Stimulation of Fibroblast Collagen and Total Protein Formation by an Endothelial Cell–Derived Factor

Andrew G. Villanueva, Harrison W. Farber, Sharon Rounds, and Ronald H. Goldstein

We investigated the effect of medium conditioned by bovine aortic endothelial cells on collagen accumulation and total protein formation by human embryonic fibroblasts or bovine smooth muscle cells in cultures. The conditioned medium at a 1:10 dilution induced a twofold increase in collagen and total protein accumulation in fibroblast cultures. At low concentration (1:50 dilution), the conditioned medium stimulated collagen accumulation preferentially; at high concentration (1:10 dilution), overall protein synthesis also was increased. The increase in type I collagen accumulation was associated with an increase in the steady-state level of α1(I) mRNA for collagen. The conditioned medium increased the production of types I and III collagen without affecting the proportion of collagen types in both fibroblast and smooth muscle cell cultures. Partial purification of the endothelial cell–derived factor disclosed it to be a heat-stable protein with an apparent molecular weight of 8–10 kDa. The stimulation of protein formation by this substance was not inhibited by antibodies against transforming growth factor-β or the insulinlike growth factor I receptor. The partially purified factor stimulated protein production without affecting fibroblast proliferation. This endothelial cell–derived protein may play a role in the remodeling of vascular connective tissue by stimulating collagen synthesis. (Circulation Research 1991;69:134–141)

Endothelial cells synthesize substances that increase cell proliferation by fibroblasts and smooth muscle cells. In addition, several studies in vivo provide evidence that endothelial cells modulate the synthesis of the extracellular matrix perhaps through the production of specific effector substances. An increase in wall thickness of the pulmonary and systemic arteries results from increased intravascular pressure. Langille and O’Donnell observed that vascular remodeling required an intact endothelium and postulated that this remodeling was mediated by an endothelial cell–derived factor. Tozzi et al demonstrated that increases in static mechanical tension applied to intact pulmonary arteries caused an increase in collagen and elastin production in segments with intact endothelium but not in segments denuded of endothelium. These studies suggest that the endothelium modulates connective tissue accumulation by fibroblasts and smooth muscle cells.

Accumulating evidence indicates that connective tissue remodeling is regulated by effector substances functioning through different cellular pathways. Certain effector molecules such as transforming growth factor-β (TGF-β) stimulate collagen formation without activating fibroblast proliferation. Other substances such as insulin-related peptides stimulate collagen formation but also act in concert with other molecules to stimulate cell division. In these studies, we investigated the effect of endothelial cell–derived conditioned medium on collagen and total protein production by lung fibroblasts. We demonstrate that bovine aortic endothelial cells (BAECs) in culture release a substance that activates collagen and total protein production by fibroblasts and smooth muscle cells.

Materials and Methods

Powdered tissue culture media and all supplements were obtained from Grand Island Biological Co., Grand Island, N.Y., except where otherwise noted. Bovine serum was obtained from Hyclone Laboratories, Inc., Logan, Utah, and fetal calf serum was obtained from Sigma Chemical Co., St. Louis. Phenylmethylsulfonyl fluoride, hydroxymercurobenzoate, and EDTA also were obtained from Sigma. [methyl-3H]Thymidine was obtained from New En-
gland Nuclear, Boston, and 1-[3H]proline was obtained from Amersham Corp., Arlington Heights, Ill. Sephadex G-50 gel chromatography beads were obtained from Pharmacia LKB Biotechnology, Piscataway, N.J. Materials for polyacrylamide gel electrophoresis were obtained from Bio-Rad, Richmond, Calif. Purified platelet-derived growth factor (PDGF, AB isoform), anti-PDGF immunoglobulin G, TGF-β, and anti-TGF-β were obtained from Research and Diagnostic Systems, Minneapolis, Minn. αR-3 antibody was a gift from Dr. Steve Jacobs.

**Cell Cultures**

Bovine endothelial cells were obtained from calf aortas. Briefly, endothelial cells were obtained by lightly scraping the luminal surface of longitudinally opened vessels. The cells were suspended in minimal essential medium (MEM; Flow Laboratories, Inc., McLean, Va.) containing 15% bovine serum and initially were seeded in 25 cm² flasks (Corning Glass Inc., Corning, N.Y.). Cultures were incubated at 37°C in 95% air-5% CO₂ and were passed after treatment with viocase (0.25%, GIBCO, Grand Island, N.Y.). Cells were verified as endothelial cells by phase microscopic “cobblestone appearance,” immunofluorescent staining for factor VIII antigen, and angiotensin converting enzyme activity. Experiments were performed by using endothelial cells of passage 3–12 from several different primary cell lines.

Human embryonic lung fibroblasts (IMR-90, Institute for Medical Research, Camden, N.J.) or bovine aortic smooth muscle cells (kindly provided by G. Sonenschein) were grown in Dulbecco’s modified Eagle’s medium with 0.37 g sodium bicarbonate/100 ml, 10% fetal bovine serum, 100 units/ml penicillin, 10 µg/ml streptomycin, and 0.1 mM nonessential amino acids. Cells were maintained in a humidified 5% CO₂-95% air incubator at 37°C and grown to confluence in 35-mm plastic dishes (Falcon Plastics, Los Angeles). After confluence was reached, the cells were placed into the quiescent state by reducing the serum content of the medium to 0.4%. These cells remained viable and retained the ability to divide on resuscitation.

**Preparation of Conditioned Medium**

BAECs were grown to confluence on 100-mm plastic dishes (Corning). Confluence, assured by phase microscopy, occurred approximately 4 days after the dishes were seeded. The confluent monolayers, containing approximately 10⁷ cells, were washed with phosphate buffered saline (pH 7.35), and the medium was replaced with serum-free MEM. At the end of the incubation period, the medium from culture plates was pooled and centrifuged at 800g for 15 minutes at 4°C to remove cellular debris. The supernatants were used immediately or frozen at −20°C until further assay was performed. Endothelial cell monolayers were assessed for injury by phase microscopic appearance, adherent cell counts, and ⁵¹Cr release. There was no observable cell injury when cells were kept in serum-free conditions for 24 hours.

**Assay for Mitogenic Activity**

Confluent quiescent fibroblast cultures were treated with serum-free medium in the presence or absence of medium conditioned by BAECs. Cell proliferation was assessed in fibroblast cultures by determining the incorporation of 2 µCi/ml [³H]thymidine (80 Ci/mmol) into a trichloroacetic acid-insoluble cell fraction. The medium was removed and the cell layer washed twice with saline, exposed to 1% Triton X-100, and centrifuged after the addition of trichloroacetic acid (10% final concentration). The trichloroacetic acid-precipitated material was solubilized by heating to 70°C for 20 minutes, and radioactivity was determined by scintillation counting. Statistical evaluation of the data was performed using Student’s t test for means of equal and unequal size. Values of p<0.05 were considered significant.

**Assay for Collagen and Total Protein Production**

Collagen and total protein production were evaluated by determining the amount of nondialyzable [³H]hydroxyproline (collagen) and [³H]proline (total protein) present in the culture medium and cell layer. The medium of confluent quiescent cell cultures was replaced with serum-free medium, ascorbate (50 µg/ml), and 2 µCi/ml [³H]proline in the presence or absence of conditioned medium. After 6 or 24 hours, the medium and cell layer were removed and dialyzed against H₂O at 4°C. The samples then were lyophilized and subsequently hydrolyzed in 6N HCl for 20 hours at 106°C. The hydrolysates were analyzed for [³H]hydroxyproline and [³H]proline using an automatic amino acid analyzer (Beckman Instruments Inc., Fullerton, Calif.) fitted with a stream-splitting device and a fraction collector. The hydroxyproline and proline peaks were separated by several fractions without overlap. Fractions were placed in scintillation fluid, and radioactivity was determined. To determine the amounts of hydroxyproline in the hydrolysates (nanomoles per sample), the amino acids were reacted with ninhydrin and assayed colorimetrically.

**Polyacrylamide Gel Electrophoresis**

Confluent quiescent fibroblast cultures were labeled with [³H]proline (2 µCi/ml) in the presence or absence of conditioned medium for 24 hours. The media from two dishes were pooled, and a solution of protease inhibitors yielding a final concentration of 10⁻⁴ M phenylmethylsulfonyl fluoride, 10⁻³ M hydroxymercuribenzoate, and 2×10⁻⁷ M EDTA was immediately added. The samples were dialyzed against H₂O at 4°C and then lyophilized. Polyacrylamide gel electrophoresis of equal aliquots of samples from untreated and conditioned medium–treated cultures was performed in the presence or absence of dithiothreitol on a 6% gel. Autoradiography was performed according to the method of Bonner and Laskey.
RNA Isolation and Northern Analyses

Total cellular RNA was isolated by the methods outlined by Chirgwin et al.22 RNA was quantitated by absorbance at 260 nm. Purity was assessed by absorbance at 280 and 310 nm. Quantitation was confirmed by size determination of ribosomal bands that had been fractionated on agarose–formaldehyde gels stained with ethidium bromide. RNA (10 μg) was electrophoresed on a 1% agarose, 6% formaldehyde gel and transferred to a nitrocellulose filter. The filter was prehybridized for 4–24 hours at 42°C. Hybridization was performed using 0.5–1.0×106 cpm per lane of labeled probe (specific activity, 4–10×106 cpm/μg). The cDNA probes used in these experiments were pαR1 for α(I) collagen and pαR2 for α(II) collagen (kindly provided by Dr. D. Rowe)23 and a β-actin probe (kindly provided by Dr. S. Farmer).24 After hybridization, the nitrocellulose filter was washed according to methods described by Thomas.25 The filter was autoradiographed onto x-ray film for 1–2 days. Densitometric analysis was performed with an Ultrosan XL (Pharmacia LKB, Broma, Sweden) densitometer.

Characterization of Endothelial Cell–Derived Collagen Stimulating Factor

To determine the biochemical characteristics of the endothelial cell–derived factor that stimulates collagen production by lung fibroblasts, the conditioned medium was subjected to dialysis, microfiltration, incubation with trypsin, heating, lipid extraction, and gel chromatography. Extensive dialysis against H2O was performed using Spectra/Por molecular porous membranes (Spectrum Medical Industries, Inc., Los Angeles) with a molecular weight cutoff size of 6–8 kDa. For microfiltration, the conditioned medium was filtered through a Millex-GS 0.22-μm filter unit (Millipore Corp., Bedford, Mass.). For trypsin incubation, we used trypsin attached to sepharose beads (TPCK-Trypsin and Sepharose CL-4B; Pierce Chemical Co., Rockford, Ill.).

The endothelial cell supernatants were acidified (pH 3.5) with 2 M citric acid, and lipids were extracted with 1.5 vol diethyl ether. After vigorous mixing and centrifugation, the resulting organic and aqueous phases were isolated, evaporated to dryness under nitrogen, resuspended in equal volumes of MEM, and then dialyzed extensively against distilled water. The aqueous fraction then was separated using a Sephadex G-50 90×1.5 cm column and an eluting solution of phosphate buffered saline, pH 7.4. Each fraction was dialyzed against H2O, lyophilized, resuspended in MEM, and assayed for biological activity.

Results

Effect of Endothelial Cell–Derived Conditioned Medium on Fibroblast Proliferation and Collagen Accumulation

We determined proliferation of lung fibroblasts by measuring the incorporation of [3H]thymidine in quiescent confluent fibroblast cultures stimulated for 24 hours by nondialyzable medium or medium conditioned by BAECs. There was a twofold to threefold increase in [3H]thymidine incorporation into DNA when the fibroblasts were treated with conditioned medium at a 1:1 dilution (vol/vol) with serum-free medium (Figure 1). There was a small increase in the incorporation of [3H]thymidine into DNA in fibroblasts treated with conditioned medium at a 1:10 dilution.

Figure 2 shows the effect of endothelial cell–derived conditioned medium on collagen and total protein accumulation. The amount of collagen was estimated by nondialyzable [3H]hydroxyproline, and the amount of total protein was estimated by nondialyzable [3H]proline. The conditioned medium at a 1:10 dilution induced a twofold to threefold increase in collagen and total protein accumulation. Approximately 15% of total collagen accumulation was found in the cell layer in untreated or conditioned medium–treated cultures.

Table 1 shows the effect of time of exposure and dose of conditioned medium on collagen and total protein accumulation. A twofold increase in collagen and total protein formation was found 6 hours after the addition of conditioned medium (1:10 dilution). A similar twofold increase was found at 24 hours after the addition of conditioned medium (1:10 dilution). At a lower dose (1:50), conditioned medium stimulated a small increase (20%) in collagen accumulation without stimulating overall protein synthesis. To ensure that the increase in nondialyzable [3H]proline reflected actual increases in protein ac-
cumulation rather than alterations in proline pool sizes, we measured the amount of total nondialyzable proline (nanomoles per milliliter) in the medium. Cultures incubated with conditioned medium (1:10)

![Graph 2](image2.png)

**Figure 2.** Effect of conditioned medium from bovine aortic endothelial cell (BAEC) cultures on collagen and total protein accumulation by lung fibroblasts. Confluent quiescent fibroblast cultures received either serum-free nonconditioned medium (C) or serum-free medium conditioned by BAEC cultures (CM) diluted in a 1:10 ratio with a medium containing [3H]proline. After 24 hours, the medium and cell layer were harvested separately, and the amounts of nondialyzable [3H]hydroxyproline and nondialyzable [3H]proline were determined. Data are expressed as mean ± SEM for four determinations. Asterisk denotes significant differences (p < 0.05).

**Table 1.** Effect of Endothelial Cell–Derived Conditioned Medium on Collagen and Total Protein Accumulation by Lung Fibroblasts

<table>
<thead>
<tr>
<th></th>
<th>[3H]Hydroxyproline (cpm×10^{-2})</th>
<th>[3H]Proline (cpm×10^{-2})</th>
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<tbody>
<tr>
<td>6-Hour incubation</td>
<td></td>
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<tr>
<td>No additions (control)</td>
<td>21.6±1.4</td>
<td>34.8±0.4</td>
</tr>
<tr>
<td>CM (1:10)</td>
<td>38.1±1.0*</td>
<td>65.8±6.6*</td>
</tr>
<tr>
<td>24-Hour incubation</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No additions (control)</td>
<td>68.2±0.1</td>
<td>203.9±7.5</td>
</tr>
<tr>
<td>CM (1:10)</td>
<td>122.5±10.9*</td>
<td>462.9±34.7*</td>
</tr>
<tr>
<td>CM (1:50)</td>
<td>82.9±3.0*</td>
<td>183.7±14.8</td>
</tr>
<tr>
<td>CM (1:100)</td>
<td>69.1±1.6</td>
<td>205.7±20.8</td>
</tr>
</tbody>
</table>

Conditioned medium (CM) was obtained from bovine aortic cells cultured in serum-free medium for 24 hours and then dialyzed against H_{2}O. Confluent quiescent fibroblasts were incubated with medium containing [3H]proline with no other additions or with medium containing various dilutions of CM (vol/vol). Medium was removed and exhaustively dialyzed against H_{2}O, and the amounts of nondialyzable [3H]hydroxyproline and [3H]proline were determined. Values are mean ± SEM.

*Significant differences from corresponding control (p < 0.05).

![Graph 3](image3.png)

**Figure 3.** Autoradiographs of 6% polyacrylamide gel electrophoresis (PAGE) of fibroblast and vascular smooth muscle cell medium after limited pepsin digestion. Confluent quiescent cultures were pulsed with [3H]proline for 24 hours. The medium was dialyzed and pepsin treated before equal aliquots from individual dishes of lung fibroblasts were separated on 6% PAGE for analysis by autoradiography. Left panel: Lane 1 received sample from untreated fibroblast cultures; lane 2 received sample from cultures treated with conditioned medium (1:10 dilution). Right panel: Lane 1 received sample from untreated smooth muscle cell cultures; lane 2 received sample from cultures treated with conditioned medium (1:10 dilution).

Production of intact collagen chains was assessed by polyacrylamide gel electrophoresis and autoradiography. The densitometric scanning indicated that the fibroblast cultures synthesized predominantly type I collagen (95%) and a small quantity of type III collagen (5%) (Figure 3, left panel). The identity of type III collagen was verified by the shift in the position of the high molecular weight α_{1}(III), after delayed reduction. The proportion of collagen types was similar in cultures treated with nonconditioned medium and cultures treated with conditioned medium. The densitometric scans also indicated that the
conditioned medium induced more than a twofold increase in the total amounts of collagen synthesized. We used bovine aortic smooth muscle cells to assess whether collagen production in these cells also was stimulated by the endothelial conditioned medium. We found that the conditioned medium (1:10 dilution) stimulated type I and type III collagen formation by vascular smooth muscle cells in culture (Figure 3, right panel). Densitometric scanning indicated that collagen formation was increased threefold by the addition of conditioned medium.

The effect of endothelial cell–derived conditioned medium on the expression of mRNA levels for α1(I) collagen chains in fibroblast cultures was examined. Total RNA was extracted after a 24-hour incubation, and equal loading on agarose–formaldehyde gels was verified by ethidium bromide staining of ribosomal bands. The steady-state level of α1(I) mRNA was determined in cultures without treatment or cultures treated with endothelial conditioned medium (1:10 dilution) by hybridization of extracted total cellular RNA with a cDNA probe. Figure 4A shows multiple transcripts for the α1 collagen gene that result from multiple polyadenylation attachment sites at the 3’ untranslated region.26 Densitometric scanning revealed a twofold to threefold increase in α1(I) mRNA in cultures treated with conditioned medium. The α1(I) collagen mRNA is constitutively expressed in these cells27 and was not increased by conditioned medium (Figure 4B). However, the conditioned medium induced large increases in the steady-state levels of β-actin (Figure 4C).

To determine whether the stimulation of collagen production was specific for medium conditioned by endothelial cells, we assessed the effect of serum-free medium conditioned by fibroblast cultures for 24 hours on collagen and total protein production by fetal lung fibroblasts. This conditioned medium did not stimulate the production of collagen or total protein. The amount of nondialyzable [3H]hydroxyproline was 4.6±0.5×103 cpm (mean±1 SEM), for four determinations, in fibroblast cultures with unconditioned medium and 4.4±0.9×103 cpm in cultures with conditioned medium. The amount of nondialyzable [3H]proline was 14.3±0.4×103 cpm in fibroblast cultures incubated with unconditioned medium and 16.3±3.1×103 cpm in cultures incubated with conditioned medium.

**Partial Characterization of the Endothelial Cell–Derived Fibroblast Stimulating Factor**

We undertook characterization of the substance by treating aliquots of conditioned medium with trypsin, heat, dialysis, microfiltration, or lipid extraction, followed by testing for biological activity (Figure 5). Incubation with trypsin eliminated the collagen-stimulating activity of the conditioned medium. Heating at either 56°C for 30 minutes or 100°C for 3 minutes did not reduce the activity of the conditioned medium. Dialysis against H2O using molecular porous membranes with a molecular weight cutoff size of 6–8 kDa did not reduce the activity, whereas microfiltration through a Millex-GS 0.22-μm filter unit (Millipore) reduced the activity in the filtrate to the level of the control group. When the conditioned medium was extracted with diethyl ether, all the biological activity appeared in the aqueous phase, and none appeared in the organic phase. Further purification of the conditioned medium using a Sephade G-50 column showed that the activity occurred in fractions corresponding to an apparent molecular weight of 8–10 kDa (Figure 6). When the substance was partially purified using a Sephade G-50 column, it did not activate cell proliferation as measured by [3H]thymidine incorporation into DNA (Figure 7). Furthermore, the partially purified factor did not stimulate further growth in the presence of insulin or epidermal growth factor.

**Relation of the Endothelial Factor to PDGF, TGF-β, and Insulinlike Growth Factors**

We examined whether purified human platelet PDGF (AB isoform) would activate collagen and

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**FIGURE 4. Effect of endothelial cell–conditioned medium on the steady-state level of α1(I), α2(I), and β-actin mRNA in fibroblast cultures.** Confluent quiescent lung fibroblasts were untreated (lane 1) or stimulated with conditioned medium at 1:10 dilution (lane 2). Total cellular RNA was extracted, electrophoresed, and transferred to a nitrocellulose filter. Ethidium bromide staining after Northern analysis demonstrated that approximately equal amounts of RNA were loaded into each lane. cDNA probes were labeled, incubated with the nitrocellulose filter for 40 hours, and autoradiograms were obtained for α1(I) collagen (panel A), α2(I) collagen (panel B), and β-actin (panel C). Location of the ribosomal RNA (28S or 18S) is indicated by the arrows.
total protein production by lung fibroblasts. Our results show that PDGF (5 ng/ml) stimulated protein production, but there was no increase in collagen accumulation; nondialyzable \([^3H]\)hydroxyproline was 32.8±4×10² cpm for three determinations in untreated cultures and 32.8±3×10³ cpm in PDGF-treated cultures, whereas \([^3H]\)proline was 109±9×10³ cpm in untreated cultures and 160±2×10³ cpm in PDGF-treated cultures.

We determined whether the endothelial cell-derived factor was related to TGF-\(\beta\) or to insulin-like growth factors (IGFs) by using specific blocking antibodies. The addition of these antibodies alone did not affect protein formation (data not shown). The presence of anti-TGF-\(\beta\) antibody at 10 \(\mu\)g/ml caused a 53% inhibition of TGF-\(\beta\)-induced protein formation (Table 2). The antibody did not inhibit the increase in protein formation stimulated by the conditioned medium. To determine whether the endothelial cell-derived factor was related to insulin or IGF-I, we used an antibody specific for the IGF-I receptor (αIR-3). This antibody inhibits the protein-stimulating effect of both insulin and IGF-I on lung fibroblasts.17 The presence of this antibody also did not inhibit conditioned medium-induced protein formation.

Figure 5. Effect of heat, trypsin, dialysis, microfiltration, and lipid extraction on the activity of conditioned medium. Serum-free medium conditioned by bovine aortic endothelial cells was untreated (CM), treated with trypsin for 2 hours (TRYP), heated at 56°C for 30 minutes, heated at 100°C for 3 minutes, dialyzed against H₂O using molecular porous membranes with a molecular weight cutoff size of 6–8 kDa (DIAL), or passed through a Millex-GS 0.22-µm filtration unit (FIL). Conditioned medium was extracted into aqueous (AQ) and organic (ORG) phases. Confluent quiescent fibroblast cultures received medium with no additions (C), conditioned medium (CM), or conditioned medium subjected to the various treatments, all diluted in a 1:1 ratio with a serum-free medium containing \([^3H]\)proline. After 24 hours, the medium and cell layer were harvested, and the amount of \([^3H]\)proline incorporated into proteins was measured. Data are expressed as percent control±1 SEM for four determinations.

Figure 6. Sephadex G-50 chromatography of medium conditioned by bovine aortic endothelial cell cultures. After extraction, the aqueous phase was extensively dialyzed against H₂O and lyophilized. A lyophilized aliquot containing 10 \(\mu\)g protein was resuspended in phosphate buffered saline, pH 7.4, and applied to a Sephadex G-50 column (90×1.5 cm) equilibrated in the same buffer. Effect of each fraction on protein accumulation was assayed using quiescent confluent fibroblast cultures that received \([^3H]\)proline and by measuring the amount of \([^3H]\)proline in the cell layer precipitated by 10% trichloroacetic acid. Chromatography of standards was performed in an identical manner and assayed by measuring UV absorbance at 280 nm. Molecular weight standards were chymotrypsinogen (25 kD), ribonuclease A (14 kD), cytochrome c (12.3 kD), and vitamin B₁₂ (1.2 kD).
Endothelial cells produce several substances that increase mesenchymal cell proliferation.\textsuperscript{1,2} We found that endothelial cell–derived conditioned medium stimulated an increase in cell division in confluent quiescent fibroblast cultures. This increase may be related to the production of PDGF by the endothelial cells. Gajdusek et al\textsuperscript{2} reported that endothelial cells produce a protein that inhibits the binding of radioiodinated PDGF to its receptor on target cells. PDGF consists of two chains, one of which is transcribed and synthesized by both human and bovine endothelial cells in culture.\textsuperscript{3,4} Although basic fibroblast growth factor is synthesized by endothelial cells, this mitogen is not secreted into the culture medium.\textsuperscript{5,28,29}

The mitogenic activity found in the conditioned medium is distinct from that stimulating collagen and total protein accumulation. When the factor was partially purified using gel chromatography, the 10-kDa fraction stimulated protein production but did not stimulate proliferation. This substance was trypsin sensitive, was completely partitioned into the aqueous phase after extraction, and unlike basic fibroblast growth factor, was heat stable.\textsuperscript{29} The material has an apparent molecular weight of 8–10 kDa and is released by endothelial cells in culture but not by human embryonic lung fibroblasts.

The ratio of collagen to total protein formation varied with the concentration of endothelial cell–derived conditioned medium added to fibroblast cultures. At low concentration (1:50 dilution), conditioned medium preferentially stimulated collagen accumulation; at high concentration (1:10 dilution), the increase in collagen accumulation was nonselective as compared with other proteins. The dose–response findings suggest that the increased collagen formation was not the result of overall stimulation of protein synthesis. This difference in the dose–response relation for stimulation of collagen and total protein formation was described previously for both TGF-\(\beta\) and IGF.\textsuperscript{13,17} This effect could result from binding to a multifunctional receptor or binding to receptors of different affinity.\textsuperscript{30} We observed that the conditioned medium induced increases in steady-state levels of collagen \(\alpha_1(I)\) and \(\beta\)-actin mRNA. Taken together, these results suggest that conditioned medium stimulates the production of a group of intracellular and extracellular structural proteins.

TGF-\(\beta\)\textsuperscript{13} and IGF-1,\textsuperscript{17} but not PDGF, stimulate collagen formation by lung fibroblasts in culture. TGF-\(\beta\) is a dimeric molecule with a molecular weight of 24 kDa and is inactive in its monomeric form.\textsuperscript{12} The addition of a blocking antibody to TGF-\(\beta\) did not inhibit the action of the endothelial cell–derived substance. We reported previously that \(\alpha\)IR-3, an

**Table 2. Effect of Antibodies to Transforming Growth Factor-\(\beta\) and the Insulin-like Growth Factor 1 Receptor on Protein Stimulation by Conditioned Medium**

<table>
<thead>
<tr>
<th>Addition</th>
<th>[(^{14})C]Proline (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (control)</td>
<td>1,476±134</td>
</tr>
<tr>
<td>TGF-(\beta) (1 ng/ml)</td>
<td>3,332±48</td>
</tr>
<tr>
<td>TGF-(\beta) (1 ng/ml)+anti-TGF-(\beta) (10 (\mu)g/ml)</td>
<td>2,340±163*</td>
</tr>
<tr>
<td>CM (1:10)</td>
<td>3,167±223</td>
</tr>
<tr>
<td>CM (1:10)+anti-TGF-(\beta) (10 (\mu)g/ml)</td>
<td>2,899±192</td>
</tr>
<tr>
<td>CM (1:10)+(\alpha)IR-3 antibody (1 (\mu)g/ml)</td>
<td>3,064±175</td>
</tr>
</tbody>
</table>

Conditioned medium (CM) was obtained from bovine aortic endothelial cells cultured in serum-free medium for 24 hours and then dialyzed against H\(_2\)O. Confluent quiescent fibroblasts were incubated with medium containing [\(^{14}\)C]proline with various additions for 24 hours. Medium was removed, and the amount of radioactivity in the trichloroacetic acid–insoluble fraction was determined. Values are mean±1 SD from three determinations. TGF-\(\beta\), transforming growth factor-\(\beta\).

*Significant difference from TGF-\(\beta\)-treated cultures without antibody (\(p<0.05\)).

**FIGURE 7.** Effect of partially purified endothelial cell factor on growth of lung fibroblasts. Confluent quiescent fibroblast cultures received medium containing [\(^3\)H]thymidine with no additions (CONTROL) or with the endothelial cell–derived factor (ECF) partially purified using a Sephadex G-50 column. Factor was added either alone or in combination with insulin (2 \(\mu\)g/ml) or epidermal growth factor (EGF, 10 ng/ml). In another set of cultures, cells were stimulated with EGF and insulin either alone or in combination. Amount of [\(^3\)H]thymidine incorporated in DNA was determined 24 hours later. Data are expressed as mean±1 SEM for four determinations.
antibody to the IGF-I receptor, inhibited insulin and IGF-I–induced collagen formation. This antibody did not block protein formation stimulated by the endothelial cell–derived factor. These results show that this substance is probably distinct from either TGF-β or insulin-related peptides. Whether this substance is a unique factor is uncertain at this time.

Collagen accumulation occurs as the result of increases in tangential pressure in pulmonary and systemic arteries. It is noteworthy that pressure-induced connective tissue accumulation in the pulmonary artery is dependent on the presence of endothelium. Our results indicate that endothelial cells produce a substance that stimulates collagen formation by fibroblasts and smooth muscle cells in culture. The stimulation of collagen formation by a protein derived from endothelial cells may be an important feature of connective tissue remodeling.

References

Key Words: endothelial cells \* collagen formation \* protein formation \* fibroblasts
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