

# Endothelial Cells Secrete a Factor That Promotes Fibroblast Contraction of Hydrated Collagen Gels

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**Abstract.** Bovine aortic endothelial cells (BAEC), grown *in vitro*, are shown to synthesize and secrete factor(s) that stimulate fibroblasts to contract collagen matrices. The amount of contraction-promoting activity in the conditioned media is dependent on conditioning time and the number of cells in the culture. Production of the contraction-promoting activity continues at a high stable level for at least 5 d in serum-free medium but is abolished when the cells are exposed to an inhibitor of protein synthesis. The mechanism of action of the contraction factor(s) derived from endothelial cells was compared with that of unidentified serum factors. The endothelial

cell-secreted factor(s) depends on active protein synthesis by the target cell but does not need to be present during the contraction process. The serum factors on the other hand promote collagen contraction in the absence of *de novo* protein synthesis but need to be continuously present. Preliminary biochemical characterization of the contraction-promoting factors produced by endothelial cells revealed properties similar to those of previously identified growth factors. However, the BAEC-secreted factor was found to be distinct from a previously identified contraction-promoting transforming growth factor beta.

**F**IBROBLASTS cultured on hydrated collagen gels develop a bipolar morphology typical of fibroblasts found in skin and tendon compared with the flattened morphology of fibroblasts cultured on planar substrata (6, 19). Additionally, during culture the fibroblasts contract the collagen gel into a dermis-like structure (1) which has in fact been successfully transplanted and integrated as a dermal equivalent (9). This has resulted in considerable interest in fibroblast contraction *in vitro* as a model system for the study of connective tissue development, wound healing, and fibrosis.

We have examined fibroblast contraction of collagen using short incubations which permit the study of collagen contraction as a process separate from fibroblast growth. It was previously shown that fibroblast contraction involves little collagen synthesis or degradation and does not involve enzymatic alteration or covalent cross-linking of the preexisting collagen (7). Additionally, contraction can occur only on a matrix capable of propagating contractile force generated by the fibroblasts (8). While the continuity of a collagen matrix and thus contractability of the matrix can be modulated by addition of heparin, fibroblast contraction is not dependent upon exogenous noncollagenous matrix components such as fibronectin. However, contraction is dependent, even in the early stages, on the addition of exogenous factors that stimulate fibroblast contractile activity (7). Serum has been demonstrated to contain contraction promoters, but these have not yet been characterized (18).

The wound environment also must contain factors that in-

duce fibroblast growth, migration, and matrix contraction. Serum components from clotted blood are undoubtedly present in the early stages of wound repair. It is unlikely, however, that these molecules persist for the length of time necessary for closure of the wound since this may take several weeks. It seems more likely that fibroblast contraction is promoted from within granulation tissue by resident cell populations.

Wound contraction occurs in a complex multicellular environment involving both mesenchymal and inflammatory cells. Fibroblasts and capillary endothelium progressively infiltrate the wound bed from the margins of the wound (16). Neutrophils are present in great numbers in the early stages of wound repair but, in the absence of infection, diminish rapidly (17). Macrophages do persist throughout the repair process (10). This limits the probable sources of contraction-promoting activity to invading endothelium and resident macrophages. A recent report suggested that transforming growth factor beta (TGF $\beta$ )<sup>1</sup> from macrophages functions as a fibroblast contraction promoter (12) in addition to its previously characterized effect of promoting matrix synthesis (14). We have examined the other resident cells, namely endothelium, for secretion of factors that promote fibroblast contraction of collagen gels *in vitro*. The results of this study are reported herein.

1. *Abbreviations used in this paper:* BAEC, bovine aortic endothelial cells; TGF $\beta$ , transforming growth factor beta.

## Material and Methods

### Cells

Human skin fibroblasts were obtained from the American Type Culture Collection (Rockville, MD). Human choriocarcinoma (BeWo, ATCC-CCL 98), murine sarcoma virus-transformed and control 3T3 fibroblasts were a gift from Dr. Hans Peter Hohn; rat glial cells were a gift from Dr. Etty Benveniste; rat embryo fibroblasts were provided by Anne Woods; human umbilical vein endothelial cells were provided by Dr. Francois Booyse; and bovine aortic endothelial cells (BAEC) were provided by Dr. Joanne Murphy-Ullrich all from the University of Alabama at Birmingham. Cells were cultured in 75-cm<sup>2</sup> tissue culture flasks (Corning Glass Works, Corning, NY) using growth media composed of DME (Gibco Laboratories, Grand Island, NY), 20 mM Hepes, and 10% FBS (Flow Laboratories, Inc., McLean, VA). The cultures were incubated in a humidified incubator containing an atmosphere of 5% CO<sub>2</sub> and 95% air. Cultures were harvested using an 0.5% trypsin/0.02% EDTA solution (Flow Laboratories, Inc.). Culture conditions for the endothelial cells were identical except that the growth media contained 20% FBS and 2 mM glutamine.

### Preparation of Conditioned Media

Cells were grown to confluency in 75-cm<sup>2</sup> tissue culture flasks in DME with 10 or 20% FBS, depending on the cell type. At confluency, the flasks were rinsed twice with serum-free DME, and the culture was continued at 37°C for an additional 24 h with 10 ml of serum-free DME. After incubation, the media were removed, centrifuged to remove cell debris, and either used immediately or frozen at -20°C until used. Radiolabeled conditioned medium was prepared as above except for the addition of 10  $\mu$ Ci/ml [<sup>35</sup>S]methionine (1,115 Ci/mmol; New England Nuclear, Boston, MA). Serum-free conditioned media from human skin keratinocytes, rat mammary carcinoma cultures, and cultured rat tongue keratinocytes were kindly provided by Dr. Henning Birkedahl-Hanson (University of Alabama at Birmingham, Birmingham, AL).

### Preparation of Collagen Gels

Native collagen gels were prepared as described previously (7, 8). Briefly, Vitrogen 100 (Collagen Corp., Palo Alto, CA) was adjusted to physiological ionic strength pH and to a concentration of 1.5 mg/ml with 10 $\times$  PBS (1.5 M NaCl, 0.1 M Na<sub>2</sub>HPO<sub>4</sub>) and 0.1 M NaOH while maintained at 4°C. An aliquot (0.2 ml) of the collagen solution was added to the center of a 10-mm circular score on the bottom of a 24-well tissue culture plate (Falcon Labware, Oxnard, CA), and the gels were allowed to polymerize at 37°C for 90 min. The resultant gel thickness was  $\sim$ 2 mm.

### Measurement of Gel Contraction

Fibroblasts harvested with trypsin/EDTA and washed with DME were suspended in DME, and small aliquots (0.05 ml containing 5  $\times$  10<sup>6</sup> cells) were placed on top of the polymerized gels. The gels were incubated at 37°C for an additional 30 min to allow the cells to attach after which the gels were covered with 1 ml of media containing the test substance. Gel contraction was measured as reduction in gel thickness by adjusting the plane of focus on an inverted phase-contrast microscope (Diaphot; Nikon Inc., Garden City, NY) from the bottom to the top of the gel and recording the distance of stage movement. Repetitive measurements proved this method to be reproducible to 25- $\mu$ m (1.25% of initial gel thickness).

### Chromatography and Electrophoresis

Samples analyzed by gel filtration were chromatographed on a Pharmacia Fine Chemicals (Piscataway, NJ) fast performance liquid chromatography HR10/30 column of Superose 12. Heparin-Sepharose CL-6B was obtained from Pharmacia Fine Chemicals. Native collagen and gelatin affinity matrices were prepared with cyanogen bromide-activated Sepharose 4B.

Samples analyzed by SDS-PAGE were electrophoresed on a 5-15% acrylamide gradient gel in the presence of SDS and in the absence of reducing agents.

### Other

Human platelet-derived TGF $\beta$  was obtained from R & D Systems Inc. (Minneapolis, MN) and reconstituted according to the manufacturer's instruc-

tions. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Protein concentrations were determined according to the method of Bradford (2).

## Results

### Media Conditioned by Endothelial Cells Stimulates Fibroblasts To Contract Collagen Gels

Cultures of BAEC were grown to confluency in the presence of serum, washed, and further incubated in serum-free DME for an additional 24 h at 37°C. The medium was harvested and tested for contraction-promoting activity on cultures of human skin fibroblasts attached to gels of type I collagen. Hourly measurements of gel thickness in the presence of BAEC-conditioned DME, DME alone, or DME containing 3% FBS, which is known to contain contraction-promoting activity (18), indicated that the conditioned medium contained factors that were able to promote fibroblast contraction of collagen gels (Fig. 1). Fibroblasts incubated in DME alone did not contract the collagen gels.

The relative potency of the contraction-promoting activity in BAEC-conditioned DME was compared with that of serum. The test substances were added in varying concentrations to fibroblasts seeded on collagen gels. After 4 h of incubation, the extent of contraction was determined for each dose (Fig. 2). Half of the maximal observed contraction ( $\sim$ 25%) was achieved with medium containing 5  $\mu$ g/ml conditioned medium protein, whereas a similar amount of contraction required  $\sim$ 250  $\mu$ g/ml of serum protein. Hence, the conditioned medium has a specific activity 50-fold higher than that of serum.

The morphology of contracting fibroblasts varied depending upon the source of the contraction-promoting activity. Serum-stimulated fibroblasts exhibited a typical bipolarity, with relatively blunt processes and extensive cytoplasmic

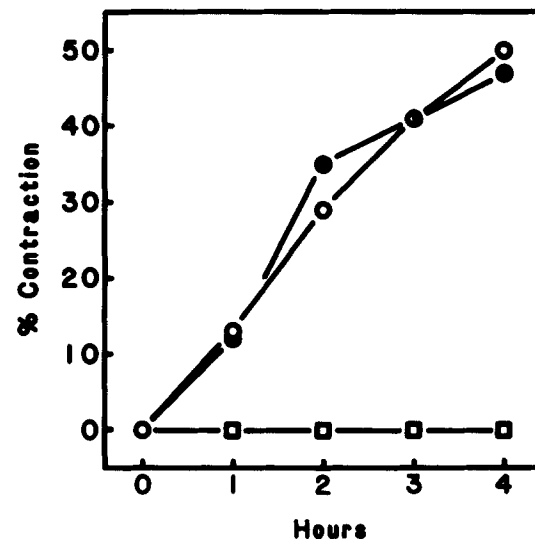
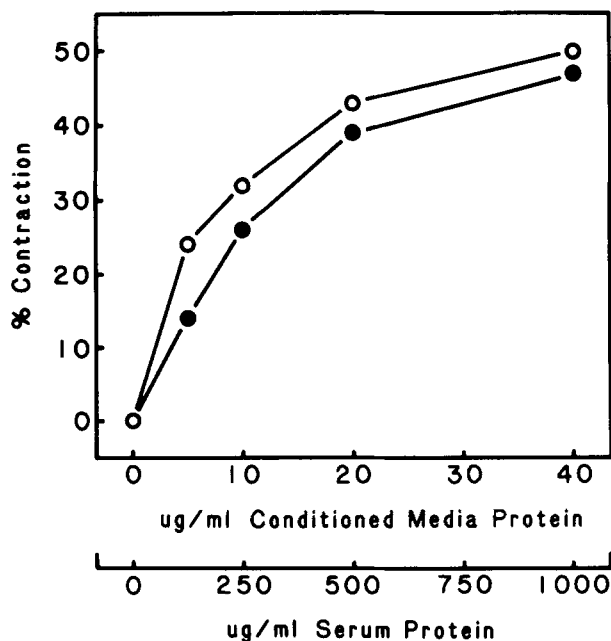


Figure 1. Fibroblast contraction of collagen gels is promoted by endothelial cell-conditioned media. Fibroblasts were seeded onto collagen gels and incubated at 37°C in DME alone (□), DME with 3% FBS (●), or DME conditioned by BAEC (○). At the indicated times, the gel thickness was determined. The data presented are the averages of results from duplicate cultures. Other details are described in Materials and Methods.



**Figure 2.** Fibroblast contraction of collagen gels promoted by varying concentrations of FBS- or BAEC-conditioned media protein. Fibroblasts on top of collagen gels were incubated in DME containing the indicated amounts of either FBS- (●) or BAEC-conditioned media protein (○). After 4 h of incubation, the percent contraction was determined. The data presented are the averages of results from duplicate cultures. Other details are described in Materials and Methods.

spreading after 4 h of incubation (Fig. 3 A). Fibroblasts in DME alone also had short processes, but little cytoplasmic spreading (Fig. 3 B). Cells incubated in BAEC-conditioned DME were also well spread, but had longer more numerous processes (Fig. 3 D, arrows). These observations suggested that serum- and BAEC-conditioned media affected the fibroblasts differently. The morphology of cells incubated in BAEC-conditioned DME also containing added serum was similar to that of cells incubated in conditioned medium alone (Fig. 3 C). This observation suggests that the BAEC-conditioned medium contains factors not present in serum.

### **The Contractile Factor Is an Endothelial Cell Secretory Product**

It is conceivable that serum components with contraction-promoting activity are retained by BAEC or adsorbed to the tissue culture plastic and subsequently released to the medium during conditioning. To address this concern, a number of experiments were performed to establish the fact that the contraction promoter in conditioned medium is an endothelial cell secretory product. We first examined the rate at which contraction-promoting factors are secreted into the medium. Confluent cultures of BAEC were incubated with DME for times varying from 0 to 24 h. These media were harvested and assayed for contraction-promoting activity (Fig. 4 A). The rate and extent of contraction increased with conditioning time throughout the entire period examined.

The effect of BAEC number on the contraction-promoting activity in conditioned medium was examined by incubating with BAEC cultures varying in cell density from confluency

( $10^6$  cells/35-mm dish) to 0.1% of confluent density ( $10^3$  cells/dish) for 24 h. The contraction-promoting activity in the harvested media increased with the number of cells in the conditioning culture throughout the range examined (Fig. 4 B). These data suggest that the contraction-promoting activity in the conditioned media is produced by the endothelial cells and does not represent released or desorbed serum factors.

To obtain further evidence of a BAEC origin of the contraction-promoting activity, cultures were conditioned in the presence of cycloheximide, an inhibitor of protein synthesis. Control studies indicated that 25  $\mu$ g/ml of cycloheximide reduced BAEC incorporation of [ $^{35}$ S]methionine into TCA-precipitable material >97%, while the viability of the cells remained >95% after 24 h (data not shown).

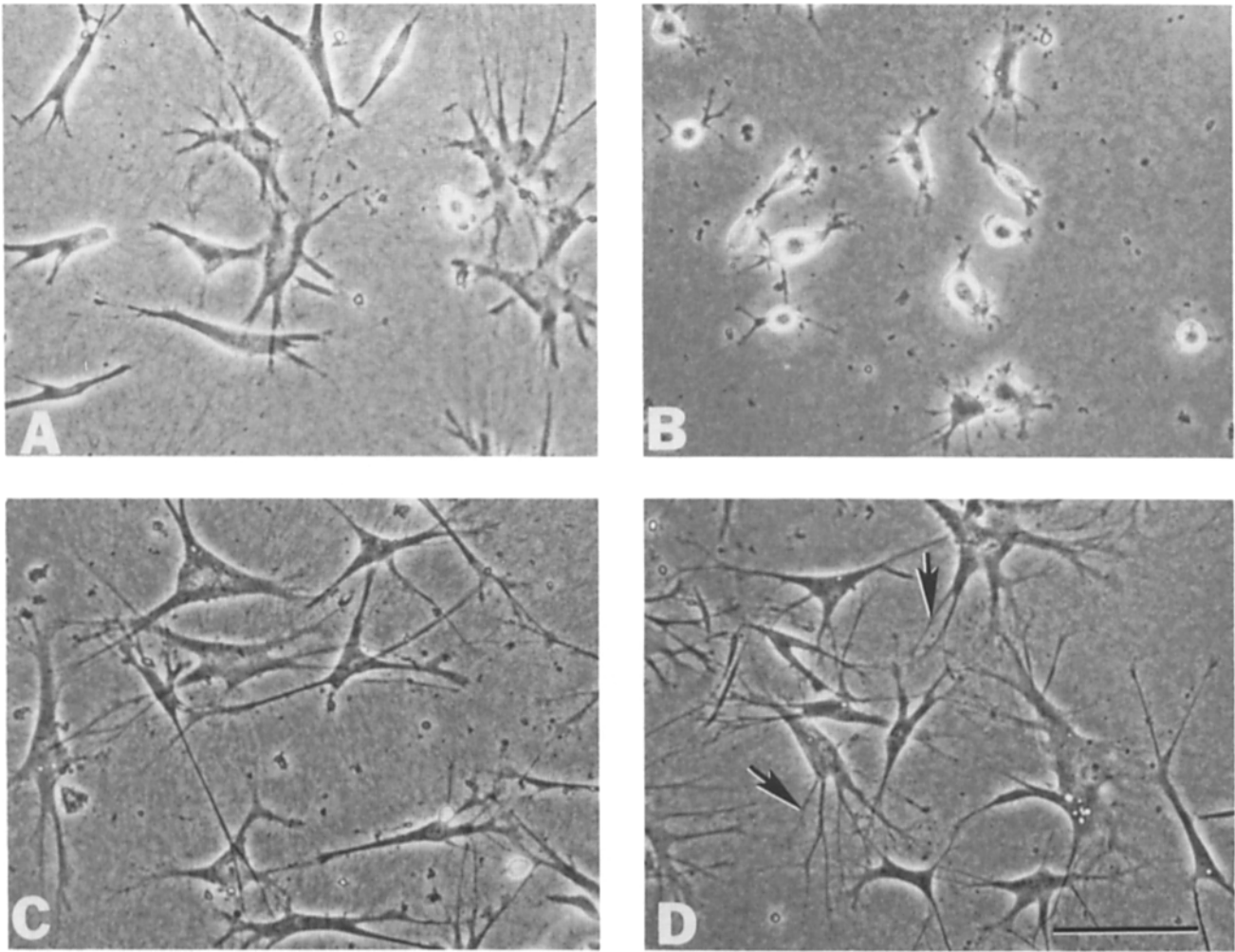
Confluent cultures of BAEC were incubated for 24 h in DME with or without added cycloheximide. These media were harvested, dialyzed against fresh medium to remove the cycloheximide, and assayed for contraction-promoting activity (Fig. 5). Medium conditioned in the presence of cycloheximide did not promote fibroblast contraction, indicating that the contraction promoters in conventionally conditioned media are in fact synthesized by BAEC. As a control, media samples were supplemented with cycloheximide after conditioning, dialyzed, and then assayed. Conditioned media treated in this way retained >85% of activity (data not shown).

### **BAEC Secretion of Contraction Promoters Is Stable over Time**

To examine the stability of the BAEC phenotype responsible for the secretion of contraction-promoting activity, confluent cultures of BAEC were incubated in DME that was changed daily for 5 d. The media collected from each day were assayed for protein content and for contraction-promoting activity. The results of this study (Table I) indicated only slight variations in the daily amounts of contraction-promoting activity and total protein secreted by the cultures over the period of the experiment. The concentration of serum albumin in each conditioned medium sample was determined in an ELISA to assess the level of contamination of serum proteins. The albumin concentration in the day-1 medium sample was highest at 135 ng/ml. Albumin concentrations in the media samples from days 2-5 were <10 ng/ml. These results show that the BAEC secretory phenotype is stable in long-term culture and that at least the serum protein albumin is not released from the cultures in appreciable quantities after 24 h. Taken together, these experiments show that the contraction-promoting activity in the conditioned media is not derived from serum but is produced by the BAEC.

### **Occurrence of Contraction-promoting Activity**

A number of different cell types were examined for the ability to secrete contraction-promoting factors (Table II). The conditioned media of most cell types tested, including fibroblasts, contained relatively low levels of activity, which confirms a previous report (7) showing that fibroblasts secrete a low but clearly detectable amount of contraction-promoting activity. Under the conditions tested, the highest specific activity was found in medium conditioned by BAEC. The specific activity of media conditioned with BAEC was



**Figure 3.** Morphology of fibroblasts contracting collagen. Phase-contrast micrographs of cells incubated as described in the legend to Fig. 1 after 4 h of incubation in DME plus FBS (A), DME alone (B), BAEC-conditioned DME plus FBS (C), or BAEC-conditioned medium alone (D). Other details are described in Materials and Methods. Bar, 100  $\mu\text{m}$ .

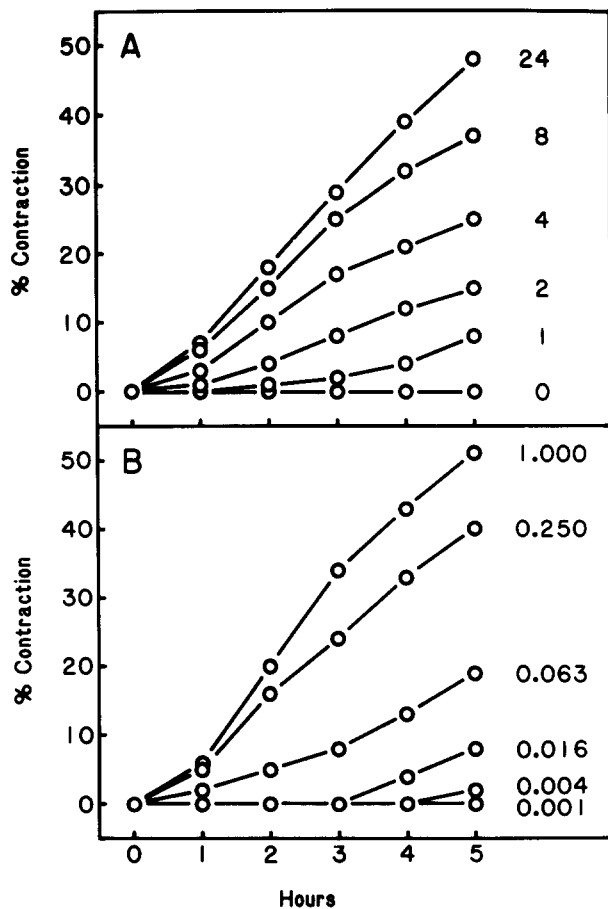
10–20-fold higher compared with that conditioned with various types of fibroblasts. Furthermore, since BAEC also secrete more protein than do fibroblasts, the total amount of contraction-promoting activity secreted is 50–100-fold higher for BAEC compared with fibroblasts (data not shown). In addition to the BAEC, high levels of contraction-promoting activity were also detected in medium conditioned with a choriocarcinoma and a mammary carcinoma cell line. These activities were not further characterized at this time.

#### ***The Contraction-promoting Factor(s) from BAEC Act on Fibroblasts***

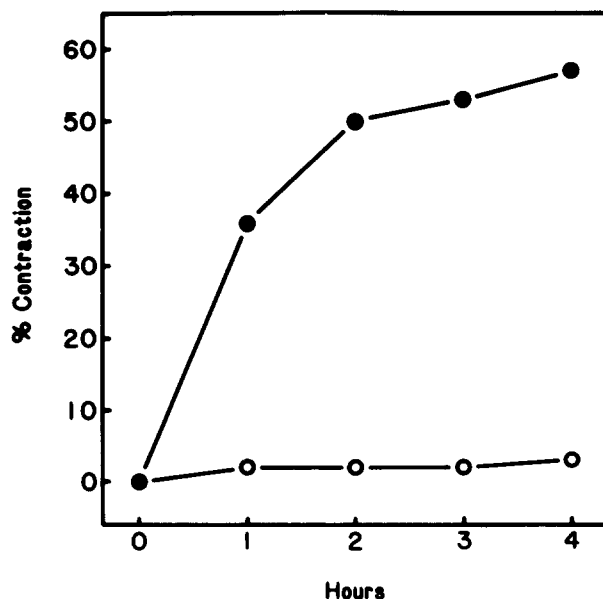
The endothelial factor(s) could conceivably stimulate the contraction of collagen gels by different mechanisms. Components in the conditioned media could chemically modify or adsorb to the collagen matrix in a way that facilitated contraction either directly or by the fibroblasts. To examine this possibility, collagen gels were preincubated for 16 h at 37°C with conditioned medium containing protein metabolically labeled with [<sup>35</sup>S]methionine. The collagen gel thickness did not change during this incubation (data not shown).

These pretreated gels were then washed for 24 h with media to remove unbound material (monitored by release of radioactivity) and seeded with fibroblasts. Pretreating the gels in this way did not promote contraction of the collagen gels. However, when serum- or BAEC-conditioned medium was present during the contraction assay, the treated gels could still be contracted by fibroblasts (data not shown). These results suggest that the contraction-promoting factors produced by BAEC do not act via a modification of the matrix but probably directly stimulate fibroblasts to contract collagen gels.

To analyze if fibroblasts retained a contractile response after pretreatment with BAEC-conditioned medium, cultures were incubated with BAEC-conditioned media for 2 h before harvest and seeding onto the collagen gels. Fibroblasts pretreated in this way did not contract the collagen gels in serum-free DME (data not shown). The pretreated cells did contract the collagen gel when serum- or BAEC-conditioned medium was added directly to the assay system, indicating that the pretreatment was not toxic to the cells. In a separate experiment, the fibroblasts were harvested and then preincubated in suspension with conditioned media for 60 min at



**Figure 4.** Acquisition of contraction-promoting activity is dependent on time and cell number. (A) DME conditioned by confluent cultures of BAEC for the number of hours indicated at the right of the figure was incubated with fibroblasts seeded onto collagen gels. At the times indicated on the abscissa, gel thickness was measured. (B) DME was incubated with BAEC cultures containing the number of cells ( $\times 10^6$ ) indicated at the right of the figure for 24 h. These media were incubated with fibroblasts seeded onto collagen gels. At the times indicated on the abscissa, gel thickness was measured. These data are the means of results from triplicate cultures. Other details are described in Materials and Methods.



**Table I. Comparison of Contraction-promoting Activity from Daily Collections of Endothelial Cell-conditioned Media**

Day	Protein	Contraction per microgram
	$\mu\text{g}$	%
1	284.0	5.20
2	261.5	6.00
3	242.0	6.44
4	275.6	6.00
5	295.5	5.40

Confluent cultures of BAEC in 150-cm<sup>2</sup> tissue culture flasks were first washed with serum-free DME and incubated with an additional 20 ml of fresh medium. After each 24-h incubation, the medium was removed and replaced with fresh serum-free medium for a total of 5 d. Other details are described in Materials and Methods.

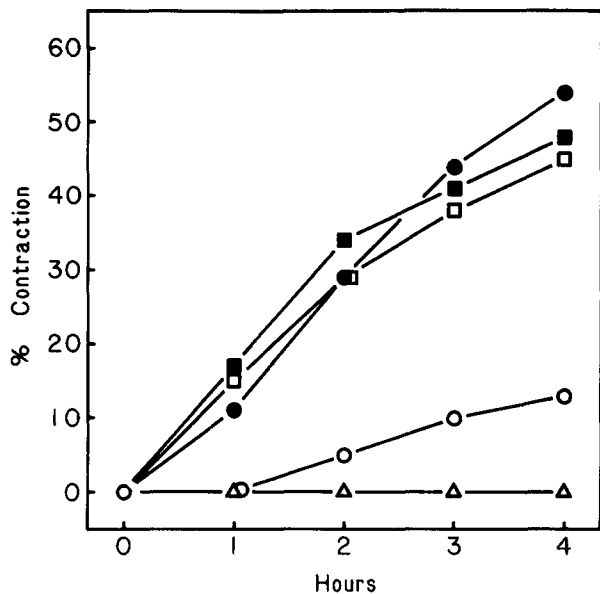
**Table II. Comparison of Contraction-promoting Activities of Cell-conditioned Media**

	Contraction per microgram
	%
<b>Controls</b>	
FBS	0.11 $\pm$ 0.01
Serum-free media	0.00 $\pm$ 0.43
BSA	>0.01 $\pm$ 0.01
<b>Conditioned media from various cultures</b>	
Bovine aortic endothelium	4.80 $\pm$ 0.80
Rat embryo fibroblasts	0.21 $\pm$ 0.21
3T3 fibroblasts	0.43 $\pm$ 0.26
<b>Murine sarcoma virus-transformed</b>	
3T3 fibroblasts	0.39 $\pm$ 0.09
BeWo choriocarcinoma	1.06 $\pm$ 0.24
Human skin fibroblasts	0.32 $\pm$ 0.35
Human umbilical endothelium	0.35 $\pm$ 0.21
Rat mammary carcinoma	1.23 $\pm$ 0.08
Rat tongue keratinocytes	0.58 $\pm$ 0.32
Human skin keratinocytes	0.27 $\pm$ 0.12
Rat brain glials	0.32 $\pm$ 0.03

Serum-free conditioned media from the varying cell types were tested for contraction-promoting activity at protein concentrations ranging between 5 and 25  $\mu\text{g}/\text{ml}$ . Samples collected in media other than DME were first dialyzed against fresh DME before protein determination and contraction assay. Other details are described in Materials and Methods.

either 4 or 37°C. After incubation, the cells were centrifuged, washed, and plated onto the collagen gels. Cells pretreated in this manner did not contract the collagen gels in unconditioned media (data not shown). Similarly treated control cells were able to contract the gel when BAEC-conditioned medium was present during the assay, again indicating that the cells remained viable during this treatment. The failure of the various pretreatments to produce a contrac-

**Figure 5.** BAEC secretion of contractile factors is inhibited by cycloheximide. DME with (○) or without (●) added cycloheximide (25  $\mu\text{g}/\text{ml}$ ) was incubated with confluent BAEC cultures for 24 h after which the media samples were dialyzed against fresh medium to remove the cycloheximide. These media were added to fibroblasts atop collagen gels. At the times indicated, gel thickness was measured. Data presented are the averages of results from duplicate cultures. Other details are described in Materials and Methods.



**Figure 6.** BAEC stimulation of fibroblasts is dependent on de novo protein synthesis. Fibroblasts were seeded onto collagen gels and incubated in DME alone ( $\Delta$ ), DME plus FBS with ( $\square$ ) or without ( $\blacksquare$ ) cycloheximide, or BAEC-conditioned media with ( $\circ$ ) or without ( $\bullet$ ) added cycloheximide. The extent of contraction was determined at the times indicated. Other details are described in Materials and Methods.

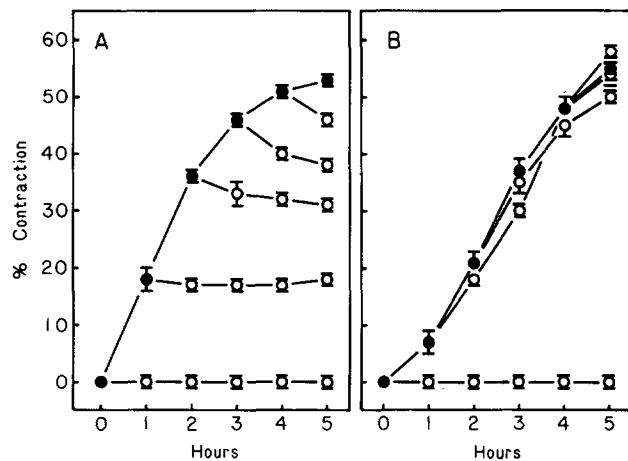
tile response by the fibroblasts suggests that the cells must be stimulated while in contact with the collagen gel.

To investigate if the contractile response by the fibroblasts required de novo protein synthesis, fibroblasts were placed on top of collagen gels and incubated in DME conditioned by BAEC in the presence of cycloheximide. The extent of gel contraction was determined at varying times during the incubation. The results (Fig. 6) indicated that cycloheximide largely blocked the BAEC-promoted contraction. Over a 4-h incubation, cycloheximide-treated cells reduced the gel thickness by 14%, whereas fibroblasts incubated with BAEC-conditioned medium in the absence of cycloheximide contracted the collagen by an average of 54% of its original thickness during the same period.

#### Comparison of Contraction-promoting Factors from BAEC and Serum

When fibroblasts seeded on collagen gels were stimulated by serum in the presence of cycloheximide, contraction proceeded essentially to the same extent as when cycloheximide was absent (Fig. 6). These data confirm previous results (7) and indicated, based on differing sensitivities to cycloheximide, that the contraction-promoting activities in serum- and BAEC-conditioned medium have different mechanisms of action.

To analyze what effect removal of contraction factors from the media would have on contraction, fibroblasts were seeded onto collagen gels and incubated at 37°C in either BAEC-conditioned medium or medium containing serum. At intervals, the media from selected wells were removed and replaced with DME, and the incubation was continued. The results of this study indicated, as previously shown (7),



**Figure 7.** The effect of removal of factor on fibroblast contraction of collagen gels stimulated by BAEC-conditioned media or DME plus FBS. Fibroblasts were seeded onto collagen gels and incubated in either DME plus FBS or BAEC-conditioned medium. At different times during the assay, the media were removed from duplicate cultures and replaced with DME alone, and the incubation was continued. (A) Fibroblast contraction in the presence of DME plus FBS: ( $\bullet$ ) before and ( $\circ$ ) after removal of the serum. (B) Fibroblast contraction in the presence of BAEC-conditioned media: ( $\bullet$ ) in the presence of BAEC-conditioned medium; ( $\circ$ ) after removal of the conditioned medium. Data presented are the averages of results from duplicate cultures. Other details are described in Materials and Methods.

that serum-stimulated contraction (Fig. 7 A) requires the continuous presence of the factor for contraction to proceed. Contraction ceased and in some cases the gels even expanded slightly when the serum factor(s) were removed. In the case of BAEC-conditioned media (Fig. 7 B), the rate of contraction was not significantly altered by removal of the stimulus, suggesting that the continuous presence of the factor is not necessary. These data reflect another difference in the mechanism of action between the serum and BAEC factors. The fact that serum-stimulated contraction occurs in the absence of protein synthesis suggests that the activity of the serum factor(s) is directed on the contractile behavior of the cell, whereas the BAEC factor(s) induce the synthesis of an intermediate messenger component. This effector could be secreted to the media or be retained with the cell. Furthermore, the synthesis of an effector, promoted by the BAEC factors, appears to continue once the fibroblasts have been exposed to the BAEC-conditioned medium for a sufficient period of time, as indicated by the sensitivity of the process to cycloheximide but not to the removal of BAEC factors.

#### Characterization of Contraction Promoter(s)

**Affinity Chromatography.** As a prelude to purification of the contraction promoters, we characterized the binding affinity of the active factors to immobilized native type I collagen, heat-denatured type I collagen (gelatin), and heparin. Samples of BAEC-conditioned media (10 ml) were run over columns (5 ml bed volume) of the different affinity matrices at room temperature. The unbound fractions were collected, and the columns were eluted with 4 M urea, 1 M NaCl in the case of the collagen columns or 0.5 and 2 M NaCl in the

**Table III. Affinity Chromatography of BAEC-conditioned Media**

Matrix	Starting activity associated with fraction
	%
Native collagen Sepharose	
Flow through	96 ± 2.0
Bound	3 ± 1.5
Gelatin Sepharose	
Flow through	98 ± 2.0
Bound	3 ± 0.0
Heparin Sepharose	
Flow through	65 ± 3.0
Bound	34 ± 1.5

Samples of BAEC-conditioned medium (10 ml) were run over 5-ml columns of each affinity matrix. Proteins bound to native collagen or gelatin were eluted with 4 M urea, 1 M NaCl, 0.02 M Tris, pH 7.4. Proteins bound to heparin were eluted sequentially with 0.5 and 2 M NaCl with 0.02 M Tris, pH 7.4. Other details are described in Materials and Methods.

case of the heparin-Sepharose. Material that did not bind or bound and was subsequently eluted from the affinity matrices was dialyzed against physiologic buffers and assayed for contraction-promoting activity (Table III). In the case of the native collagen and gelatin affinity matrices, essentially all contraction-promoting activity could be recovered in the unbound pools, indicating that these factors do not interact with native or denatured collagens. When the BAEC-conditioned medium was applied to a heparin-Sepharose matrix, ~65% of the contraction-promoting activity was recovered in the unbound pool, whereas 18 and 15% of the activity was found in the material eluted with 0.5 and 2 M NaCl, respectively. These data suggest that the contraction-promoting activity may occur in several different molecular forms. Rerunning the unbound material over the eluted column did not result in additional binding, indicating that the column was not overloaded in the initial run.

**Sensitivities.** Serial precipitations with increasing concentrations of ammonium sulfate (0–80% of saturation) indicate

**Table IV. Stability of Endothelial Cell-secreted Contraction Promoter**

Treatment	Activity remaining after treatment
	%
None	100
DME alone	0
Ammonium Sulfate 0–60%	72
DTT	0
1 M HCl	93
100°C for 10 min	94
SDS/acetone	65
Trypsin-agarose	44

Samples of BAEC-conditioned medium were subjected to the treatment shown, dialyzed against DME, and assayed for contraction-promoting activity. The percent activity remaining was calculated from a direct comparison with equal volumes of untreated conditioned medium. Samples reduced with DTT (10 mM) were also alkylated with iodoacetamide (25 mM) before dialysis. SDS/acetone-treated samples were made 1% with SDS and precipitated with 90% acetone at 4°C. Treatment with trypsin-agarose was for 2 h at 37°C with 0.5 U/ml. The data shown are the means of results from duplicate determinations. Other details are described in Materials and Methods.

that the majority of the contraction-promoting activity precipitates at 60% of saturation (Table IV). Additionally, we examined the sensitivity of the factors to denaturing conditions. The contraction-promoting activity can be completely recovered from samples heated to 100°C for 10 min or treated with 1 M HCl followed by dialysis. Partial recovery (65%) was observed with samples treated with 1% SDS followed by acetone precipitation. Reduction and alkylation completely abolished contraction-promoting activity (Table IV). Incubation with immobilized trypsin resulted in the loss of 56% of the contraction-promoting activity within 1 h (Table IV). Extending the incubation up to 8 h did not further reduce the recoverable activity (data not shown).

**Gel Permeation.** To estimate the molecular size of molecules with contraction-promoting activity, 30 ml of BAEC-conditioned medium protein was precipitated with 80% ammonium sulfate, dissolved in tris-saline, and chromatographed on a Superose 12 gel filtration column equilibrated with the same buffer. Individual fractions were analyzed for protein content, contraction-promoting activity, and finally by SDS-PAGE (Fig. 8). Contraction-promoting activity was recovered in fractions throughout the chromatogram (Fig. 8 B), indicating that this activity is either associated with many molecular species that vary in size or that the contraction promoter forms complexes with other molecules under these conditions. Analysis by SDS-PAGE (Fig. 8 C) did not demonstrate a major common component present in all active fractions.

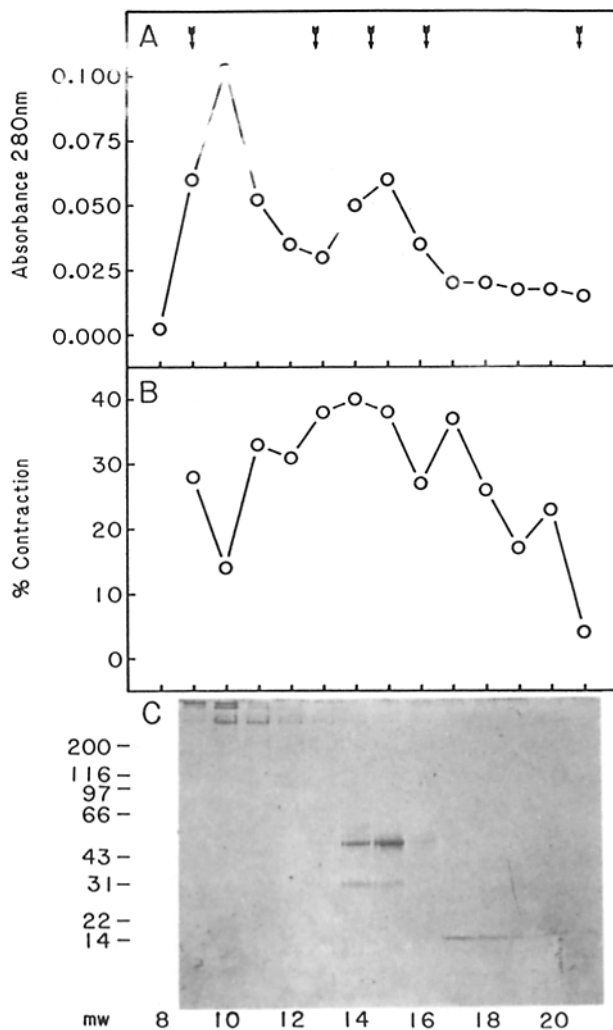
#### *The BAEC-derived Contraction Factor Is Not TGFβ*

The properties of the contraction-promoting activity in BAEC-conditioned medium are similar, in some respects to previously characterized growth factors. Furthermore, a previous report indicated that TGFβ is able to promote collagen gel contraction in an experimental system similar to ours (12). Additionally, TGFβ is secreted by many cell types including endothelium (21). For these reasons we examined the contraction-promoting potential of TGFβ and compared it with that of BAEC-conditioned medium.

Serum-free medium containing TGFβ in concentrations ranging from 0.1 μg to 1 pg/ml was tested in a contraction assay. The extent of contraction, after 4 h of incubation (Fig. 9), indicated that TGFβ in the doses tested promoted contraction of not >10% of the original collagen thickness, which is substantially less than that observed with the BAEC-conditioned medium. Extended incubations in the presence of TGFβ, as used in the previous report (12), were not examined. These data do show, however, that the factors in BAEC-conditioned medium which promote contraction are different from TGFβ.

#### *Discussion*

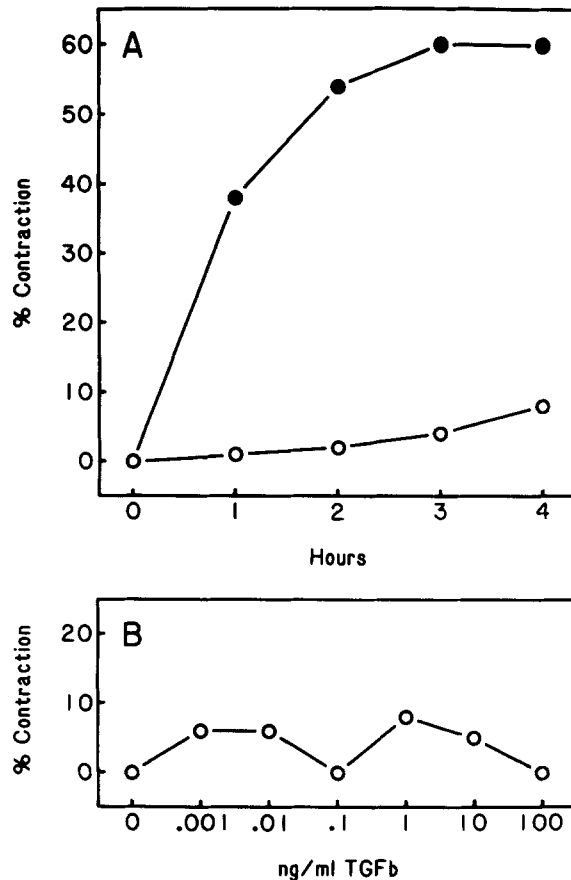
We have shown that BAEC secretory products can stimulate fibroblasts to contract collagen gels. Previous studies have demonstrated the presence of contraction-promoting activity in serum (18). Unfractionated BAEC-conditioned media promoted contraction of the collagen gels to 48% of its original thickness within 4 h of incubation, whereas the presence of 3% FBS promoted contraction to 52% of the original gel thickness during the same period. Contraction in the pres-



**Figure 8.** Gel filtration of BAEC-conditioned media proteins. Proteins from 25 ml of BAEC-conditioned medium were precipitated with 80% ammonium sulfate, dialyzed, and applied to a Superose 12 gel permeation column in Tris-saline. 2-ml fractions were collected and analyzed. (A) Absorbance at 280 nm of individual fractions. Arrows indicate the positions of gel filtration standards from left to right: 670, 158, 44, 17, and 1.4 kD, respectively. (B) Percent contraction promoted by 0.25 ml of individual fractions after dialysis. The percent contraction was determined after 3 h of incubation. Fraction 8 was not analyzed. (C) SDS-PAGE of individual fractions under nonreducing conditions. Bars on the left indicate positions of protein standards of known molecular weight. Fraction 8 was not analyzed.

ence of dilutions of these media indicate that contraction promotion is dose dependent and that BAEC-conditioned media has a specific activity, on a protein basis, some 50-fold higher than unfractionated serum.

Studies of the conditioning process show that the amount of contraction-promoting activity secreted by BAEC cultures is dependent upon length of conditioning time and cell number. Furthermore, conditioned media from cycloheximide-treated cultures do not contain contraction-promoting activity. These observations demonstrate that the contraction-promoting activity is produced and secreted by BAEC. Additionally, the amount of contraction-promoting activity



**Figure 9.** Fibroblast contraction is not promoted by TGFb. Fibroblasts atop collagen gels were incubated in either serum-free media containing the indicated doses of TGFb or BAEC-conditioned medium. All media tested were made 1 mg/ml with BSA. (A) The kinetics of fibroblast contraction promoted by BAEC-conditioned medium (●) or 1 ng/ml TGFb (○). (B) The extent of fibroblast contraction promoted by the concentrations of TGFb is indicated on the abscissa after 4 h of incubation. These data are the averages of results from duplicate cultures. Variance between duplicates was <3% contraction. Other details are described in Materials and Methods.

secreted by confluent cultures of BAEC remains constant for at least 5 d in serum-free medium, indicating that the phenotype responsible for secretion of contraction factors is stable when cells are removed from serum.

When conditioned media from a number of other transformed and nontransformed cell types were analyzed for contraction-promoting activity, most cell types produced low amounts of activity. Of the cells tested, substantial amounts of contraction-promoting activity were produced by, in addition to BAEC, a mouse mammary carcinoma and a human choriocarcinoma cell line. These observations indicate a relative specificity for the cell types producing contraction-promoting factors.

A preliminary characterization of the biochemical properties of the contraction-promoting factors indicate substantial heterogeneity: 30% of the activity bound to heparin-Sepharose; 55% was lost on trypsin digestion; 70% of the activity could be precipitated with ammonium sulfate and could be recovered over a wide range of molecular weights when conditioned media protein was fractionated by gel filtration. On



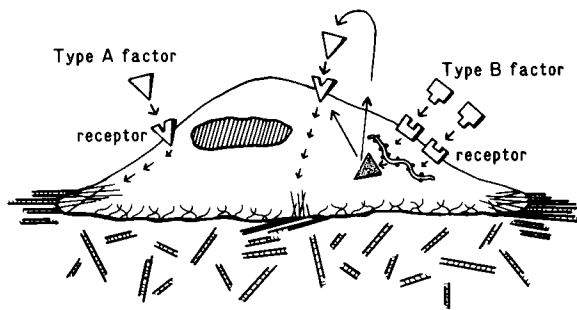


Figure 10. Proposed model for mechanism by which type A and B contraction factors stimulate fibroblasts.

the surface, these data suggest that a variety of molecular species have contraction-promoting activity. On the other hand, the contraction-promoting activity was remarkably resistant to treatments with 1 M HCl or high temperature (100°C), whereas treatment with a reducing agent resulted in complete loss of activity. Small proteins with growth-promoting activities have been previously demonstrated to have similar behavior (5, 20). These proteins also frequently associate with "carrier" molecules, such as glycosaminoglycans (15) and other proteins (13). Such associations may explain the apparent heterogeneity described above.

The mechanism of action of the BAEC factors is different from that of the serum factors. Both types of factors promote contraction of collagen gels by acting on the target fibroblasts rather than on the collagen matrices. However, BAEC contraction promoters depend on active protein synthesis in the target cell for activity, whereas serum factors can initially act in the presence of cycloheximide. Also, the serum factors need to be continuously present during the contraction process, whereas once fibroblasts seeded on the collagen gel have been treated with BAEC factors they remain contractile. A model of the different mechanisms of action is presented in Fig. 10.

For matters of simplicity, we refer to the two contraction factors as type A (FBS derived) and type B (BAEC secreted). The type A factor directly enhances the contractile behavior of the target cells, whereas the type B factor stimulates the synthesis of an effector protein which in turn is responsible for stimulation of contraction. It is possible that the effector protein may be identical to the type A factor. Alternatively, the effector could represent a separate protein that acts within the cell. Because the end result of fibroblast stimulation with type A or type B contraction factors is the same, it seems reasonable to assume that the two mechanistic pathways would merge at some point.

A previous report indicated that TGF $\beta$  can act as such a promoter over longer incubations (12). In our system, TGF $\beta$  did not promote substantial amounts of contraction and is probably unrelated to the factors we are studying.

Most recently, studies presented in a rapid publication suggested that PDGF can function as a fibroblast contraction promoter (3). Additionally, another recent study detected PDGF-related activity in fluids from healing wounds (11). This activity, however, could not be attributed to authentic PDGF but rather to PDGF-related peptides that probably are secretory products of cells within the wound.

As endothelial cells are known to secrete PDGF-related

molecules (4), it seems likely that these may account for at least a portion of the BAEC-secreted contraction-promoting activity we have observed.

We have shown that cultured endothelial cells synthesize and secrete factors that are able to promote fibroblast contraction of collagen matrices *in vitro*. Given the proximity of budding capillaries and invading fibroblasts into granulation tissue and the fact that these areas of the wound generate contractile force, it is tempting to speculate that the secretion and action of these factors reflect events occurring in wound contraction *in vivo*.

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