Endothelins Produced by Endothelial Cells Promote Collagen Gel Contraction by Fibroblasts

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Abstract. Endothelial 1 (E1) is identified as an endothelial cell secreted factor that stimulates collagen gel contraction by fibroblasts. This identification is based on (*a*) co-localization of stimulatory activity in endothelial cell conditioned media with synthetic E1 in reversed phase analysis; (*b*) removal of the activity from conditioned media with antiserum directed against E1; and (*c*) the activity of synthetic E1. Treatment of endothelial cell conditioned media with immobilized anti-E1 antibodies removed 59% of the activity from the pool suggesting that E1 is the major contraction promoter in endothelial cell conditioned medium. The mechanism of action of E1 is shown to be different from serum in that E1-promoted contraction is dependent upon the synthesis of an unknown effector protein. Synthetic E1 is shown to be a potent promoter of gel contraction with half-maximal activity occurring at 32 pM. Two other endothelins, E2 and VIC, are slightly less active than E1. A fourth endothelin species, E3, is substantially less active. A comparison of E1 with other contraction promoting peptides revealed that E1 and platelet-derived growth factor are essentially equal in specific activity, whereas TGF_β is ~50-fold more potent.

FIBROBLASTS incubated on gels formed of native type I collagen develop a morphology typical of cells in dermis and tendon (7, 31). Additionally, over time the fibroblasts will reorganize the collagen fibrils into a structure which closely resembles dense connective tissue (2). Collagen matrices, condensed in this fashion, have been successfully transplanted and integrated as dermal replacements (15). This in vitro phenomenon resembles a number of pathologic as well as normal processes including dermal and tendonous contracture during wound healing (2), the development of contractile connective tissue membranes above and below the retina in proliferative vitreoretinopathy (19), and the matrix reorganization observed during connective tissue development (29).

Collagen gel contraction by fibroblasts involves the actin cytoskeleton and can be inhibited by agents such as cytochalasins (28, 11). It can involve cell surface integrin-type receptors since an antibody to the beta-1 subunit can inhibit this process when stimulated by PDGF (14). Gel contraction is not dependent upon de novo synthesis of collagens and apparently does not require enzymatic cross-linking, degradation or other covalent modifications of the collagens (11). Heparin can modulate the contraction process by altering the tensile strength or continuity of the collagen matrix, though this effect is on the matrix rather than the cells (12). Gel contraction is dependent upon the presence of exogenous factors that stimulate cells to exert contractile forces on the matrix.

We have examined fibroblast–collagen interactions with the aim of defining the exogenous factors that stimulate collagen gel contraction by fibroblasts and other nonmuscle cells. Serum, for example, stimulates matrix contraction by fibroblasts, but the molecular identities of the active species are not yet known (28). The secretory products of endothelial cells, collected as conditioned medium, contain potent promoters of gel contraction which, as a pool, are more than 50-fold more potent than serum and act through mechanisms different from that of serum (13). Type A promoters, serum derived, can directly stimulate the cells to contract matrix through a mechanism which does not require de novo protein synthesis. Type B promoters, such as that described within endothelial cell conditioned medium, are not active in the presence of an inhibitor of protein synthesis (13). In this report, we have identified the endothelins as the major endothelial cell-secreted peptide promoter of collagen matrix contraction by fibroblasts.

The endothelins are a recently described family of acidic, highly homologous peptides initially identified as potent vasoconstrictors secreted by vascular endothelial cells (32). Four known members of the endothelin family, E1, E2, E3, and vasoactive intestinal contractor (VIC)[1], the mature forms of which are 21 amino acids in length, were subsequently found to have many effects on both vascular and nonvascular tissues and cultured cells including fibroblasts (for review see reference 27). E1 and E2 bind to high affinity receptors on both murine and human fibroblast cell surfaces (30, 5), rapidly increasing the intracellular free Ca^{2+} con-

1. *Abbreviations used in this paper*: TFA, trifluoroacetate; VIC, vasoactive intestinal contractor.

centration (30, 23), stimulating phospholipase C (30, 22), and protein kinase C activities (30, 22) in a dose-dependent fashion. E3 binding to fibroblasts is negligible (23). The endothelins appear to elicit little mitotic response alone, but they act synergistically to amplify fibroblast responses to growth factors such as PDGF, basic fibroblast growth factor, and insulin-like growth factor-I (17, 3, 30). These observations lead us to speculate that the endothelins may serve as peptide hormones mediating communication between endothelial cells and local fibroblasts and function to amplify fibroblast growth and to promote matrix contraction by fibroblasts.

Materials and Methods

Cells

Cultures of human skin fibroblasts were established from foreskins obtained at circumcisions and used between passages 4 and 15. Cells were cultured in 75-cm^2 tissue culture flasks using growth medium composed of DME, 20 mM Hepes, and 10% FBS all from Gibco Laboratories (Grand Island, NY) (13). The cultures were incubated in an humidified atmosphere containing 5% CO_2 and 95% air. Cells were harvested for subculture or experimentation using 0.5% trypsin/0.02% EDTA solution (Gibco Laboratories). Bovine aortic endothelial cells were provided by Dr. Joanne Murphy-Ullrich (University of Alabama at Birmingham). These were grown under conditions similar to the fibroblasts except that the media contained 20% FBS.

Preparation of Endothelial Cell Conditioned Medium

Endothelial cells were grown to confluence in 75-cm^2 tissue culture flasks in DME with 20% FBS as described previously (13). At confluence, the growth medium was removed, the cell monolayers were rinsed and incubated further in serum-free DME. Each day for five days the culture medium was removed and replaced with fresh serum-free DME. The collection from the first day was discarded to exclude potential contamination by serum. The collected media were centrifuged to remove cell debris and stored at −20°C until use.

Preparation of Collagen Gels and Measurement of Matrix Contraction by Fibroblasts

Native collagen gels were prepared as described previously (13) using Vitrogen 100 (Collagen Corp., Palo Alto, CA) adjusted to physiological ionic strength and pH with 10× PBS (1.5 M NaCl, 0.1 M Na_2HPO_4) and 0.1 M NaOH, while maintained at 4°C. Aliquots (0.2 ml) of the collagen solution were added within circular scores (12 mm) on the bottom of 24-well culture plates and polymerized at 37°C for 90 min. The resultant gel thickness was ~2 mm.

Fibroblasts harvested as for subculture were washed with growth media containing serum and again with serum-free DME before application onto the top of the polymerized gels (5×10^4 cells/gel in 0.05 ml). The cultures were incubated at 37°C for 30 min to allow the cells to attach after which the wells were flooded with 1 ml DME containing the test substances. In experiments involving transforming growth factor beta (TGF_β), all wells were flooded with DME containing 1.0 mg/ml BSA, according to the manufacturer's instructions. Collagen gel contraction is observed as a function of changes in gel thickness. These were measured on an inverted phase contrast microscope (Nikon TMS; Nikon Inc., Garden City, NY) by adjusting the plane of focus from the bottom to the top of the gel and recording the distance of objective stage movement with a Z axis digitizer (LaSICO, Los Angeles, CA). This method of measurement is reproducible to 25 μm or 1.25% of the initial gel thickness (11). Photomicrographs of fibroblasts were taken using an inverted phase contrast microscope equipped with a 10× objective.

Reversed Phase Fractionation of Endothelial Cell Conditioned Medium

The proteins in conditioned medium (50 ml), prepared as described above, were concentrated over a 1.0-ml C_{18} syringe cartridge (Analtech Inc., Newark, DE). The cartridge was washed with 10 ml H_2O/0.1% trifluoroacetate (TFA) and eluted with 10 ml 60% acetonitrile/H_2O/0.1% TFA. The eluted pool was lyophilized, reconstituted with H_2O/0.1% TFA, loaded over a Vydac C_{18} analytical column, and eluted at 1.0 ml/min with a linear gradient of 0–60% acetonitrile containing 0.1% TFA. Eluted proteins were detected at 225 nm. Fractions corresponding to 2 min of elution were collected in polypropylene tubes. Samples (50 μl) of each fraction were lyophilized and reconstituted with 0.1 ml DME containing 1 mg/ml BSA before assay for stimulatory activity.

Reagents

Synthetic E1, E2, E3, and VIC were obtained from Peninsula Laboratories Inc. (Belmont, CA). Rabbit anti-human E1 antiserum was obtained from Dr. Eng Tau (Peninsula Laboratories). This antiserum, raised against synthetic E1 was reported by the supplier to have 7% cross-reactivity with E2 and E3. Human $TGF_{\beta 1}$ was purchased from Oncomembrane Inc. (Seattle, WA). Human recombinant PDGF-BB homodimer was purchased from Upstate Biotechnology Inc. (Lake Placid, NY). All peptides were reconstituted according to the manufacturers' instructions. Protein A Agarose was purchased from Pierce Chemical Co. (Rockford, IL). Other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

Identification of Endothelin-1 as an Endothelial Cell Secreted Promoter of Collagen Gel Contraction

Endothelial cell conditioned media proteins (2 mg in 50 ml) were fractionated by reversed phase HPLC using a gradient of 0–60% acetonitrile/0.1% TFA/H_2O over 40 min as described in Materials and Methods (Figs. 1 *A*, *solid line*). Fractions (2.0 ml) corresponding to two minutes were collected. Lyophilized aliquots of each fraction (50 μl) were reconstituted with 1 ml DME containing 1 mg/ml BSA and added to cultures of fibroblasts seeded on collagen gels. These were incubated for 4 h at 37°C after which changes in gel thickness were recorded (Fig. 1 *B*). The two fractions eluted between minutes 26 and 30 were active in promoting collagen gel contraction. Electrophoretic analysis of the active fractions using 5–15% acrylamide gradient gels did not reveal peptide(s) whose presence corresponded with the activity. There were, however, several small (<14 kD), poorly resolved bands near the ion front (not shown). As the molecular masses of the endothelins are in the range of 2.5 kD and E1 is known to be synthesized and secreted by endothelial cells we analyzed the possible relation between E1 and the activity we observed in the reversed phase fractions. Synthetic E1 (3 μg) was loaded and eluted from the same C_{18} column under the same conditions as the conditioned media proteins. The elution position of the synthetic peptide corresponded to the major contraction-promoting activity of endothelial cell conditioned medium (Fig. 1 *A*, *dotted line*).

Attempts to identify E1 within endothelial cell conditioned media by western blotting were unsuccessful. This was primarily because of the difficulties we encountered in resolving the low molecular weight peptide by gel electrophoresis. To confirm that the contraction-promoting activity observed was in fact because of E1 we attempted to remove the activity using an antiserum raised against synthetic E1. Direct neutralization of the activity with the antiserum was not possible given that serum also contains contraction-promoting factors. It was necessary to first separate the IgG fraction from the rest of the serum proteins. To accomplish this,

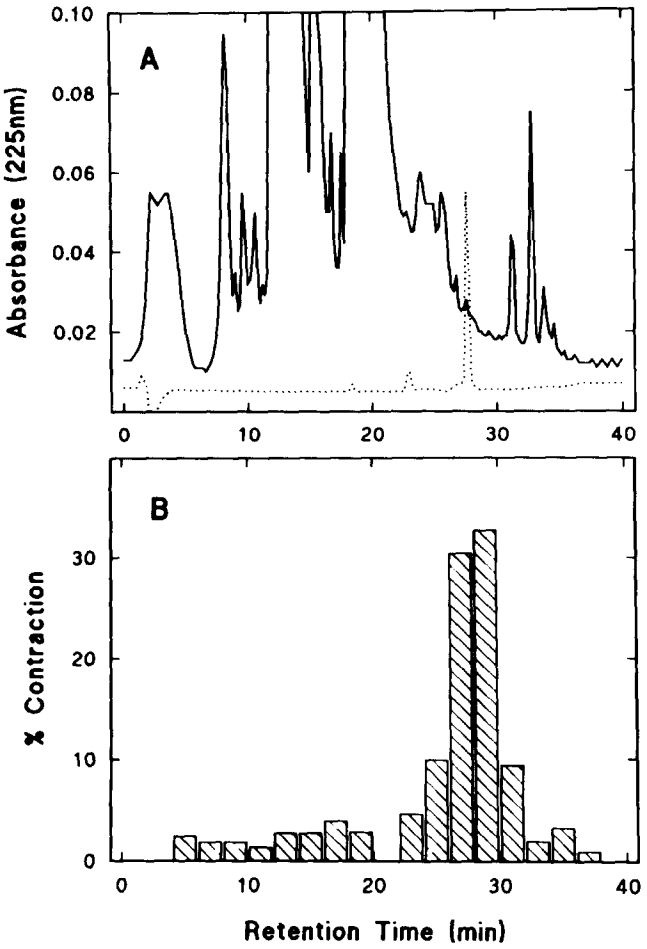

Figure 1. Reversed phase fractionation of endothelial cell conditioned media proteins. Conditioned medium proteins (~2.0 mg) were concentrated over a C_{18} cartridge, eluted with 60% acetonitrile/0.1% TFA, and lyophilized. This pool was reconstituted with H_2O/0.1% TFA and fractionated by reversed phase HPLC over a C_{18} column using a linear gradient of acetonitrile (0–60%) as described in Materials and Methods (*A, solid line*). 2.0-ml fractions, corresponding to 2 min, were collected and assayed individually for contraction-promoting activity on fibroblasts attached to collagen gels. The extent of contraction observed for 50 μl of each fraction after 4 h of incubation is shown in *B*. The retention time of the active fractions is compared to the retention time of synthetic E1 (3 μg) eluted under identical conditions (*A, dotted line*).

50-μl aliquots of protein A agarose were preincubated for 60 min with 50 μl anti-E1 antiserum or, as a control, nonimmune rabbit serum. These affinity matrices were washed with PBS (3 × 1 ml) and then incubated with 5.0 ml of unfractionated endothelial cell conditioned medium for 60 min at room temperature while mixing. After the incubation the affinity matrices were removed by centrifugation and the supernatants were tested in dilutions for contraction-promoting activity on fibroblasts attached to collagen gels. The results of this experiment, shown in Fig. 2, indicate that the anti-E1 matrix removed a substantial portion of the contraction-promoting activity observed after 4 h of incubation. A second treatment of the supernatants with additional anti-E1 matrix did not reduce the remaining contraction-promoting activity further (data not shown).

Linear regression analysis of the data in Fig. 2 yielded the functions $y = 30.09(\log x) + 6.80$ and $y = 33.31(\log x) - 8.22$ for nonimmune and immune serum-treated samples, respectively, with r_{corr} of 0.993 and 0.991 (Fig. 2, *dotted lines*). Defining one unit of contraction-promoting activity as the amount necessary to induce fibroblasts to reduce collagen gel thickness by 25%, we can calculate the activities of the two pools as 24.80 U/ml for nonimmune serum-treated and 10.06 U/ml for immune serum-treated media. These calculations indicate that the immunoreactive material accounts for 59% of the contraction promoting activity present in endothelial cell conditioned medium.

E1 Promotes Collagen Gel Contraction by Fibroblasts

To demonstrate that E1 can stimulate collagen gel contraction we performed a series of experiments using synthetic E1 added to serum-free culture medium. Fibroblasts attached to collagen gels were incubated in DME containing FBS (positive control), 1.0 μg/ml E1, or DME alone (negative control). Hourly measurements of gel thickness indicated that the synthetic E1 was able to stimulate gel contraction (Fig. 3). The onset of E1-promoted contraction, however, was delayed 2–3 h, compared to serum.

Fibroblast morphologies differed only upon the presence or absence of a contraction promoter. Fibroblasts incubated in DME alone remained rounded. Only thin cytoplasmic processes were visible in photomicrographs taken after 2 and 8 h of incubation (Fig. 4, *A* and *D*). The morphologies of fibroblasts incubated in E1 or serum were not significantly different at any stage of the assay. The lack of morphological difference between the serum and E1-stimulated cells were particularly remarkable given the large differences in colla-

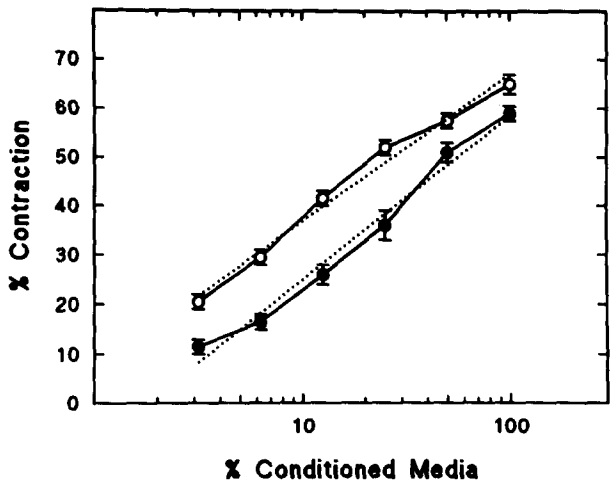

Figure 2. Immunoprecipitation of E1 from endothelial cell conditioned medium. 5-ml aliquots of endothelial cell conditioned medium were incubated for 60 min at room temperature with 50 μl protein A Sepharose preloaded with 50 μl rabbit anti-E1 serum (•) or 50 μl nonimmune rabbit serum (○). After incubation the affinity beads were pelleted by centrifugation and the supernatants were tested at the indicated dilutions for contraction-promoting activity on fibroblasts seeded onto collagen gels. The data shown for each concentration are the means and ranges of results from duplicate cultures after 4 h of incubation. The dotted lines are plots of the functions derived from linear regression analysis of the data.

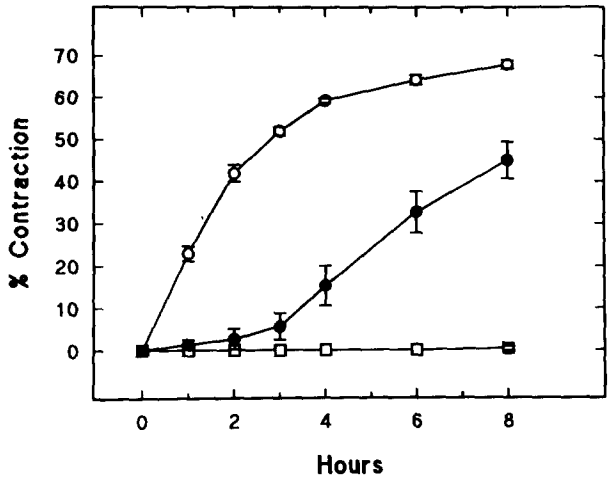

Figure 3. Fibroblast contraction is promoted by E1. Fibroblasts seeded onto collagen gels were incubated at 37°C in DME alone (□), DME with 1.0 μg/ml of E1 (●), or 1.0 mg/ml FBS (○). At the times indicated, gel thickness was measured. The data shown are the means and standard deviations of results from triplicate cultures. Other details are described in Materials and Methods.

gen gel thickness at the times photographed. After two hours of incubation, E1-stimulated cells (Fig. 4 *B*) had not yet reduced the gel thickness, but exhibited cytoplasmic spreading and extended large processes. These features, normally associated with fibroblasts actively contracting collagen were also visible in serum-stimulated fibroblasts, which had reduced the thickness of the matrices by greater than 40% during the same period (Fig. 4 *C*). The lack of morphological difference between the differentially stimulated cells persisted and was evident for both E1 (Fig. 4 *E*) and serum (Fig. 4 *F*) stimulated cells after 8 h of incubation when E1-stimulated contraction was more pronounced (45%).

Experiments were performed to measure the potency of E1 in stimulating collagen gel contraction by fibroblasts. Fibroblasts attached to collagen gels were incubated in DME containing E1 at concentrations ranging from 4×10^{-6}–4×10^{-14} M. The resulting collagen gel thicknesses were measured after 10 h of incubation (Fig. 5). The extent of matrix contraction remained essentially constant from the highest dose down to 4.0×10^{-10} M after which the activity rapidly decreased. The E1 concentration which would result in 25% reduction in gel thickness (approximately half-maximal) was calculated from a linear regression analysis of the data in Fig. 5, as 3.2×10^{-11} M. This is comparable to other reports of endothelin-induced effects on fibroblasts in vitro (30, 3).

E1 Is A Type B Contraction Promoter

To investigate if E1-promoted contraction required de novo protein synthesis, fibroblasts were seeded onto collagen gels and incubated in DME with 4 nM E1 or FBS, with or without 25 μg/ml cycloheximide. This concentration of cycloheximide was found to inhibit incorporation of ^{35}[S]methionine by >95% without decreasing cell viability (data not shown). At varying times during the incubation gel thickness was measured (Fig. 6 *A*). FBS-promoted gel contraction was essentially unaffected by the addition of cycloheximide, which was consistent with our previous observations (13). There was, however, a substantial effect on E1-promoted contraction. E1 stimulation of cycloheximide-blocked fibroblasts resulted in only 5% reduction in gel thickness during 8 h of incubation. Gels containing nonblocked cells were reduced in thickness by 45% during the same period.

To assess what effect removal of E1 would have on gel contraction, fibroblasts were seeded onto collagen gels and incubated in DME containing 4 nM E1 (Fig. 6 *B*, ●). After each hour the media from triplicate wells were removed and replaced with fresh serum-free DME and incubated further (Fig. 6 *B*, ○). The rate of gel contraction was not significantly reduced by removal of the peptide indicating that after the initial exposure, E1 is no longer required.

Together, these two experiments indicate that E1-promoted gel contraction is via an effector protein. Additionally, it appears that E1 is a type B contraction promoter with characteristics similar to that observed previously with unfractionated endothelial cell conditioned medium (13).

Comparison of E1 Activity with Other Known Endothelins

While there is considerable sequence homology among the different endothelins, there are differences in the target receptors and distributions of the different peptides (23, 5). These observations suggest potential differences in the biological activities of the different endothelins. For these reasons it was of interest to examine the potential contraction promoting activities of the other endothelins. Fibroblasts attached to collagen gels were incubated in DME containing 0.4 μM E1, E2, E3, or VIC. Hourly measurements of gel thickness revealed that, at this concentration, all of the endothelins were active in promoting gel contraction (Fig. 7 *A*). E1, E2, and VIC were essentially identical in both rate and extent of contraction observed. E3, however, appeared to be less active than the other three species.

Experiments to measure the potencies of the different endothelins were performed in a manner similar to that described in the legend to Fig. 5. However, because of the large numbers of samples required for these experiments it was necessary to test each peptide separately. To facilitate direct comparison the results from each experiment were normalized to internal positive controls (FBS) by dividing the peptide-promoted responses by the serum-promoted response. The results of these assays, all taken after 10 h of incubation indicate that E2 and VIC are also active at picomolar concentrations (Fig. 7 *B*). E3 was active only at the highest concentration tested.

Comparison of Endothelin-promoted Gel Contraction with PDGF and TGF$_\beta$

Two growth factors which have been reported to promote collagen gel contraction by fibroblasts are transforming growth factor beta (TGF$_\beta$) (21) and PDGF (1, 4, 14). Previously we reported that the extent of TGF$_\beta$-stimulated gel contraction was <10% during 4 h of incubation (13). Because E1-stimulated gel contraction required longer incubations we again examined the stimulatory activity of TGF$_\beta$ using incubations of up to 10 h. E1 was also compared to PDGF-BB, the form determined to be the most active in stimulating gel contraction (4). Fibroblasts placed on top of collagen gels were

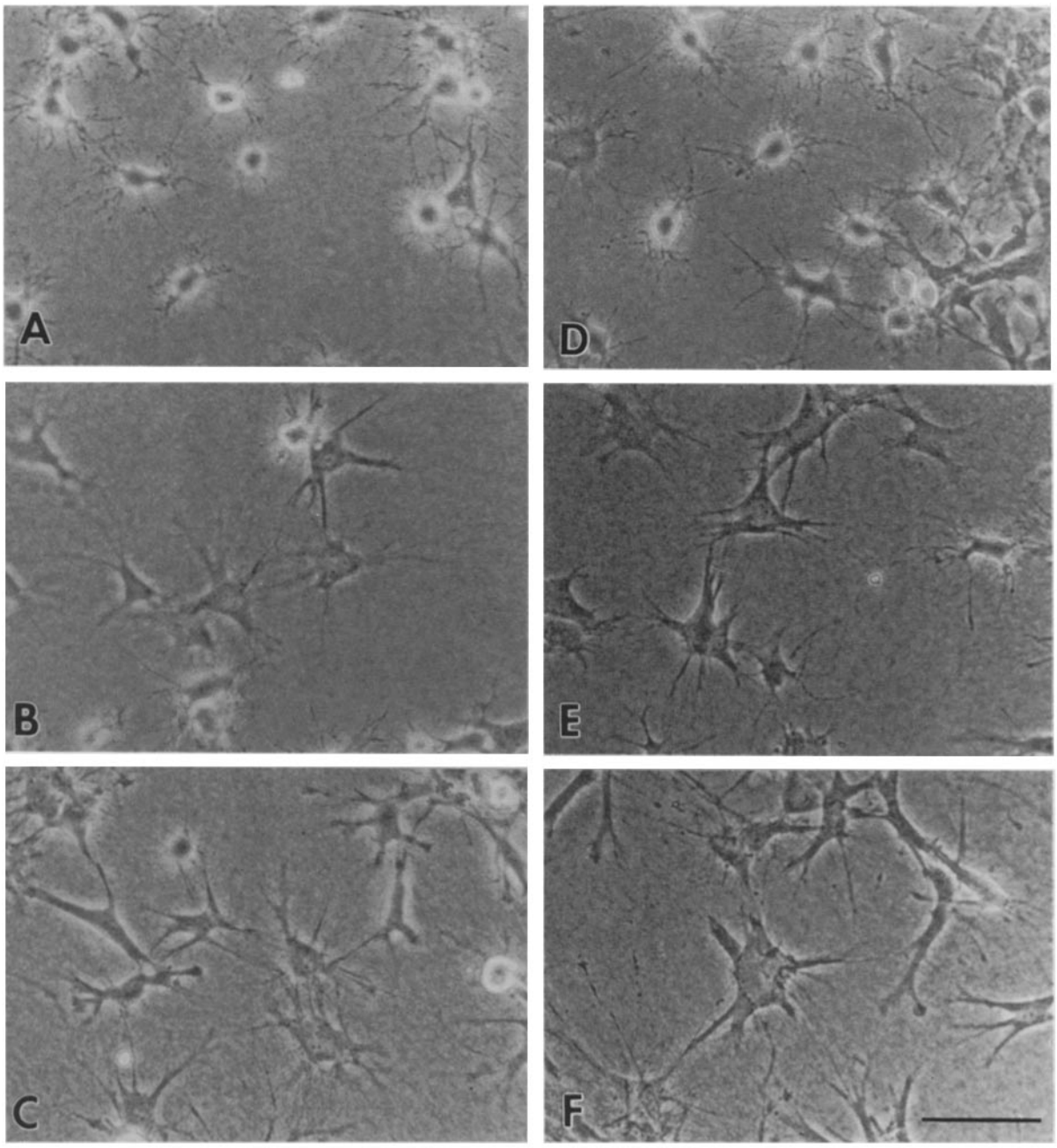

Figure 4. Morphology of fibroblasts contracting collagen. Phase-contrast micrographs of cells incubated as described in the legend to Fig. 3 after 2 (*A*, *B*, and *C*) and 8 (*D*, *E* and *F*) h of incubation in DME alone (*A* and *D*), E1 (*B* and *E*), or FBS (*C* and *F*). Other details are described in Materials and Methods. Bar, 100 μm.

incubated in DME–BSA (1 mg/ml) containing 4 nM E1, $TGF_{\beta 1}$, or PDGF–BB, and incubated at 37°C. Hourly measurements of gel thickness indicate that of the three factors, gel contraction promoted by PDGF–BB had the most rapid onset (Fig. 8 *A*). The onset of $TGF_{\beta 1}$-promoted gel contraction was the most delayed among the three.

The potencies of each promoter were examined by incubating fibroblasts in varying concentrations of each promoter. The results of these assays, normalized to internal controls as in Fig. 7 *B*, suggest that E1 and PDGF–BB are of essentially equal potency (Fig. 8 *B*). $TGF_{\beta 1}$ appears to be considerably more active. Activity calculations from linear regression analyses yield 25% contraction-promoting doses of 26.9 pM for PDGF–BB and 0.51 pM for $TGF_{\beta 1}$, respectively, compared to 32 pM for E1.

Discussion

We have identified E1 as an endothelial cell secreted factor that stimulates collagen gel contraction by fibroblasts. This was accomplished by: (*a*) colocalization of synthetic E1 with the contraction-promoting activity of endothelial cell condi-

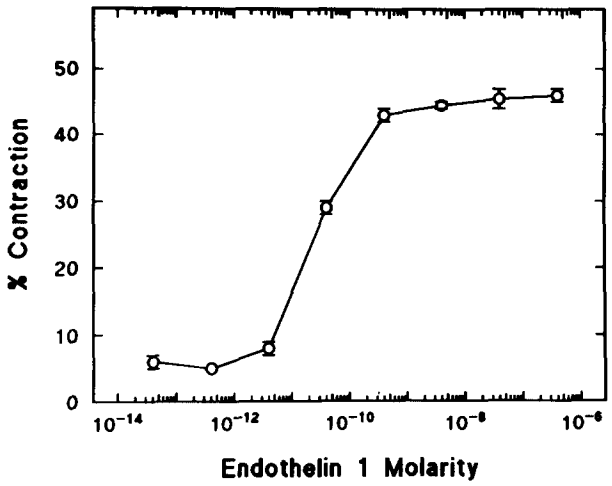

Figure 5. Dose response of E1-promoted fibroblast contraction. Fibroblasts seeded onto collagen gels were incubated at 37°C in DME containing E1 (○) at the indicated concentrations. After 10 h of incubation, gel thickness was measured. The data presented are the average and range of results obtained from duplicate cultures at each concentration. Other details are described in Materials and Methods.

tioned medium in reversed phase analysis; (*b*) removal of contraction-promoting activity from conditioned medium with antiserum raised against synthetic E1; and (*c*) demonstration of contraction-promoting activity with synthetic E1. Analysis of the data from the anti-E1 treated and control conditioned media samples indicated that removal of E1 decreased the contraction-promoting activity of the media by 59%. From this we conclude that E1 is the major contraction-promoting factor in the conditioned medium pool.

Dose response analysis of E1-stimulated contraction indicated that E1 is active at picomolar concentrations with half-maximal effect (25% reduction in gel thickness) occurring at ~32 pM. Similar studies performed with three other endothelins indicate that the different species are not equal in stimulatory activity. E2 and VIC were observed to be slightly less active than E1. E3, however, was approximately four orders of magnitude less active than E1. These data are in agreement with studies of the relative affinities of the various endothelins for fibroblast receptors (23, 5).

Experiments to characterize E1-stimulated contraction suggest that mechanistically it is a type B promoter. Type A promoters (FBS derived) stimulate contraction directly in contrast to type B promoters which require synthesis of an effector protein (13). E1 stimulation was observed to be dependent upon de novo protein synthesis and continued after removal of the promoter.

Finally, E1-promoted contraction was compared with that of two growth factors, PDGF (1, 4, 14), and TGF_β (21, 25), each of which is reported to stimulate matrix contraction by fibroblasts. These experiments demonstrated that the specific activities of E1 and the most active isomer of platelet-derived growth factor (PDGF-BB), were essentially equal. Half-maximal contraction (25%) was calculated to occur at a concentration of 26.9 pM PDGF-BB. $TGF_{\beta 1}$ was found to be ~50-fold more active with half-maximal contraction calculated to occur at 0.51 pM.

These results are somewhat different from those obtained

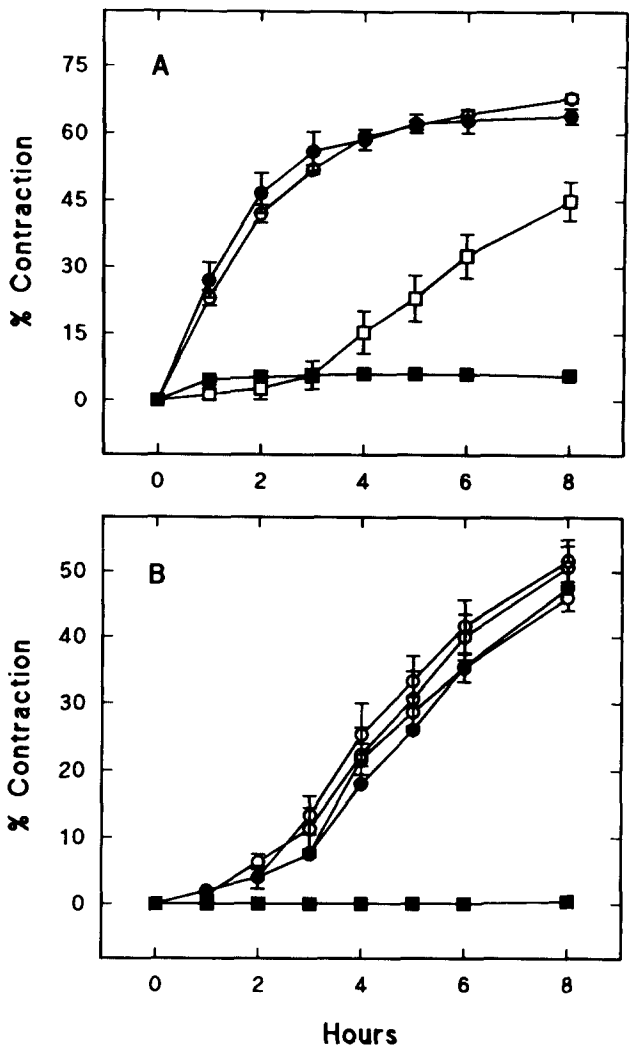

Figure 6. Characterization of E1 as a type B promoter. (*A*) Fibroblasts seeded onto collagen gels were incubated in DME containing 1.0 mg/ml FBS (○,●) or 4 nM E1 (□,■) with (*closed symbols*) or without (*open symbols*) 25 μg/ml cycloheximide with triplicate cultures under each condition. At the times indicated, gel thickness was measured. (*B*) Fibroblasts seeded onto collagen gels were incubated in DME alone (■) or containing 4 nM E1 (●). At hours 1, 2, and 3 the media in triplicate cultures were exchanged for fresh serum free DME without additives (○) and the incubation continued. At the times indicated gel thickness was measured.

by other investigators. Clark and co-workers observed little or no collagen gel contraction by TGF_β-stimulated fibroblasts during the first 24 h of incubation (4). While we have also observed a significant delay in the onset of TGF_β-stimulated gel contraction, substantial changes in gel thickness were observed after 6 h of incubation. The reasons for these differences are unclear. Our assay system is different in that the cells are placed on the top of the gel rather than polymerized within the gel. However, an earlier study revealed that cells in both locations are equally contractile (10). Other differences include cell numbers, collagen concentrations as well as method to measure gel contraction.

The delays in onset of E1, as well as TGF_β and PDGF, promoted gel contraction are somewhat confusing. While the fibroblasts do develop a contractile morphology rela-

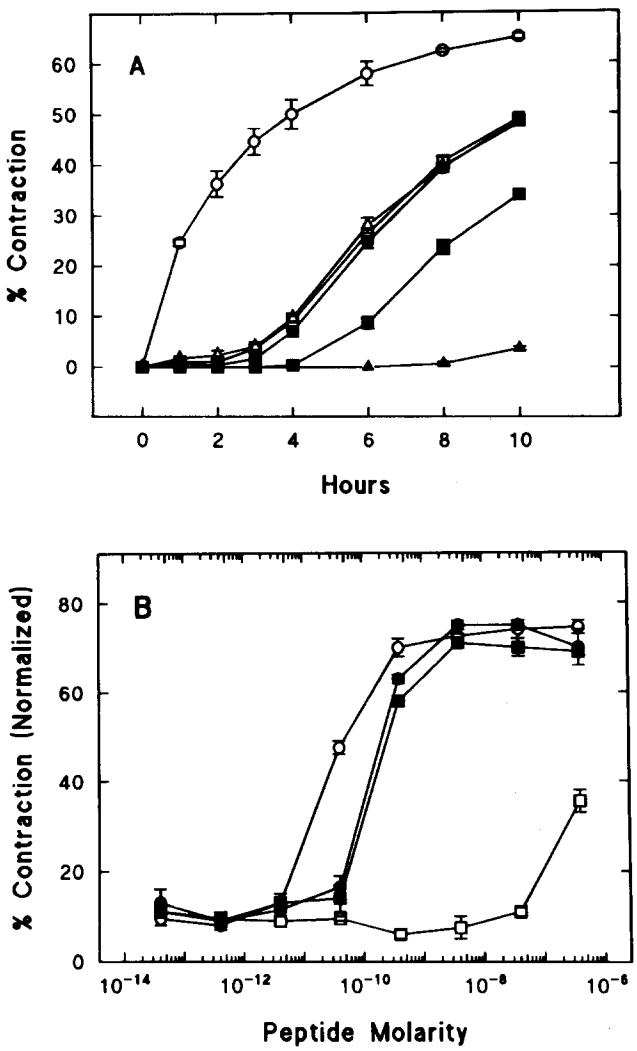

Figure 7. Fibroblast contraction is differentially promoted by the various endothelins. (*A*) Fibroblasts seeded onto collagen gels were incubated at 37°C in DME alone (▲) or containing 0.4 μM E1 (●), E2 (□), E3 (■), VIC (△), or 1.0 mg/ml FBS (○) At the times indicated gel thickness was measured. (*B*) In separate assays, fibroblasts seeded onto collagen gels were incubated at 37°C in DME containing E1 (○), E2 (■), E3 (□), or VIC (●) at the indicated concentrations. After 10 h of incubation gel thickness was measured. The data shown are the mean and range of results obtained from triplicate cultures in *A* and duplicate cultures in *B* at each concentration tested. For direct comparison the data from each experiment in *B* were normalized by dividing the peptide-promoted response by the serum-promoted internal standard from each assay.

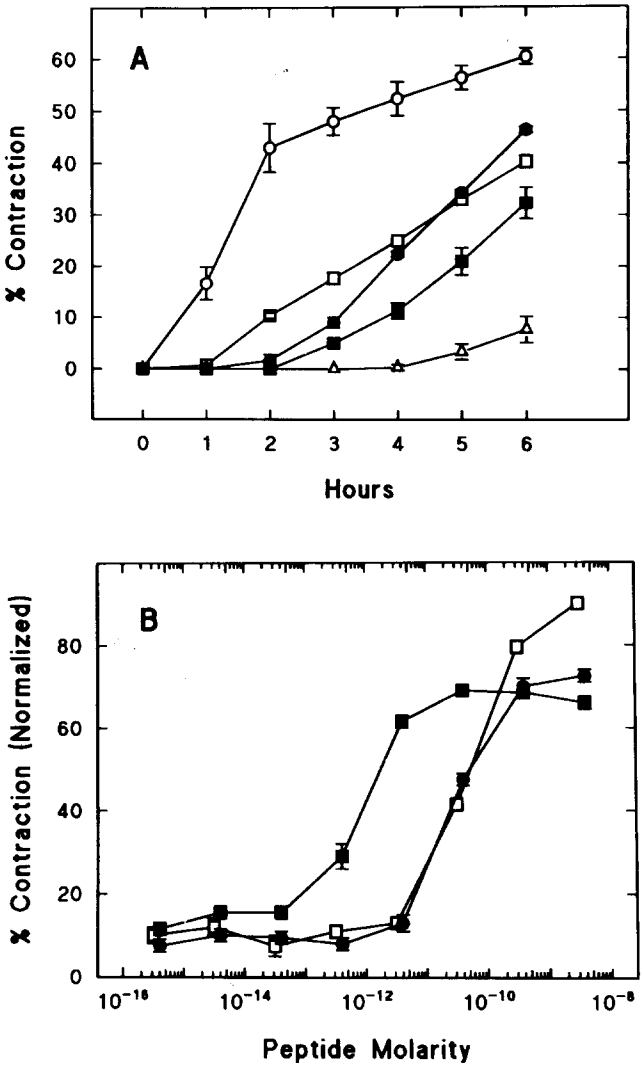

Figure 8. Kinetics and dose response for fibroblast contraction promoted by E1, PDGF-BB, and $TGF_{\beta 1}$. (*A*) Fibroblasts seeded onto collagen gels were incubated in DME + BSA alone (△), with 0.2 nM E1 (●) PDGF-BB (□), $TGF_{\beta 1}$ (■), or 1.0 mg/ml FBS (○). At the times indicated gel thickness was measured. The data shown are the means and standard deviations of results from triplicate cultures. (*B*) In separate assays, fibroblasts seeded onto collagen gels were incubated at 37°C in DME + BSA containing E1 (●), PDGF-BB (□) or $TGF_{\beta 1}$ (■) as described in the legend to Fig. 7. The normalized data are the mean and range of results obtained from duplicate cultures at each concentration.

tively soon after exposure to E1, the onset of measurable contraction is delayed by 2–3 h. This can be contrasted with fibroblasts treated with either serum or complete endothelial cell conditioned medium in which substantial contraction is measurable within the first hour of exposure (13). These observations are particularly surprising in that other studies of endothelin-induced effects on fibroblasts demonstrate second messenger effects, such as protein kinase C and phospholipase activation within minutes of exposure (30, 22). Also, anti-E1–treated endothelial cell conditioned medium was diminished in contraction-promoting activity measurable within the first hour (data not shown). These observations lead us to suggest that E1 in conditioned medium acts with or as a co-factor to other peptide promoters. In support of this notion are other studies demonstrating mitogenic synergism between E1 and several growth factors (30, 17, 3). It is possible that TGF_{β}, or other promoters, might act synergistically with E1 to produce the rapid onset matrix contraction we have observed with both serum and endothelial cell–conditioned medium stimulated fibroblasts.

Another question raised by these studies concerns the second messengers involved in promoting fibroblast contractile activity. The second messengers activated by endothelins during the first hour of exposure, protein kinase C, phospholipase C (30, 22), are obviously not sufficient to produce the relatively rapid contractile response observed with either serum or endothelial cell conditioned medium (13). Potential

explanations are that contraction requires activation of different second messengers, or perhaps even multiple systems. While the activation resulting in fibroblast contraction does eventually occur with endothelins, PDGF and TGF$_\beta$, it appears to be a secondary rather than primary effect of the peptides.

The physiological significance of these observations is not yet clear. The endothelins were identified and characterized mainly as vasoactive peptides which are potent in promoting extremely rapid short-lived alterations in vascular tone, presumably through their effects on vascular smooth muscle (32). Studies in which embryonic and adult tissues were probed with radiolabeled E1 to identify receptors did not localize receptors within connective tissues (18, 16). This, however, is inconsistent with the observations that, in vitro, fibroblasts as well as a number of other nonvascular cells, are sensitive to endothelin stimulation. An attractive hypothesis is that quiescent fibroblasts in tissues do not normally express endothelin receptors, but do so during wound healing.

A number of recent studies serve to illustrate both the complexity of the wound environment as well as provide reason for caution in overinterpreting data from simplified in vitro models. Fibroblasts isolated from granulation tissue have greater sensitivity to TGF$_\beta$ than fibroblasts isolated from normal skin, suggesting that there are significant phenotypic differences in cells isolated from normal versus nonwounded skin (8). Several studies of wound exudates have determined the presence of factors which promote (20) as well as prevent matrix contraction by fibroblasts (6, 26). Two different growth factors which promote matrix contraction by fibroblasts in vitro (PDGF and TGF$_\beta$) have different effects when applied directly to developing granulation tissue (24). Continued exploration of the mechanisms of these regulatory factors at the molecular level should provide insights into their roles in wound repair.

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