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Contraction of Fibrillar Type I Collagen by Endothelial Cells: A Study In Vitro

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Abstract The formation of microvascular sprouts during angiogenesis requires that endothelial cells move through an extracellular matrix. Endothelial cells that migrate in vitro generate forces of traction that compress (i.e., contract) and reorganize vicinial extracellular matrix, a process that might be important for angiogenic invasion and morphogenesis in vivo. To study potential relationships between traction and angiogenesis, we have measured the contraction of fibrillar type I collagen gels by endothelial cells in vitro. We found that the capacity of bovine aortic endothelial (BAE) cells to remodel type I collagen was similar to that of human dermal fibroblasts—a cell type that generates high levels of traction. Contraction of collagen by BAE cells was stimulated by fetal bovine serum, human plasma-derived serum, bovine serum albumin, and the angiogenic factors phorbol myristate acetate and basic fibroblast growth factor (bFGF). In contrast, fibronectin and immunoglobulin from bovine serum, several nonserum proteins, and polyvinyl pyrrolidone (a nonproteinaceous substitute for albumin in artificial plasma) were not stimulatory. Contraction of collagen by BAE cells was diminished by an inhibitor of metalloproteinases (1,10-phenanthroline) at concentrations that were not obviously cytotoxic. Zymography of proteins secreted by BAE cells that had contracted collagen gels revealed matrix metalloproteinase 2. Subconfluent BAE cells that were migratory and proliferating were more effective contractors of collagen than were quiescent, confluent cells of the same strain. Moreover, bovine capillary endothelial cells contracted collagen gels to a greater degree than was seen with BAE cells. Collectively, our observations indicate that traction-driven reorganization of fibrillar type I collagen by endothelial cells is sensitive to different mediators, some of which, e.g., bFGF, are known regulators of angiogenesis in vivo.

Key words: angiogenesis, basic fibroblast growth factor, bovine, capillary, collagen gel, phorbol ester, traction

The generation of new microvessels from extant vasculature (angiogenesis) is an important constituent of embryonic development, repair of wounds, and various pathological processes that include the growth of tumors. Angiogenesis, at all stages, is characterized by the migration of endothelial cells through an extracellular matrix (ECM). The formation of new vascular buds or "sprouts" begins when endothelial cells of the parent vessel detach from the vascular wall, penetrate the basal lamina that invests them, and invade the surrounding interstitial ECM. The subsequent migration of endothelial cells through the interstitial ECM is associated with increases in the length of individual sprouts and with the anastomosis of adjacent sprouts to form vascular loops and networks [Clark and Clark, 1939; Cliff, 1963; Schoefl, 1963; Schoefl and Majno, 1964; Ausprunk and Folkman, 1977; Paweletz and Knierim, 1989; Paku and Paweletz, 1991].

Studies in vitro reveal that the migration of endothelial cells and certain other cell types (e.g., fibroblasts) over deformable surfaces is accompanied by a significant distortion of the substrate [Harris et al., 1980, 1981; Vernon et al., 1992]. For example, endothelial cells that are moving on top of a thin film of silicone rubber pull on the surrounding film and compress the rubber that lies beneath them into a series of wrinkles [Vernon et al., 1992]. A similar but more dramatic phenomenon occurs when endothelial cells or fibroblasts are cultured on top of a malleable ECM (e.g., gels of basement membrane matrix or type I collagen): each cell pulls ECM toward itself in a continuous manner and thereby accumulates ECM in its immediate vicinity. The process by which cells pull on the substrate that supports them is referred to as

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traction [Harris et al., 1981; Stopak and Harris, 1982; Vernon et al., 1992].

Traction and progressive motility both require intact cytoplasmic actin microfilaments and therefore are likely to be driven by similar processes within the cytoskeleton—in fact, it has been proposed that traction is a component of cellular propulsion [Harris et al., 1981]. In addition to a direct function in the motility of endothelial cells, traction might influence vascular morphogenesis via its effects on the spatial organization of ECM. For example, we observe that the traction that bovine aortic endothelial (BAE) cells apply to planar substrates of either type I collagen or basement membrane matrix in vitro aligns the fibers of ECM into a network of linear "tracks" on which endothelial cells migrate and establish a cellular network that resembles a microvascular bed [Vernon et al., 1992].

The fact that endothelial cells apply traction to ECM in vitro has been established; however, much remains to be learned about the purpose of traction and about factors that mediate and regulate the process. The traction that a population of cells exerts collectively can be studied by use of a collagen gel contraction (CGC) assay [Guidry and Grinnell, 1987; Clark et al., 1989; Gullberg et al., 1990; Asaga et al., 1991; Guidry and Hook, 1991; Kitamura et al., 1991, 1992; Vernon et al., 1992; Reed et al., 1994], in which dispersed cells are embedded in a floating, disk-shaped gel of fibrillar type I collagen (type I collagen is usually selected because it is the primary collagen of interstitial connective tissue and because it can be easily polymerized in vitro to form a tissue-like matrix). With time in culture (typically 12–72 h), the gathering and compression of collagen fibers around individual cells causes a reduction in diameter of the collagen disk. The degree to which the disk is contracted reflects the effectiveness of the traction process. By use of the collagen contraction parameter, traction can be compared among different populations of cells. Moreover, molecular factors that mediate, enhance, or inhibit traction can be identified.

In the present communication, we employ CGC assays to identify factors that influence the traction of endothelial cells in vitro. We find that the reorganization of collagen by endothelial cells is influenced by differences in cellular origin, states of confluence, serum factors, inhibition of metalloproteinases, and by the presence of specific angiogenic molecules.

MATERIALS AND METHODS

Cells and Cell Culture

BAE cells were isolated from adult aortae as described previously [Iruela-Arispe et al., 1991]. Capillary endothelial cells from bovine adrenal glands were provided by P. D'Amore (Department of Pathology, Children's Hospital/Harvard Medical School). Endothelial cells were maintained routinely in 75-cm^2 plastic tissue-culture flasks in Dulbecco's Modified Eagle Medium (DMEM) (Sigma Chemical Co., St. Louis, MO) with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT), 100 U/ml penicillin-G, and 100 μg/ml streptomycin-SO_4. Cells were used between passages 10–15.

Phorbol myristate acetate (PMA) (Sigma) was made as a 1-mg/ml stock solution in dimethyl sulfoxide (DMSO). BSA and human plasma-derived serum (hPDS) that were essentially free of platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), as determined by enzyme-linked immunoabsorbent assays (ELISA), were provided by E. Raines (Department of Pathology, University of Washington). Cycloheximide, lactalbumin hydrolysate, ovalbumin, 1,10-phenanthroline, and polyvinylpyrrolidone were obtained from Sigma Chemical Co. Purified bovine serum fibronectin, purified bovine serum immunoglobulin type G (IgG), and human recombinant basic fibroblast growth factor (bFGF) were obtained from Telios (San Diego, CA), Cappel/Organon Teknika Corp. (Durham, NC), and Upstate Biotechnology Inc. (Lake Placid, NY), respectively.

CGC Assays

The traction-mediated reorganization of type I collagen by endothelial cells was analyzed with CGC assays as described previously [Vernon et al., 1995]. Briefly, wells of 24-well or 48-well plastic tissue-culture plates (wells were 15 mm and 11 mm in diameter, respectively) were coated with a thin layer of 1% agarose (Sea-Kem LE; FMC BioProducts, Rockland, ME). One vol of 3 mg/ml type I collagen from bovine skin, dissolved in 0.012N HCl (Vitrogen) (Celtrix Corp., Palo Alto, CA), was brought to physiological ionic strength and pH with 1/6 vol of 7X DMEM, and supplemented with 1X DMEM to yield gels with final collagen concentrations (after addition of cells and other supplements) of 1, 0.75, 0.5, 0.375, and 0.25 mg/ml. Nine vol of DMEM-type I collagen solution and 1 vol of DMEM that

contained suspended endothelial cells were combined, dispensed in quadruplicate into the agarose-coated wells (wells of 11-mm and 15-mm diameter received 200 μl and 500 μl, respectively), and allowed to polymerize for 2 h at 37°C. A volume of DMEM equal to the volume of the collagen gel was then added to each well to float the collagen disks. When applicable, biological agents (e.g., FBS, BSA, bFGF) were added to the collagen solution prior to gelation and to the DMEM in which the gels were floated. After incubation of the plates for 18 h at 37°C, the degree of contraction of each collagen disk was determined by measurement of the diameter of the cellular ring that formed at the periphery of the gel as a consequence of the contraction process [Gullberg et al., 1990; Vernon et al., 1995]. Measurements for the five series of quadruplicate wells were plotted as average diameters of disks (±SEM) versus concentrations of collagen. In experiments that measured the response of endothelial cells to varied doses of biological agents, the gels were made from collagen at a single concentration. In these situations, the average diameters of disks were plotted against the concentration of the specific agent. The potential for differential proliferation of endothelial cells within collagen gels was examined. At the conclusion of incubation, cells were counted following their release from gels by treatment with collagenase [Reed et al., 1994]. We found that in no circumstance did endothelial cells proliferate within floating collagen gels within the 16- to 18-h period of incubation.

Zymography

BAE cells (3×10^5/ml final concentration) were embedded in 0.375 mg/ml type I collagen (24 gels of 15 mm diameter). Twelve gels contained 0.22 mg/ml BSA and 12 gels were made with DMEM only. Gels that contained BSA were suspended in 500 μl of DMEM/0.22 mg/ml BSA; gels that lacked BSA were suspended in 500 μl of DMEM only. All gels were subsequently cultured for 24 h. Supernates from 6 wells that contained BSA (average diameter of the collagen gels was 3.85 mm) were collected and pooled, as were supernates from 6 wells that lacked BSA (average diameter of gels was 12.25 mm). Supernates were diluted with an equal volume of buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [Laemmli, 1970] and were subjected to electrophoresis through a polyacrylamide gel that contained 0.1% gelatin. Stacking and resolving portions of the gel were made from 5% and 10% acrylamide, respectively. Subsequent to electrophoresis, the polyacrylamide gel was incubated in two changes of 2.5% Triton X-100 for 2 h and divided in half to separate duplicate samples. Half-gels were incubated separately for 21 h at 37°C in a solution of 50 mM Tris (pH 8), 5 mM $CaCl_2$, and 0.02% NaN_3 with or without 10 mM EDTA. Gels were subsequently stained with Coomassie Brilliant Blue R-250.

RESULTS

The strong traction that fibroblasts apply to collagen gels in vitro correlates with the function of this cell type as a remodeler of interstitial ECM in vivo (e.g., during development or during the repair of wounds). Our initial studies employed human dermal fibroblasts as a standard against which the ability of BAE cells to remodel collagenous ECM was compared. Equal numbers of fibroblasts and BAE cells were cultured in the presence of 2% FBS in a series of type I collagen gels with decreasing malleabilities. Both cell types contracted collagen to a similar extent, although the fibroblasts contracted rigid gels of 0.75–1 mg/ml collagen to a somewhat greater degree than did BAE cells (Fig. 1).. In general,

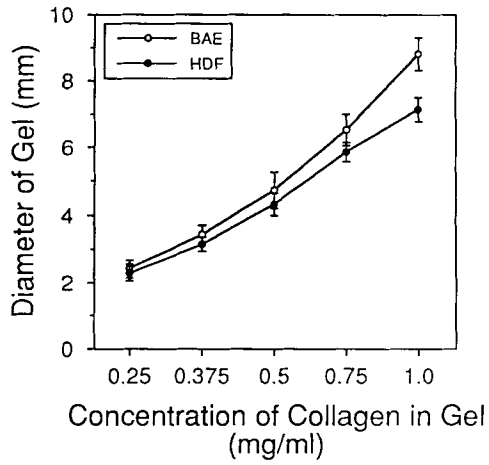

Fig. 1. The performance of endothelial cells and fibroblasts is compared in CGC assays. Equal numbers (3×10^5 cells/ml) of BAE cells and human dermal fibroblasts (HDF) were embedded in a series of 15-mm disks made from 0.25–1 mg/ml type I collagen and were incubated for 18 h in vitro in the presence of 2% FBS. Both cell types contracted malleable gels similarly; however, the fibroblasts contracted rigid gels (0.75–1 mg/ml collagen) more effectively in comparison to BAE cells. Each point of the data is the average of 4 samples. Capped bars = SEM.

both cell types remodeled type I collagen effectively: the cells contracted malleable gels (0.25–0.375 mg/ml collagen) to less than 20% of their original diameters within 18 h.

Serum is known to enhance the contraction of collagen gels by fibroblasts [Steinberg et al., 1980]. Since endothelial cells appeared to be similar to fibroblasts with respect to the generation of traction, it was of interest to test the effects of FBS on the contractile behavior of BAE cells (Fig. 2, top graph). BAE cells contracted malleable collagen to a limited extent in serum-free medium (gels averaged 78.3% of original diameter after 18 h of incubation); however, contraction was stimulated by addition of low concentrations (0.1%) of FBS. Higher concentrations of FBS (0.2–1%) induced a dose-dependent increase in contraction. Further enhancement of contraction was minimal beyond 1% FBS. Contraction of gels induced by 2% FBS was inhibited by cycloheximide in a concentration-dependent manner (Fig. 2, bottom graph); therefore, the stimulatory effects of serum were mediated via a mechanism that required protein synthesis.

As shown in Figure 3, hPDS stimulated gel contraction by BAE cells as effectively as FBS. The stimulatory effects of serum could be accounted for largely by the albumin fraction, since purified BSA stimulated contraction in a manner similar to that of intact serum (Fig. 3). We examined further the specificity of the BSA stimulation by substitution of other proteins for BSA in CGC assays (Fig. 4). Contraction of collagen was not enhanced by either fibronectin or IgG that were purified from bovine serum, by the nonserum protein ovalbumin, or by a mixture of peptides (lactalbumin hydrolysate). Moreover, contraction was not stimulated by polyvinyl pyrrolidone, a compound that has been tested clinically as a substitute for albumin in artificial plasma as a consequence of its viscosity, surface-active characteristics, and osmotic properties [Moffit, 1975].

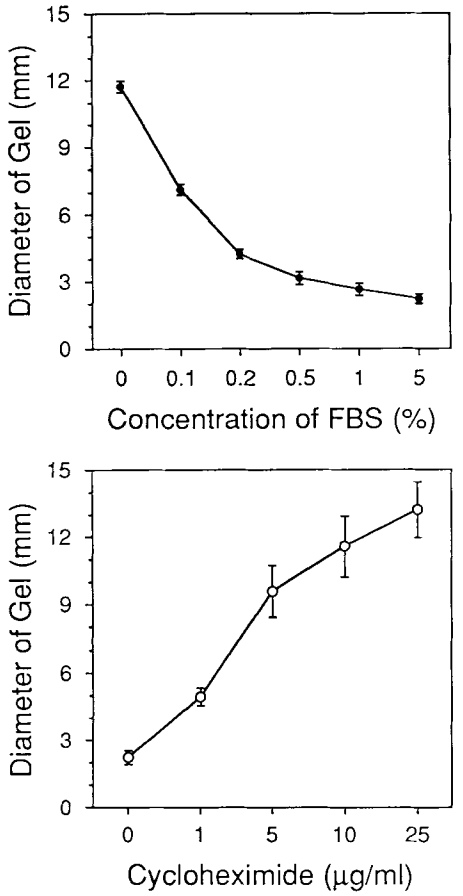

Fig. 2. Top graph: BAE cells at 3 × 10^5/ml were embedded in 15-mm disks made from 0.375 mg/ml type I collagen and were incubated for 18 h in the presence of graded concentrations of FBS. **Bottom graph:** BAE cells at 3 × 10^5/ml were embedded in 15-mm disks made from 0.375 mg/ml type I collagen and were incubated for 18 h in the presence of 2% FBS that was supplemented with graded concentrations of cycloheximide. In top and bottom graphs, each point of the data is the average of 4 samples. Capped bars = SEM.

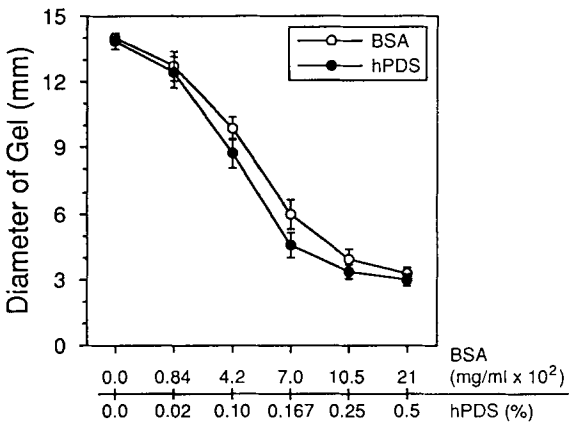

Fig. 3. hPDS and BSA induce the contraction of collagen by endothelial cells. BAE cells at 3 × 10^5/ml were embedded in 15-mm disks made from 0.375 mg/ml type I collagen and were incubated for 18 h in the presence of increasing concentrations of hPDS or purified BSA. Both hPDS and BSA stimulated contraction of collagen with similar dose-response profiles. Concentrations of purified BSA (in mg/ml) were compared with dilutions of hPDS (in %) with equivalent concentrations of albumin. Each point of the data is the average of 4 samples. Capped bars = SEM.

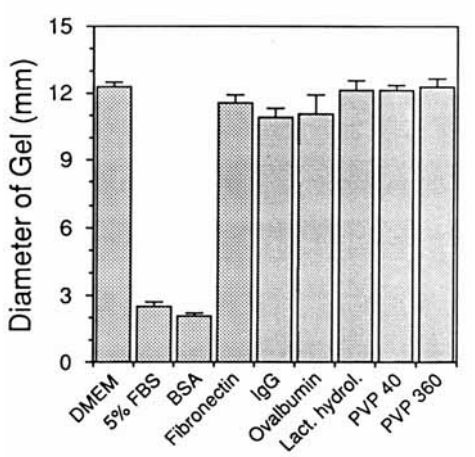

Fig. 4. Effects of serum proteins, nonserum proteins, and protein analogs in CGC assays. BAE cells at 3 × 10^5/ml were embedded in 15-mm disks made from 0.375 mg/ml type I collagen and were incubated for 18 h in the presence of DMEM only, DMEM supplemented with 5% FBS, or DMEM supplemented with the following additives: BSA, bovine fibronectin, bovine IgG, ovalbumin, lactalbumin hydrolysate (lact. hydrol.), or polyvinyl pyrrolidone (PVP) of two molecular weights: 40,000 (PVP 40) and 360,000 (PVP 360). BSA at 1.1 mg/ml, which equals the concentration of albumin in 5% FBS, stimulated the contraction of collagen as effectively as 5% FBS. By contrast, the other supplements were not stimulatory. All supplements were tested at 1.1 mg/ml with the exception of fibronectin, which was tested at 10 μg/ml—the concentration of fibronectin in 5% serum [Mosesson and Umfleet, 1970]. Each point of the data is the average of 4 samples. Capped bars = SEM.

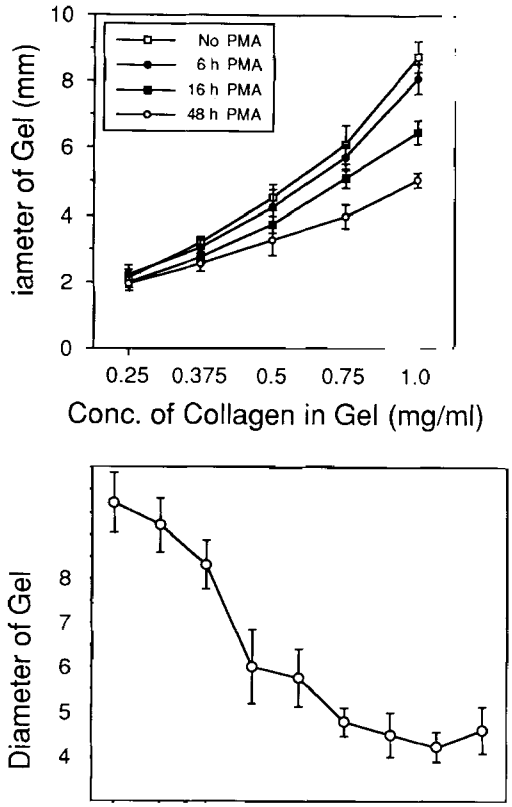

Fig. 5. PMA stimulates endothelial cells to contract type I collagen. **Top graph:** Confluent monolayers of BAE cells, grown on plastic, were exposed to 50 ng/ml PMA for 6 h, 16 h, or 48 h and were subsequently tested in a CGC assay. The assay was performed as described in Figure 1 but with 0.22 mg/ml BSA substituted for FBS. PMA was not present during the assay. Contraction of collagen increased as the time of exposure of BAE cells to PMA was lengthened. The stimulatory effect, relative to control, was greatest in gels made from higher concentrations of collagen. **Bottom graph:** BAE cells, grown as confluent monolayers on plastic, were exposed to graded concentrations of PMA for 48 h and were subjected to a CGC assay (3 × 10^4 BAE cells/ml were embedded in 15-mm disks made from 0.375 mg/ml type I collagen and were incubated for 18 h). The collagen gels and culture media were supplemented with 0.22 mg/ml BSA and with the concentration of PMA that was equal to the prior level of exposure. In top and bottom graphs, each point of the data is the average of 4 samples. Capped bars = SEM.

Angiogenesis in vivo is stimulated by a variety of humoral factors that are released either by tumors or by normal cells [Paweletz and Knierim, 1989; Klagsbrun and D'Amore, 1991]. The effects of angiogenic factors on vascular development can be simulated in vitro. For example, PMA, which mimics the effects of certain hormones and growth factors via the activation of protein kinase C, induces endothelial monolayers to invade underlying matrices of type I collagen to form tubular, capillary-like structures [Montesano and Orci, 1985, 1987]. We sought to determine whether altered cellular traction was a component of the response of endothelial cells to angiogenic factors: we exposed monolayers of BAE cells (grown to confluence on tissue-culture plastic) to 50 ng/ml PMA for varying lengths of time and subsequently transferred the cells into collagen gels that lacked PMA (Fig. 5, top graph). Exposure of BAE cells to PMA for 6 h resulted in a minimal enhancement of gel contraction in comparison to control cultures not exposed to PMA. Contraction of collagen was significantly enhanced by a 16-h or 48-h exposure to PMA: gels of 1 mg/ml collagen were reduced to approximately 74% and 57% of control diameters, respectively. Subsequently, we examined the influence of PMA concentration on contraction of collagen gels by BAE cells (Fig. 5, bottom graph). A 48 h exposure to 0.1 ng/ml PMA did not stimulate contraction in compari-

son to controls that lacked PMA. Contraction was stimulated in a dose-dependent manner by 1-, 5-, 10-, or 20 ng/ml PMA. Further enhancement of contraction was not observed at concentrations of PMA above 20 ng/ml.

bFGF, a member of a family of heparin-binding growth factors, stimulates neovascularization in vivo [e.g., Olivo et al., 1992] and induces angiogenic behaviors among endothelial cells in vitro that resemble the effects of PMA [Esch et al., 1985; Montesano et al., 1986; Pepper et al., 1994]. Unlike phorbol esters, which are not found in vivo, bFGF is present in a variety of tissues that include sites of active angiogenesis [Schulze-Osthoff et al., 1990; Guthridge et al., 1992; Bechtner et al., 1993; Ferriani et al., 1993]. We observed that a 48 h exposure of confluent BAE cells (on plastic) to bFGF stimulated contraction of collagen gels (Fig. 6). bFGF at 0.1–0.3 ng/ml was not stimulatory; however, a dose-dependent stimulation was observed when cells were exposed to bFGF at 1-, 3-, and 10 ng/ml. A further enhancement of contraction was not observed at concentrations of bFGF above 10 ng/ml.

Proteolytic modification of ECM is thought to play a role in vascular morphogenesis [Montesano and Orci, 1985; Moses and Langer, 1992; Pepper et al., 1992; Fisher et al., 1994]. Moreover, angiogenic factors that include PMA and bFGF are known to increase the synthesis and release of plasminogen activators and collagenase [Gross et al., 1982; Montesano and Orci, 1985; Montesano et al., 1986; Pepper et al., 1990, 1994; Fisher et al., 1994]. We studied the potential influence of endogenous proteolysis on the traction-mediated reorganization of collagen by performing CGC assays in the presence of 1,10-phenanthroline (an inhibitor of metalloproteinases) or aprotinin (an inhibitor of serine proteinases) (Fig. 7, top graph). Contraction of collagen gels by BAE cells grown in the absence of angiogenic factors was effectively inhibited by 10^{-5} M or 10^{-4} M 1,10-phenanthroline. We observed that exposure of a confluent monolayer of BAE cells (cultured on a plastic substrate) to 10^{-5} M or 10^{-4} M 1,10-phenanthroline for 18 h was not obviously toxic, as measured by exclusion of trypan blue—a finding in accordance with Mignatti et al. [1989], who reported an absence of cytotoxic effects among bovine capillary endothelial cells exposed for 72 h to 10 μg/ml (5.04×10^{-5} M) phenanthroline. We observed that contraction of collagen gels by BAE cells was not inhibited by high concentrations ($\leq$400 KIU/ml) of aprotinin. Aprotinin and 1,10-phenanthroline administered in combination did not inhibit contraction more than did 1,10-phenanthroline alone. Inhibition of collagen gel contraction by 1,10-phenanthroline was not influenced by the addition of bFGF (Fig. 7, middle graph). PAGE zymography (Fig. 7, bottom panel) indicated that the BSA employed in the CGC assays lacked endogenous gelatinolytic activity. By contrast, medium isolated from cultures of BAE cells that had contracted collagen gels in the presence of BSA contained a significant amount of gelatinolytic activity in a single band with a molecular weight of 67 kDa. Based on this molecular weight and characterization of the band as a metalloproteinase (gelatinolysis was inhibited by removal of divalent cations with EDTA), the band was identified as matrix metalloproteinase 2 (MMP-2) [Seltzer et al., 1990; Wysocki et al., 1993]. It was noteworthy that similar levels of MMP-2 were observed in medium from cultures of BAE cells in uncontracted gels that lacked BSA.

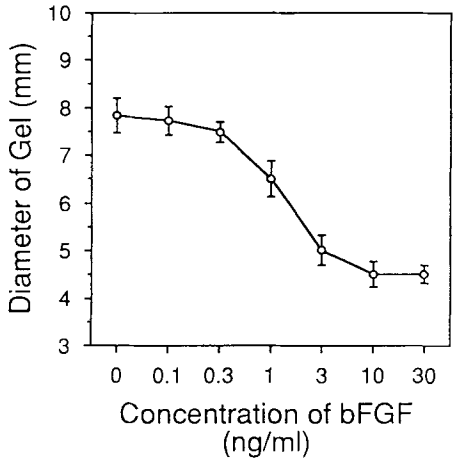

Fig. 6. bFGF stimulates endothelial cells to contract type I collagen. The design of the experiment was similar to that described in Figure 5, bottom graph; however, bFGF was substituted for PMA and the disks of collagen were 11 mm in diameter. Each point of the data is the average of 4 samples. Capped bars = SEM.

The facility with which endothelial cells accomplish angiogenesis might be influenced not only by the presence of specific angiogenic factors, but also by behaviors that derive from environmental cues or cellular origins. For example, endothelial cells cultured as subconfluent mono-

layers on plastic substrates proliferate and migrate in the manner of endothelial cells that participate in angiogenesis or repair of vascular injury in vivo. By contrast, confluent monolayers of endothelial cells in vitro are nondividing and nonmotile in the manner of endothelial cells of mature, uninjured vasculature. In regard to cellular origin, cultures of endothelial cells derived from microvasculature have been shown to differ from cultures of macrovascular endothelial cells with respect to cellular morphology, biosynthesis, and the propensity to form vessel-like structures in vitro. We compared the contraction of collagen between quiescent, confluent BAE cells and (a) active, subconfluent BAE cells or (b) quiescent, confluent bovine microvascular (capillary) endothelial cells. BAE cells cultured for 3 days at 25% or 75% confluence contracted collagen gels similarly, but with greater effectiveness than observed for BAE cells maintained at confluence for 3 days (Fig. 8, top graph). The suppressive effect of confluence was elevated with time: BAE cells confluent for 3 days were significantly more effective contractors of collagen in comparison to BAE cells that had been confluent for two weeks. Bovine capillary endothelial cells that were confluent on plastic for the same length of time as BAE cells (i.e., 5 days) contracted collagen to a greater degree than was seen for BAE cells (Fig. 8, bottom graph).

DISCUSSION

Why do endothelial cells exert traction? Fibroblasts are typically chosen for studies of cellular traction because the traction of fibroblasts is important to the repair of dermal wounds in vivo [Guidry and Grinnell, 1987; Clark et al.,

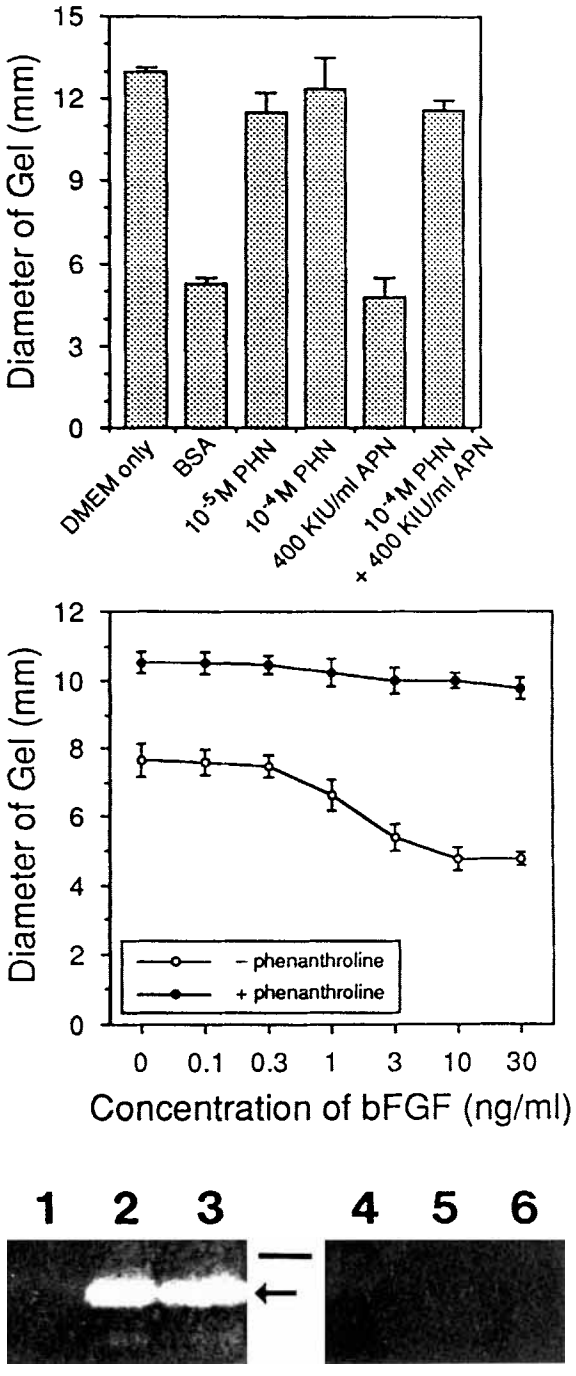

Fig. 7. Inhibition of metalloproteinases suppresses contraction of type I collagen. **Top graph:** BAE cells at 1×10^6/ml were embedded in 15-mm disks made from 0.375 mg/ml type I collagen and were incubated for 18 h in the presence of the following additives (from left): DMEM only; 0.22 mg/ml BSA; 10^{-5} M and 10^{-4} M 1,10-phenanthroline (PHN); 400 kallikrein inactivator units (KIU)/ml of aprotinin (APN); 10^{-4} M PHN + 400 KIU/ml APN. All wells that contained PHN and/or APN also contained 0.22 mg/ml BSA. **Middle graph:** Stimulation of contraction by bFGF, assayed as described in Figure 6 with gels 11 mm in diameter, is inhibited by 10^{-4} M 1,10-phenanthroline. In top and middle graphs, each point of the data is the average of 4 samples. Capped bars = SEM. **Bottom:** Analysis by zymography of supernates from BAE cells cultured 18 h in type I collagen gels. Degradation of gelatin by resolved proteins within the polyacrylamide gel was conducted in the absence (lanes 1–3), or presence (lanes 4–6) of EDTA. *Lanes 1, 4,* 0.22 mg/ml BSA control; *lanes 2, 5,* culture supernate from BAE cells in uncontracted collagen gels that lacked BSA; *lanes 3, 6,* culture supernate from BAE cells in contracted collagen gels that contained 0.22 mg/ml BSA. Similar levels of gelatinolytic activity at 67 kDa (*arrow*) are observed in supernates from both contracted and uncontracted cultures. Since this activity was inhibited by EDTA, the 67-kDa gelatinase is a metalloproteinase. The position of a 68-kDa molecular weight standard (BSA) is indicated (bar).

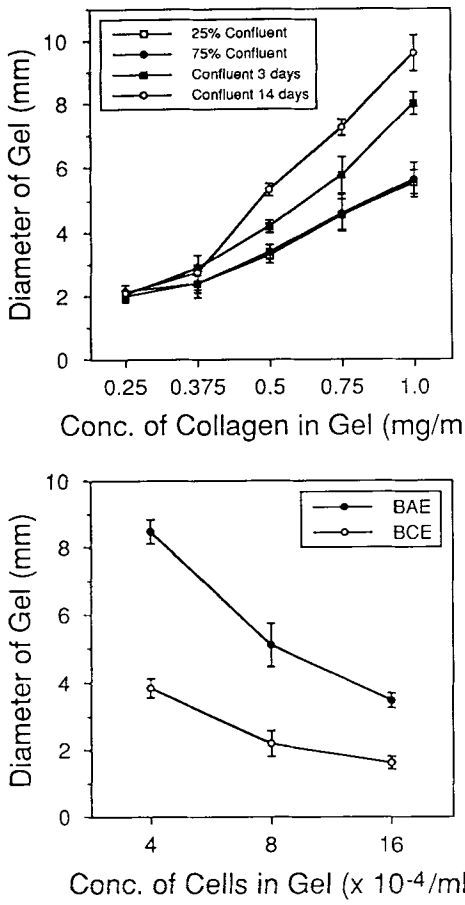

Fig. 8. Confluence and cellular origin influence the contraction of type I collagen by endothelial cells. **Top graph:** BAE cells were cultured on plastic for 3 days at 25%, 75%, and 100% confluence, or for 14 days at 100% confluence, and were subsequently analyzed by CGC assay. The assay was performed as described in Figure 1 but with 1.1 mg/ml BSA substituted for FBS. Cells from the subconfluent cultures contracted collagen similarly and to a greater degree than did cells from the confluent cultures. **Bottom graph:** BAE cells and bovine capillary endothelial (BCE) cells, confluent on plastic for 5 days, were compared by a CGC assay in which the variable was cell concentration. Cells were embedded in 15-mm disks made from 0.375 mg/ml type I collagen and were incubated for 18 h in the presence of DMEM that was supplemented with 0.22 mg/ml BSA. At all concentrations, BCE cells were significantly better contractors of collagen than were BAE cells. In top and bottom graphs, each point of the data is the average of 4 samples. Capped bars = SEM.

1989; Gullberg et al., 1990; Asaga et al., 1991; Guidry and Hook, 1991; Reed et al., 1994]. Nonfibroblastic cells, however, also generate traction in a manner characteristic of the specific cell type: macrophages, polymorphonuclear leukocytes, neurons, epithelial cells, liver parenchyma, and transformed fibroblasts exert weak or modest traction, whereas glial cells and platelets generate strong traction that is comparable to that of nontransformed fibroblasts from heart, tendon, or dermis [Harris et al., 1980, 1981]. We find that vascular endothelial cells exert strong traction: they distort silicone rubber films significantly [Vernon et al., 1992] and contract collagen lattices to an extent comparable to that of dermal fibroblasts (present study).

At first consideration, the reason for endothelial cells to exert high levels of traction in vivo is not readily apparent. Traction is not an absolute requirement for cellular migration, at least in vitro—in fact, cell types that move rapidly (e.g., macrophages, polymorphonuclear leukocytes, and neuronal growth cones) exert the weakest traction [Harris et al., 1980, 1981]. Moreover, it has been estimated that the forces of traction exerted by fibroblasts are two to three orders of magnitude greater than is needed to propel the cells at their normal speeds [Harris et al., 1981]. It has been proposed [Harris et al., 1981; Stopak and Harris, 1982] that the "excessive" traction generated by fibroblasts is applied to the rearrangement of ECM for morphogenesis in vivo, e.g., to compress ECM in dermal wounds or in other tissues that are damaged by pathological processes [Guidry and Grinnell, 1987; Clark et al., 1989; Gullberg et al., 1990; Asaga et al., 1991; Guidry and Hook, 1991; Reed et al., 1994], to facilitate the eruption of teeth [Bellows et al., 1981], and to mediate the compaction and alignment of collagen fibers within developing tendons, ligaments, periosteum, and capsules of organs (as suggested by the ability of fibroblasts to align fibers within isotropic collagen gels in vitro [Stopak and Harris, 1982]). Other cell types might use traction for similar purposes: the contraction of collagen by mesangial cells in vitro has been proposed recently as a model for scar formation during pathogenesis in the kidney [Kitamura et al., 1991, 1992]. Accordingly, we propose that the reorganization of ECM by the strong traction of endothelial cells might play a role in angiogenesis in vivo by facilitating endothelial invasion and/or morphogenesis.

Angiogenic invasion of ECM: a role for traction? Recently, it was observed that the level of traction developed by human melanoma cells was correlated with the ability of the cells to metastasize in vivo: aggressively metastatic melanoma cell lines contracted type I collagen gels in vitro to a degree that was comparable to that of fibroblasts, whereas melanoma lines of lesser metastatic potential were less effective

contractors. By contrast, normal melanocytes did not contract collagen gels [Klein et al., 1991]. Angiogenesis in vivo has been compared to the metastatic spread of cancer cells because both processes involve cell-mediated degradation of basement membranes and the invasion of cells into interstitial ECM [Stetler-Stevenson et al., 1993]. Therefore, an enhanced ability of endothelial cells to contract collagen in vitro by traction should reflect an enhancement of invasive (angiogenic) capabilities—a hypothesis which our observations support. For example, migratory endothelial cells in vitro are thought to mimic the behaviors of endothelial cells during neovascularization in vivo [Augustin-Voss and Pauli, 1992]. Accordingly, we observe that subconfluent endothelial cells that migrate on plastic prior to CGC assays are better contractors of collagen than are confluent, nonmigratory endothelial cells. Traction-mediated restructuring of ECM in vitro is influenced by the cytoskeleton, cell–ECM adhesion, and proteolysis, all of which are likely to be modified in endothelial cells that assume a migratory phenotype. Indeed, migratory endothelial cells alter their cytoskeletal structure in vitro [Ettenson and Gotlieb, 1992] and exhibit enhanced synthesis of cell-surface glycoproteins [Augustin-Voss and Pauli, 1992], sulfated proteoglycans [Kinsella and Wight, 1986], integrins [Lampugnani et al., 1991], and urokinase-type plasminogen activator [Pepper et al., 1987; Odekon et al., 1992] in vitro. It is not surprising, therefore, that PMA and bFGF stimulate the contraction of collagen by endothelial cells, since these growth factors elicit responses from endothelial cells in vitro that are typical of subconfluence, such as increased migration [Sato and Rifkin, 1988; Kurokawa et al., 1989; Rosen et al., 1991], altered expression of integrins [Enenstein et al., 1992; Klein et al., 1993; Sepp et al., 1994, 1995], and elevated synthesis of ECM proteases [e.g., Presta et al., 1986; Mignatti et al., 1989; Fisher et al., 1994]. (It is noteworthy that serum albumin binds angiogenic factors, the presence of which might account for its stimulatory effect in CGC assays, although we used BSA that lacked the known angiogenic factors PDGF and TGF-β.) The mitogenic effects of PMA, bFGF, serum, and subconfluent culture do not directly influence CGC assays, since proliferation of endothelial cells during CGC assays is minimal. Effects of proliferation on gene expression of endothelial cells prior to CGC assay cannot be ruled out; however, endothelial cells that migrate in vitro [Kinsella and Wight, 1986; Augustin-Voss and Pauli, 1992] or that participate in angiogenesis in vivo [Paweletz and Knierim, 1989] exhibit altered patterns of biosynthesis prior to proliferation.

Typically, angiogenic sprouting occurs at the level of capillaries and post-capillary venules and not of arteries and veins [Cliff, 1963; Schoefl, 1963; Ausprunk and Folkman, 1977]. Differences between macro- and microvessels with respect to either the composition of subendothelial ECM, or the presence of a smooth muscle layer, might contribute to the hierarchical specificity of angiogenesis; however, the behavior of endothelial cells might serve as an additional element of selectivity. For example, in the presence of PMA, endothelial cells isolated from either macrovasculature or capillaries will invade collagen gels to form vessel-like tubes in vitro [Montesano and Orci, 1985, 1987]; however, the phenomenon occurs at a slower pace with macrovascular endothelial cells than with capillary endothelial cells [Montesano and Orci, 1987]. Our observation that bovine capillary endothelial cells were better contractors of collagen in vitro than were BAE cells indicates that traction-associated phenomena are elements of the differential capacity for matrical invasion between macro- and microvascular endothelial cells.

The precise role that cellular traction might play in the invasion of ECM by endothelial cells or other cell types is unclear. Traction might contribute directly to the propulsion of cells through ECM and/or facilitate the clearance of ECM that lies immediately ahead of migratory cells. Moreover, traction-mediated alignment of ECM over greater distances could generate specific pathways for cellular migration. For example, we found that the traction of BAE cells aligned planar layers of basement membrane matrix in vitro into networks of narrow cables or tracks that served as scaffolds on which endothelial cells migrated and co-associated to form a network resembling a capillary mesh [Vernon et al., 1992]. Similar scaffolds, formed from type I collagen synthesized in situ, served as the pattern for planar endothelial networks in a model of spontaneous angiogenesis in vitro. Among cultures of BAE cells, strains that formed networks spontaneously contracted type I collagen gels to a greater degree than was observed for strains that did not form networks [Vernon et al., 1995; Vernon and Sage, unpublished obser-

vations]. Scaffolds of ECM might direct the migration and spatial organization of endothelial cells in vivo during vasculogenesis in avians and, potentially, during the development of other vascular systems with planar characteristics [Vernon et al., 1995].

Why does proteolysis influence vascular morphogenesis? Evidence suggests that angiogenesis is facilitated by proteinases (e.g., plasminogen activators and members of the superfamily of matrix metalloproteinases) that are secreted by endothelial cells within vascular sprouts. For example, the inhibition of endogenous metalloproteinases with specific agents blocks local angiogenesis in the developing chorioallantoic membrane of the chicken [Moses et al., 1990] and suppresses the formation of invasive, tubular sprouts by human endothelial cells cultured on gels of type I collagen in vitro [Montesano and Orci, 1985; Fisher et al., 1994]. It has been proposed that proteolysis of the vascular basement membrane and the interstitial ECM in the vicinity of sprouts lessens the mechanical restraint imposed by the ECM upon the migration of endothelial cells in vivo. In accordance with this hypothesis, we observe that 1,10-phenanthroline (an inhibitor of metalloproteinases) suppresses the traction-mediated contraction of collagen gels by endothelial cells in vitro at concentrations similar to that (5.2×10^{-5} M) which inhibits angiogenesis-like invasion of collagen gels by endothelial cells in vitro [Montesano and Orci, 1985]. We found that supernates from BAE cells cultured within collagen gels contained a significant amount of metalloproteinase MMP-2. RNA that corresponds to MMP-2 is found within endothelial cells of healing dermal wounds in vivo [Salo et al., 1994]. Moreover, MMP-2 is secreted by endothelial cells cultured in vitro on a plastic substrate [Zucker et al., 1992], on basement membrane matrix [Schnaper et al., 1993], or on gelled type I collagen [Fisher et al., 1994]. We observe that BAE cells secrete similar levels of MMP-2 in uncontracted collagen gels versus gels contracted in the presence of BSA; therefore, MMP-2 is not a primary inductor of the contractile response to BSA. MMP-2 might act permissively through a general enhancement of collagen malleability. However, MMP-2 cleaves native fibrillar collagens poorly in comparison to denatured collagens (the relative efficiency is less than 2% at 37°C) [Seltzer et al., 1981]; therefore, it is unlikely that this enzyme alone can significantly alter the structure of a collagen gel. MMP-2 might, however, further the degradation of collagen cleaved by other collagenases. Interstitial collagenase, which cleaves specifically at a single site within native collagen types I, II, and III, is secreted by human vascular endothelial cells that are grown on top of gelled type I collagen in the presence of phorbol esters [Fisher et al., 1994]. In this culture, inhibition of interstitial collagenase inhibits the angiogenesis-like invasion of the collagen by the cells. If traction-mediated reorganization of collagen is involved in endothelial invasion, the process is likely to be facilitated by interstitial collagenase, the production of which is stimulated in endothelial cells by angiogenic factors such as PMA [Fisher et al., 1994] and bFGF [Presta et al., 1986; Mignatti et al., 1989].

The degree to which ECM must be degraded to enhance angiogenesis in vivo is unclear; however, it appears that the range of optimal proteolysis has an upper limit. For example, the increased production of endogenous proteases by murine endothelial cells that express the polyoma virus middle T oncogene is correlated with their organization into large, hollow cysts within fibrin clots in vitro that resemble the cavernous hemangiomas in vivo from which the cells were isolated [Montesano et al., 1990]. In the presence of inhibitors of serine proteases, however, the cyst morphotype is replaced by branched tubes that are similar to the structures made by normal murine endothelial cells in vitro [Montesano et al., 1990]. The fact that proteolysis of ECM can inhibit the formation of endothelial tubes indicates that angiogenic sprouts are not elongated by forces derived solely from the parent vessel (e.g., by a hydraulic push): rather, the sprouts must pull themselves outward by a process (e.g., traction) that requires mechanical support from the ECM. Accordingly, "excessive" proteolytic cleavage of fibrous or crosslinking components of the ECM would disrupt the mechanical continuity of the matrix to an extent that the endothelial cells would lack a fixed substrate against which they could pull. Indeed, a disruption of the structure of type I collagen does affect its ability to serve as an anchor for traction. Fibroblasts [Guidry and Grinnell, 1987] and endothelial cells (Vernon and Sage, unpublished observations) embedded in gels that are comprised of short, poorly inter-

connected fibers of type I collagen (a consequence of the addition of heparin to the collagen) pull on and reorganize adjacent collagen fibers; however, the traction is not propagated to the fibers throughout the remainder of the gel and, therefore, contraction of the gel as a whole is inhibited.

In conclusion, there is mounting evidence that ECM participates actively in the regulation of vascular development. We believe that further investigations into the ways in which endothelial cells interact with ECM in vitro and in vivo will improve our understanding of the mechanisms that mediate angiogenic invasion and morphogenesis and might ultimately give rise to novel strategies to control neovascularization in disease.

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