol and cholesteryl ester accumulation in re-endothelialized aorta. *Am. J. Pathol.* **99:** 81–104.
6. Alavi, M., and S. Moore. 1984. Kinetics of low density lipoprotein interactions with rabbit aortic wall following balloon catheter de-endothelialization. *Arteriosclerosis.* **4:** 395–402.
7. Day, A. J., F. P. Bell, S. Moore, and R. J. Friedman. 1974. Lipid composition and metabolism of thrombo-atherosclerotic lesions produced by continued endothelial damage in normal rabbits. *Circ. Res.* **29:** 467–476.
8. Richardson, M., I. Ihnatowycz, and S. Moore. 1980. Glycosaminoglycan distribution in rabbit aortic wall following balloon catheter de-endothelialization. *Lab. Invest.* **43:** 509–516.
9. Jones, P. A. 1979. Construction of an artificial blood vessel wall from cultured endothelial and smooth muscle cells. *Proc. Natl. Acad. Sci. USA.* **76:** 1882–1886.
10. Merrilees, M. J., and L. Scott. 1981. Interaction of aortic endothelial and smooth muscle cells in culture. Effect on glycosaminoglycan levels. *Atherosclerosis.* **39:** 147–161.
11. Warren, H. B., S. E. O'Connor, B. M. Eisenhaure, and P. F. Davies. 1983. Coculture of vascular endothelium and smooth muscle cells: effects of heparin on smooth muscle cell proliferation. *J. Cell. Biol.* **97:** 87a (abstract).
12. Davies, P. F., G. A. Truskey, H. B. Warren, S. E. O'Connor, and B. Eisenhaure. 1985. Metabolic co-operation between endothelial cells and smooth muscle cells in co-culture: changes in lipoprotein metabolism. *J. Cell Biol.* In press.
13. Davies, P. F., C. Kerr, and B. Eisenhaure. 1983. The microcarrier co-culture system: interactions between endothelial and smooth muscle cells in vitro. *In* Atherosclerosis. VI. F. G. Schettler, A. M. Gotto, G. Middelhoff, A. J. R. Habenicht, and K. R. Jurutka, editors. Springer-Verlag, New York. 385–389.
14. Ross, R. 1971. The smooth muscle cell. II. Growth of smooth muscle in culture and formation of elastic fibers. *J. Cell. Biol.* **50:** 172–186.
15. Hajjar, D. P., B. B. Weksler, D. J. Falcone, J. M. Hefton, K. Tack-Goldman, and C. R. Minick. 1982. Prostacyclin modulates cholesteryl ester hydrolytic activity by its effect on cyclic adenosine monophosphate in rabbit aortic smooth muscle cells. *J. Clin. Invest.* **70:** 479–488.
16. Jaffe, E. A., R. L. Nachman, and C. G. Becker. 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. *J. Clin. Invest.* **52:** 2745–2756.
17. Martin, G. M. 1973. Human skin fibroblasts. *In* Tissue Culture Methods and Applications. P. F. Kruse and M. K. Patterson, editors. Academic Press, New York. 39–43.
18. Davies, P. F., and C. Kerr. 1982. Co-cultivation of vascular endothelial and smooth muscle cells using microcarrier techniques. *Exp. Cell. Res.* **141:** 455–459.
19. Schwartz, S. M. 1978. Selection and characterization of bovine aortic endothelial cells. *In Vitro.* **14:** 966–980.
20. Falcone, D. J., N. Mateo, H. Shio, C. R. Minick, and S. D. Fowler. 1984. Lipoprotein-heparin-fibronectin-denatured collagen complexes enhance cholesteryl ester accumulation in macrophages. *J. Cell. Biol.* **99:** 1266–1274.
21. Havel, R. J., H. A. Eder, and J. H. Bragon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
22. Haley, N. J., S. Fowler, and C. de Duve. 1980. Lysosomal acid cholesteryl esterase activity in normal and lipid-laden aortic cells. *J. Lipid Res.* **21:** 961–969.
23. Hajjar, D. P., C. R. Minick, and S. Fowler. 1983. Arterial neutral cholesteryl esterase: a hormone-sensitive enzyme distinct from lysosomal cholesteryl esterase. *J. Biol. Chem.* **258:** 192–198.
24. Vahouny, G. V., S. Weersing, and C. R. Treadwell. 1964. Micellar-solubilized substrate and cholesterol esterase activity in vitro. *Arch. Biochem. Biophys.* **107:** 7–15.
25. Belfrage, P., and M. Vaughan. 1969. Simple liquid-liquid partition system for isolation of labeled oleic acid from mixtures with glycerides. *J. Lipid Res.* **10:** 341–344.
26. Hajjar, D. P., and B. B. Weksler. 1983. Metabolic activity of cholesteryl esters in aortic smooth muscle cells is altered by prostaglandins I_2 and E_2. *J. Lipid Res.* **24:** 1176–1185.
27. Folch, J., M. Lees, and G. H. Sloane Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226:** 497–509.
28. Hojnacki, J. L., and S. C. Smith. 1974. Separation of six lipid classes on one thin-layer chromatogram. *J. Chromatogr.* **90:** 365–367.
29. Peters, T. J., M. Muller, and C. de Duve. 1972. Lysosomes of the arterial wall. I. Isolation and subcellular fractionation of cells from normal rabbit aorta. *J. Exp. Med.* **136:** 1117–1139.
30. Brown, M. S., S. E. Dana, and J. L. Goldstein. 1975. Receptor-dependent hydrolysis of cholesteryl esters contained in plasma low density lipoprotein. *Proc. Natl. Acad. Sci. USA.* **72:** 2925–2929.
31. Hara, A., and N. S. Radin. 1978. Lipid extraction of tissue with low toxicity solvent. *Anal. Biochem.* **90:** 420–426.
32. Hajjar, D. P., T. N. Wight, and S. C. Smith. 1980. Lipid accumulation and ultrastructural change within the aortic wall during early spontaneous atherogenesis. *Am. J. Pathol.* **100:** 683–706.
33. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein determination with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
34. Goldstein, J. L., S. E. Dana, and M. S. Brown. 1974. Esterification of low density lipoprotein cholesterol in human fibroblasts and its absence in homozygous familial hypercholesterolemia. *Proc. Natl. Acad. Sci. USA.* **71:** 4288–4292.
35. Burke, J. M., and R. Ross. 1977. Collagen synthesis by monkey arterial smooth muscle cells during proliferation and quiescence in culture. *Exp. Cell Res.* **107:** 387–395.
36. Davies, P. F., and R. Ross. 1980. Growth-mediated, density-dependent inhibition of endocytosis in cultured arterial smooth muscle cells. *Exp. Cell Res.* **129:** 329–336.
37. Kissane, J. M., and E. Robins. 1958. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. *J. Biol. Chem.* **233:** 184–188.
38. Zar, J. H. 1974. Biostatistical Analysis. Prentice-Hall, Inc., New York.
39. Chamley-Campbell, J. H., G. R. Campbell, and R. Ross. 1981. Phenotype-dependent response of cultured aortic smooth muscle cells to serum mitogens. *J. Cell Biol.* **89:** 379–383.
40. Willems, C. H., G. C. B. Astaldi, P. H. G. de Groot, M. C. Janssen, M. D. Gonsalvez, W. P. Zeijlemaker, J. A. Van Mourik, and W. G. Van Aken. 1982. Media conditioned by cultured human vascular endothelial cells inhibit the growth of vascular smooth muscle cells. *Exp. Cell Res.* **139:** 191–197.
41. Castellot, J. J., M. L. Addonizio, R. Rosenberg, and M. J. Karnovsky. 1981. Cultured bovine arterial endothelial cells produce a heparin-like inhibitor of smooth muscle cell growth. *J. Cell Biol.* **90:** 372–379.

42. Chamley-Campbell, J. H., and G. R. Campbell. 1981. What controls smooth muscle phenotype? *Atherosclerosis.* **40:** 347–357.

43. Gajdusek, C., P. DiCorletto, R. Ross, and S. Schwartz. 1980. An endothelial cell-derived growth factor. *J. Cell Biol.* **85:** 467–472.

44. DiCorletto, P. E., C. M. Gajdusek, S. M. Schwartz, and R. Ross. 1983. Biochemical properties of the endothelium-derived growth factor: comparison to other growth factors. *J. Cell Physiol.* **114:** 339–345.

45. Fowler, S., H. Shio, and H. Wolinsky. 1977. Subcellular fractionation and morphology of calf aortic smooth muscle cells. Studies on whole aorta, aortic explants, and subcultures grown under different conditions. *J. Cell Biol.* **75:** 166–184.

46. Yao-Young, A. O., H. Shio, and S. Fowler. 1981. Growth, biochemistry, and morphology of isolated rabbit aortic smooth muscle cells maintained in the presence or absence of serum. *J. Cell. Physiol.* **108:** 461–473.

47. Hong, S. L. 1980. Effect of bradykinin and thrombin on prostacyclin synthesis in endothelial cells from calf and pig aorta and human umbilical cord vein. *Thromb. Res.* **18:** 787–795.

48. Marcus, A. J., B. B. Weksler, and E. A. Jaffe. 1978. Enzymatic conversion of prostaglandin endoperoxide H_2 and arachidonic acid to prostacyclin by cultured human endothelial cells. *J. Biol. Chem.* **253:** 7138–7141.