Mechanochemical Switching between Growth and Differentiation during Fibroblast Growth Factor-stimulated Angiogenesis In Vitro: Role of Extracellular Matrix

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Abstract. The angiogenic factor, basic fibroblast growth factor (FGF), either stimulates endothelial cell growth or promotes capillary differentiation depending upon the microenvironment in which it acts. Analysis of various in vitro models of spontaneous angiogenesis, in combination with time-lapse cinematography, demonstrated that capillary tube formation was greatly facilitated by promoting multicellular retraction and cell elevation above the surface of the rigid culture dish or by culturing endothelial cells on malleable extracellular matrix (ECM) substrata. These observations suggested to us that mechanical (i.e., tension-dependent) interactions between endothelial cells and ECM may serve to regulate capillary development. To test this hypothesis, FGF-stimulated endothelial cells were grown in chemically defined medium on bacteriological (nonadhesive) dishes that were precoated with different densities of fibronectin. Extensive cell spreading and growth were promoted by fibronectin coating densities that were highly adhesive (>500 ng/cm²), whereas cell rounding, detachment, and loss of viability were observed on dishes coated with low fibronectin concentrations (<100 ng/cm²). Intermediate fibronectin coating densities (100–500 ng/cm²) promoted cell extension, but they could not completely resist cell tractional forces. Partial retraction of multicellular aggregates resulted in cell shortening, cessation of growth, and formation of branching tubular networks within 24–48 h. Multicellular retraction and subsequent tube formation also could be elicited on highly adhesive dishes by overcoming the mechanical resistance of the substratum using higher cell plating numbers. Dishes coated with varying concentrations of type IV collagen or gelatin produced similar results. These results suggest that ECM components may act locally to regulate the growth and pattern-regulating actions of soluble FGF based upon their ability to resist cell-generated mechanical loads. Thus, we propose that FGF-stimulated endothelial cells may be “switched” between growth, differentiation, and involution modes during angiogenesis by altering the adhesivity or mechanical integrity of their ECM.

A central question in developmental biology concerns how groups of interacting cells and molecules give rise to three-dimensional tissues that exhibit specialized form as well as function. We are interested in the process by which endothelial cell growth and capillary tube formation are controlled during angiogenesis. Capillary development is an excellent system for study of histodifferentiation because cloned endothelial cells retain the ability to form branching tubular networks; i.e., to undergo “angiogenesis in vitro” (Folkman and Haudenschild, 1980). Morphogenesis of the embryonic vasculature involves two modes of vessel formation: (a) accumulation of endothelial cells into networks composed of loosely associated cellular cords that eventually form into tubes; or (b) neovascularization by sprouting from these early vessel rudiments (Coffin and Poole, 1988). In vitro angiogenesis systems best model the former.

In vivo studies clearly demonstrate that neovascularization can be initiated by soluble endothelial mitogens such as basic fibroblast growth factor (FGF; Shing et al., 1985; Esch et al., 1985). However, the regulatory signals that determine whether capillary endothelial cells will grow, branch, differentiate, or involute in response to FGF appear to be provided by the local tissue microenvironment. For example, during initiation of the first capillary branches, one endothelial cell grows in response to mitogenic stimulation while its neighbors, only microns away, do not (Auprunk and Folkman, 1977). Furthermore, during later stages of neovascularization, rapidly growing capillary sprouts appear juxtaposed to regressing capillaries as well as differentiating tubes that
have become quiescent (Clark and Clark, 1938). FGF similarly retains its multifunctionality in vitro: FGF both stimulates endothelial cell growth (Shing et al., 1985; Esch et al., 1985) and promotes formation of differentiated capillary tubes (Montesano et al., 1986).

The local regulatory signals that modulate FGF action and control capillary development may be conveyed by extracellular matrix (ECM) molecules. Localization of alterations in ECM composition and integrity parallel changes of vascular form during capillary initiation, elongation, differentiation, and involution (Auspunck and Folkman, 1977; Folkman, 1982; Sariola et al., 1984; Ingber et al., 1986; Form et al., 1986). Purified matrix components also modulate the effects of angiogenic factors on endothelial cell growth (Schor et al., 1979; Ingber et al., 1987) and capillary differentiation in vitro (Maciag et al., 1982; Madri and Williams, 1983; Schor et al., 1983; Montesano et al., 1986). Yet, little is known about the mechanism by which insoluble ECM molecules transmit regulatory information to endothelial cells.

Matrix proteins alter cell behavior as a result of specific binding interactions with distinct types of cell surface ECM receptors, such as the integrin family of receptors (Hyneas, 1987; Ruoslahti and Pierschbacher, 1987). However, ECM molecules cannot transmit growth and pattern-regulating signals based solely upon occupancy of cell surface ECM receptors since the biological effects of matrix components vary greatly depending upon their structural configuration. For example, a variety of cells proliferate on rigid, collagen-coated dishes (Wicha et al., 1979; Madri and Williams, 1983; Ben Ze'ev et al., 1988), but differentiate when cultured on or within malleable collagen gels (Emerman and Pitekka, 1977; Schor et al., 1983; Montesano et al., 1983; Ben Ze'ev et al., 1988). The differentiation-inducing effects of collagen gels and complex ECM substrata (e.g., laminin gels, matrigel) also can be varied by altering their mechanical integrity (Lee et al., 1984; Li et al., 1987). Cell shape is determined through the action of tensile forces that are generated within the intracellular cytoskeleton and resisted by ECM attachment points (Harris et al., 1980; Ingber and Jamieson, 1985). Thus, one of the major effects of altering ECM structural integrity is induction of cell shape changes; rigid dishes support cell extension whereas malleable substrata promote rounding (Emerman and Pitekka, 1977; Ingber and Jamieson, 1985). Endothelial cells also take on different forms on rigid dishes depending upon the type of ECM molecules used for cell attachment (Ingber et al., 1987).

ECM molecules may modulate cell growth and differentiation in response to soluble factors based upon their ability to alter cell shape. Anchorage-dependent cells, such as endothelial cells, proliferate more rapidly in serum-containing medium as they become more flattened and cease growing as they take on increasingly rounded forms (Folkman and Moscona, 1978; Gospodarowicz et al., 1978). Similarly, the growth-promoting effects of different ECM molecules increase in parallel with their relative ability to support capillary cell extension in serum-free medium supplemented with FGF (Ingber et al., 1987). Matrix-dependent changes of cell shape that inhibit growth may also promote differentiation. For example, hepatocytes and mammary epithelial cells cease growing and increase their expression of differentiation-specific genes when cultured on substrata that promote cell rounding (Lee et al., 1984; Li et al., 1987; Ben Ze'ev et al., 1988). The differentiated phenotype of chondrocytes (Glowacki et al., 1983), adipocyte precursors (Spiegelman and Ginty, 1983), and pheochromocytoma cells (Bethea and Kozak, 1984) can be similarly altered by modulating substrate adhesivity and controlling cell form.

In this article, we explore the possibility that the ability of FGF to stimulate endothelial cell growth in one microenvironment and promote capillary differentiation in another depends upon the mechanical context in which it acts. We focus on the role of ECM and tension-dependent changes of endothelial cell shape during regulation of FGF-stimulated angiogenesis in vitro.

Materials and Methods

In Vitro Culture Systems for Study of Angiogenesis

Capillary endothelial cells were isolated from bovine adrenal cortex or human foreskin, cloned, and passaged as previously described (Folkman et al., 1979). Capillary endothelial cells from both species produce tubular networks of similar size and shape when cultured under similar conditions (Folkman and Haudenschild, 1980). In our time-lapse cinematographic studies, spontaneous formation of capillary tubes was promoted by refedding human capillary cells every other day with DME (Gibco Laboratories, Grand Island, NY) supplemented with 15% human serum, endothelial cell growth supplement (5.4 mg/ml; Collaborative Research Incorporated, Bedford, MA), and tumor cell-conditioned medium (Folkman et al., 1979) mixed 1:1 with conditioned medium obtained from confluent cultures of bovine aortic endothelial cells. Tubes formed within ~1 h after refeding on gelatinized dishes.

In our other spontaneous angiogenesis models, bovine capillary endothelial cells were cultured in complete medium comprised of DME supplemented with 10% calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin, and 5 µl/ml retinal extract (Gillman, 1981). Similar results were also obtained using tumor-conditioned medium (Folkman et al., 1979) in place of retinal extract as a source of endothelial mitogens. In one set of experiments, cultures were refed every 3 d until the cell monolayers spontaneously detached from the gelatinized surfaces of 6 well culture plates (Costar, Cambridge, MA). We found that different lots of gelatin had a marked effect on their ability to support monolayer retraction; best results were obtained with gelatin from Difco Laboratories, Inc., Detroit, MI (lot No. 748797). In our second set of experiments, capillary tube formation was induced by plating bovine capillary endothelial cells (2.5 x 10^5 cells/well) in complete medium within small (1.3-mm-diam) wells of microtiter plates (Terasaki Plate, Nunc, Naperville, IL). In a third set of experiments, type I collagen-coated microcarrier beads (160 µm diameter; Cyodex-3; Pharma- stics Fine Chemicals, Piscataway, NJ) were added to the upper surface of confluent endothelial cell monolayers (7.5 mg/35-mm dish). Complete medium was gently removed and replaced every 3 d.

To develop a controllable angiogenesis model, we used a previously described method for adsorption of ECM proteins to bacteriological plastic dishes that is reliable and highly efficient (Madri and Williams, 1983; Ingber et al., 1987). Fibroserin (Cappel Laboratories, Malvern, PA) and type IV collagen (Calbiochem-Behring Corp., San Diego, CA) were diluted in 0.1 M carbonate buffer, pH 9.4, at different concentrations, plated at 2 ml/35-mm dish (No. 1008 plates; Falcon Labware, Oxnard, CA), and allowed to incubate overnight at 4°C. Coated dishes were washed with DME containing 1% BSA (fraction V; Armour Pharmaceutical Co., Tarrytown, NY) before use.

Bovine capillary endothelial cells were obtained from confluent endothelial monolayers that had not been refed (i.e., exposed to new endothelial mitogens) for at least 2 d before harvesting. Quiescent monolayers were dissociated into single cells by brief exposure (1-2 min) to trypsin-EDTA (Gibco Laboratories), transferred to DME containing 1% BSA, pelleted by centrifugation, and washed repeatedly in serum-containing DME. Cell numbers were measured using a counter (Coulter Electronics Inc., Hialeah, FL), aliquots were pelleted, and cells were resuspended in defined medium consisting of DME supplemented with 5 µg/ml transferrin (Col-
Morphological Techniques

Phase-contrast images of living cells were recorded using an inverted microscope (Diaphot; Nikon Inc., Garden City, NY) with film (Plus-X Pan; Eastman Kodak Co., Rochester, NY). Time-lapse, phase-contrast recording of human capillary endothelial cells (1 min = 13 h) was carried out for a period of 4 d beginning after the third week of primary culture using a 16-mm camera (Bolex, Yverdon, Switzerland) in conjunction with a photomicroscope (No. II; Carl Zeiss, Inc., Thornwood, NY). Black and white negatives were prepared from single 16-mm film frames by Spectrum Color Laboratory (Boston, MA). For electron microscopic analysis, reorganized capillary tubes were fixed in 2.5% glutaraldehyde/2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, postfixed in 1% osmium tetroxide, and studied under an electron microscope (No. 100B; JEOL USA). Small Kodak Co., Rochester, NY). Time-lapse, phase-contrast recording of endothelial cells with FGF-containing medium resulted in spontaneous retraction and detachment of the cell layer from the underlying gelatinized substratum. The high degree of resting tension inherent in the "quiescent" monolayer was made evident by its rapid contraction upon detachment to ~15% of its original area. This effect was very reproducible; usually between 4-6 wells of a 6-well plate would spontaneously retract within the same 24 h period, ~1-2 wk after confluence. Retracted monolayers often remained attached to the plastic dish at selected sites and formed large multicellular aggregates (Fig. 2 a). These retracted cells consistently reorganized into tubular networks within 4-9 d (Fig. 2 b). Capillary organization appeared to be suppressed by culture conditions that inhibited cell retraction since tubes were never observed within regions of the original monolayer that remained in contact with the rigid dish, even though cultured for the identical time in the same medium.

We carried out a simple experiment to ask whether induction of tube formation was related to the ability of capillary cells to physically release themselves from the surface of the plastic dish. Capillary endothelial cells were plated into small wells of microtiter plates that contained beveled rather than perpendicular side walls. Under these conditions, endothelial cells at the periphery of the monolayer migrated up along the sidewalls, while maintaining lateral contacts with neighboring cells. Portions of the endothelial cell monolayer soon became elevated above the surface of the culture substratum, apparently as a result of cell tractional forces. Elevation of the endothelial cell layer and subsequent multicellular retraction once again promoted tube formation within 3-6 d after reaching confluency (Fig. 2 c).

Capillary tube formation was also induced by adding collagen-coated microcarrier beads to the upper surfaces of confluent endothelial cell monolayers. Again, cell elevation and retraction appeared to be central to the organization process. Light microscopic analysis of the same cultures at different times after addition of beads revealed that endothelial cells initially migrated up from the confluent monolayer and became adherent to the overlying microcarrier beads within a period of 1-3 d. Once elevated, the cells on beads formed long processes that stretched through the culture medium to contact the apical surfaces of cells within the underlying monolayer as well as cells on other beads (Fig. 3 a). Over the next few days, long sprouts appeared that were completely elevated above the surface of the monolayer and surrounded by culture medium. These solid cellular cords were composed of capillary endothelial cells aligned in tandem and suspended between adjacent beads that were often separated by multiple cell diameters; i.e., distances >500 μm (Fig. 3 b). Alignment of cells in this oriented fashion apparently facilitated multicellular contraction and lat-
Figure 1. Time-lapse cinematographic analysis of the early stages of spontaneous angiogenesis in vitro. This series of phase-contrast views shows the elastic nature of the filamentous web. (a) Time 0. Tips of arrows abut on adhesive tendrils that have accumulated on top of cultured human capillary endothelial cells after $\sim$3 wk in culture. The tendrils have joined and appear as a web in the form of an inverted letter "Y." (b) 4.2 h. The filaments were retracted to the right resulting in straightening of the left portion of the web and commensurate contortion of the right. (c, d, and e) 7.4, 7.8, and 8.2 h. Small arrow indicates a spread cell on the culture dish that became adherent to the fibrillar web as it underwent mitosis. The web also continued to undergo elastic transformations. (e) Double small arrows indicate two daughter cells that were produced by the cell division; one remained associated with the filament while the other eventually migrated onto the plastic dish. (f, g, h, and i) 31.6, 35.8, 36.8, and 38.4 h. Tip of small arrow abuts on the nucleus of a spread cell that migrated from right to left directly beneath a suspended filament. The web also became highly contorted during this period. (i) Large arrows indicate a region along one filament that temporarily became free of associated cells. (j) 77.4 h. The web extended once again and took on a more linear form. Associated cells appeared to form a continuous luminal space in the form of an inverted "Y" at the center of the web. Scale unit, 10 $\mu$m.
eral movement of the microcarrier beads since the beads became grouped within tight clusters over the next few days. Examination of these clusters of beads revealed that they were interlinked by a dense network of branching cellular cords (Fig. 3 c). This network subsequently remodeled into a highly developed system of branching capillary tubes containing well-defined lumina (Fig. 3 d). The total time required for tube formation was \( \approx 1-2 \) wk after addition of beads, and, once again, hollow tubular networks only appeared after multicellular retraction and bead aggregation had occurred.

In fact, when we reviewed the literature on angiogenesis in vitro, we found that capillary tubes formed on tissue culture plastic (\( \pm \) ECM coating) only under conditions that permitted endothelial cell retraction, elevation of multicellular cords, and thus partial release from contact with the rigid culture dish (Table I). Certain matrix molecules (e.g., laminin) did not support capillary differentiation when adsorbed to tissue culture dish; yet they induced rapid tube formation when presented as a three-dimensional gel (Kubota et al., 1988). In general, we noticed that the rate of capillary tube formation was accelerated by culture on malleable substrata, regardless of the type of ECM molecule utilized. For example, while capillary organization took place over a period of weeks on collagen-coated plastic dishes (Folkman and Haudenschild, 1980; Madri, 1982), tubes formed within days in native collagen gels (Schor et al., 1983; Montesano et al., 1983).

![Figure 2](image-url)

**Figure 2.** Capillary tube formation triggered by endothelial cell retraction. (a) Phase-contrast view of a region of a retracted cell monolayer showing a large multicellular aggregate that spontaneously reorganized (magnification of 50). (b) Higher magnification view of the same cellular aggregate. Only endothelial cells that became elevated on top of the adherent cell monolayer reorganized and formed capillary networks (magnification of 100). (c) Cells at the periphery of the endothelial cell monolayer migrated up along the beveled side wall of the microtiter well. This resulted in elevation of the cell layer, retraction of multicellular cords, and formation of capillary tubes. The tubes that became elevated were in a focal plane different from that of the few cells that remained adherent to the culture dish surface; thus, the adherent cells appear out of focus (magnification of 60).

In summary, results from a variety of model systems suggested that adhesion to a highly adhesive, rigid substratum served to inhibit capillary tube formation whereas attachment to a substratum that permitted multicellular retraction appeared to be stimulatory. However, the complexity of these experimental systems made analysis of the molecular and biophysical determinants of capillary tube formation very difficult, if not impossible. For example, it was not possible to determine whether capillary cells were induced to form tubular networks as a result of being suspended above the apical surfaces of a confluent cell monolayer, surrounded by culture medium, allowed to form three-dimensional cell aggregates, or whether tube formation was triggered by mechanical alterations (e.g., multicellular retraction and cell shortening). Thus, we set out to devise a more well-defined system for study of the role of cell tension and ECM-dependent changes of cell shape during angiogenesis in vitro.

Capillary endothelial cells were plated at moderate densities (2-5 \( \times 10^4 \) cells/cm\(^2\)) on bacteriological plastic dishes of varying adhesivity to control cell extension. Adhesivity was controlled by precoating the dishes with different amounts of a purified ECM molecule, fibronectin. These studies were carried out in chemically defined medium containing saturating amounts of recombinant FGF, a potent endothelial mitogen. Serum had to be excluded from the culture medium because it contains attachment factors, such as fibronectin and vitronectin, as well as undefined amounts of other growth factors. Capillary endothelial cells can not attach to bacteriological dishes in the absence of adsorbed ECM proteins or serum. Thus, any attachment and spreading that we observed were initiated through specific cell-ECM interactions.

Using this experimental system, capillary cells could be artificially induced either to spread, remain round, or form capillary tubes in the presence of saturating amounts of FGF simply by varying fibronectin molecular coating densities (Fig. 4). Endothelial cells avidly attached and spread on dishes coated with high densities of fibronectin (>500 ng/cm\(^2\)), but no tubes were observed. When cells were plated...
Figure 3. Induction of capillary tube formation by addition of microcarrier beads to an endothelial cell monolayer. (a) Day 4. Endothelial cells that have migrated onto the surfaces of overlying microcarrier beads extend cell processes that contact the surface of the monolayer below as well as other beads. (b) Day 6. Long multicellular cords appear that are suspended between neighboring beads and stretch for many cell diameters. (c) Day 10. Aggregates of closely apposed microcarrier beads form that are interlinked by a continuous network of cellular cords. (d) Day 12. Remodeling of the cellular cords has resulted in formation of branching capillaries containing well-developed lumina (magnification of 115).

Tube formation was the result of a dynamic remodeling process. During the first 8 h of culture on moderately adhesive dishes, attached endothelial cells extended cell processes, formed cell–cell contacts, and established a branched network comprised of elongated, bipolar cells. Network formation was followed by multicellular retraction over the next 16 h of culture. This mechanical shift resulted in formation of free-floating cellular cords that were attached to the culture dish at intermittent points through contacts with adjoining multicellular aggregates. When viewed at high magnification after 48 h of culture, these multicellular cords appeared as tubes that contained a central lumen along their length (Fig. 5 a). This was confirmed by EM, which revealed groups of endothelial cells that were joined by interdigitated cell processes and enclosed a central luminal space (Fig. 5 b). Residual fibrillar material appeared in many lumina that was similar to the contents found within tubes formed in other in vitro angiogenesis models (Folkman and Haudenschild, 1980; Maciag et al., 1982; Feder et al., 1983). Occasional tubes also exhibited wisps of electron-dense material at similar densities on dishes containing less than 100 ng fibronectin/cm², the cells attached but remained round. Once multicellular aggregates formed, they spontaneously retracted and completely detached themselves from these poorly adhesive dishes. In contrast, extensive branching capillary networks consistently formed within 24–48 h when capillary endothelial cells were plated on dishes of intermediate adhesivity (100–500 ng fibronectin/cm²).
### Table I. Chemical and Mechanical Requirements for Spontaneous Angiogenesis In Vitro

<table>
<thead>
<tr>
<th>Cell</th>
<th>Time</th>
<th>ECM</th>
<th>Rigid</th>
<th>Elevation</th>
<th>Reference</th>
</tr>
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<tr>
<td>HUVE</td>
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<td>FN</td>
<td>+</td>
<td>+</td>
<td>Maciag et al., 1982</td>
</tr>
<tr>
<td>HUVE</td>
<td>4-6 wk</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Maciag et al., 1982</td>
</tr>
<tr>
<td>BCE, HCE</td>
<td>3-6 wk</td>
<td>GEL</td>
<td>+</td>
<td>+</td>
<td>Folkman and Haudenschild, 1980</td>
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<tr>
<td>RCE</td>
<td>3-4 wk</td>
<td>Strroma</td>
<td>-</td>
<td>-</td>
<td>Madri and Williams, 1983</td>
</tr>
<tr>
<td>RCE</td>
<td>2-4 wk</td>
<td>I, III</td>
<td>+</td>
<td>+</td>
<td>Madri, 1982</td>
</tr>
<tr>
<td>RAEx</td>
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<td>Pl-Clot</td>
<td>-</td>
<td>-</td>
<td>Nicosia et al., 1982</td>
</tr>
<tr>
<td>HUVE</td>
<td>1-2 wk</td>
<td>P-FN</td>
<td>+</td>
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<td>Maciag et al., 1982</td>
</tr>
<tr>
<td>BAE</td>
<td>1-2 wk</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>Feder et al., 1983</td>
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<tr>
<td>RCE</td>
<td>4 d</td>
<td>IV, V</td>
<td>+</td>
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<td>Madri and Williams, 1983</td>
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<tr>
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<td>BM</td>
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<td>I-Gel</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>I-Gel</td>
<td>-</td>
<td>-</td>
<td>Montesano et al., 1983</td>
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<tr>
<td>HUVE, HCE</td>
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<td>LM-Gel</td>
<td>-</td>
<td>-</td>
<td>Kubota et al., 1988</td>
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<tr>
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<td>1-2 d</td>
<td>FN, IV, Gel</td>
<td>+</td>
<td>+</td>
<td>The present study</td>
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The cell type, time for tubes to form, and type of matrix coating used are presented for each study cited. Endothelial cells were derived from human umbilical vein (HUVE), bovine aorta (BAE), rat aortic explants (RAEx), or capillaries isolated from bovine (BCE), human (HCE), or rat (RCE) tissues. Rigid plastic dishes were used either alone (−) or coated with fibronectin (FN), proteolyzed fibronectin (P-FN), gelatin (GEL), type I collagen (I), type III collagen (III), type IV collagen (IV), or type V collagen (V). Pl-Clot, clotted chick plasma; I-Gel, native type I collagen gel; Strroma, amniotic stroma; BM, intact amniotic basement membrane; LM-Gel, laminin gel. Substrata were viewed as rigid (+) if their form could not be altered by adherent cells. Malleable substrata were rated as partially flexible (−/+); if they were held in place by inflexible holders during experiments. Capillary tubes were viewed as elevated (+) if they were separated from their substratum by 100 nm or more. In one study, tubes formed both in direct contact and suspended above the substratum (−/+).

*Figure 4.* Matrix-dependent control of angiogenesis in defined medium containing FGF. Bacteriological dishes were precoated with 10, 50, 100, or 2500 ng/cm² (from left to right) of fibronectin (FN) or type IV collagen (Type IV). The highest concentration shown was saturating for both cell attachment and spreading. These phase-contrast views show that cell attachment and spreading increased in parallel with the number of matrix molecules available for cell attachment. Tube formation was only observed on dishes of intermediate adhesivity when cells were plated at a moderate density (4 × 10⁴ cells/cm²). Endothelial cells formed extensive capillary networks on the highest FN coating density when higher numbers were plated (2 × 10⁵ cells/cm²; top right). Tube formation was observed on lower coating densities on type IV than on FN; type IV promoted more extensive cell attachment and spreading at all coating concentrations (magnification of 50).
Figure 5. Formation of tubular networks in defined medium supplemented with FGF. (a) Phase-contrast view of a network of capillary tubes that formed on a moderately adhesive fibronectin-coated dish (48 h). The tubes became elevated above the surface of the culture dish as they stretched from one multicellular cell aggregate to another. Tips of arrows abut on a central lumen that appeared to stretch the entire length of a suspended capillary tube (magnification of 150). (b) Electron microscopic view demonstrating the presence of a central lumenal space within a reorganized capillary tube. Small amounts of residual amorphous electron-dense material can be seen within the capillary lumen (magnification of 8,250).

along their periphery that were reminiscent of the discontinuous basal lamina seen surrounding forming capillaries in vivo (Ausprunk et al., 1981).

It is difficult to quantitate the amount or frequency of a pattern-generating event, such as angiogenesis. However, in our experiments, tubular networks appeared to be evenly distributed across more than 50% of the surface of dishes coated with the permissive fibronectin coating density. Nevertheless, small regions containing moderately spread cells as well as fully retracted cell aggregates were also present. Similar results were obtained in five different experiments with only minor variation of the permissive fibronectin coating concentration; tubes always formed on either 100, 250, or 500 ng/cm² even though different lots of commercial fibronectin were used. Furthermore, between 75-100% of dishes coated with the permissive fibronectin density induced tube formation within any individual experiment. Cell spreading increased in a concentration-dependent fashion as the fibronectin coating density was raised above 500 ng/cm²; however, tube formation was never observed at any of these concentrations.

Importantly, we could overcome the suppression of capillary differentiation imposed by highly adhesive dishes by increasing endothelial cell plating numbers (Fig. 4). As in less dense cultures, endothelial cells attached and spread extensively on the highly adhesive dishes (2,500 ng FN/cm²) during the first 6 h after plating. Efficient cell adhesion resulted in formation of a confluent endothelial cell layer in direct contact with the dish as well as groups of overlying cells that only contacted and exerted tractional forces on other cells. However, spontaneous retraction of dense multicellular cords at later times (6-24 h) resulted in formation of extensive tubular arrays that became elevated within the culture medium. These capillary nets were grossly visible as networks comprised of fine, white threads and often covered the entire surface of a 35-mm dish (>10 cm²).

These effects on capillary differentiation were not limited to fibronectin-coated dishes. Similar control of tube formation was obtained using plates coated with varying amounts of type IV collagen (Fig. 4) or gelatin (not shown). However, tube formation was induced by lower matrix coating concentrations on type IV collagen than on fibronectin-coated dishes (25-75 ng/cm² versus 100-500 ng/cm²). Type IV collagen, which is more adhesive for bovine adrenal capillary endothelial cells than fibronectin (Ingber et al., 1987), promoted more extensive cell spreading at all coating densities. Capillary tube formation also was induced by a variety of ECM molecules in past studies, however, the rate of capillary tube formation that we obtained using this method (1-2 d) was among the most rapid observed to date (Table I).

ECM-coated substrata that switched on differentiation in FGF-containing medium also turned off cell growth (Fig. 6). When we carried out thymidine autoradiography, we found that the ability of endothelial cells to grow in response to
stimulation by FGF, a potent endothelial mitogen, was suppressed in cells that had become organized within differentiating capillary tubes. In contrast, nearby cells that remained adherent and spread on the rigid fibronectin-coated dish continued to synthesize DNA. Quiescent cells within newly formed tubes were much smaller than their actively growing neighbors. Capillary endothelial cell spreading and growth also increased in parallel as fibronectin coating den-
Concentrations of fibronectin did not grow and lost viability over a period of days; these cells failed to attach when replated on highly adhesive dishes.

Discussion

Mechanochemical Interactions during Spontaneous Capillary Tube Formation

Capillary tube formation can be induced in vitro using a variety of different methods, many of which accelerate capillary organization by culturing endothelial cells on purified ECM molecules (Table I). However, the mechanism by which ECM components switch growing capillary cells into a differentiation mode remains unknown. This is an especially difficult problem considering that capillary differentiation can occur in the presence of a soluble angiogenic mitogen; FGF has been previously shown to promote angiogenesis in vitro (Montesano et al., 1986).

Our studies revealed that capillary tube formation resulted from a complex series of mechanochemical interactions that required formation of multiple cell–matrix and cell–cell contacts. Capillary endothelial cells initiated tube formation in longer term cultures on rigid substrata by accumulating adhesive matrix tendrils and then applying tension to their attachment points. This mechanical interaction resulted in multicellular retraction, elevation of the matrix web into the culture medium, and production of isolated cords of shortened endothelial cells surrounding central matrix filaments. Formation of new cell–matrix contacts was limited to potential adhesive sites on the suspended web because cells were no longer in contact with the rigid planar dish. Thus, capillary endothelial cells elongated with the long axis of the web and became oriented in tandem. Cell alignment within forming tubes most likely involves specific interactions between elements of the cytoskeletal contractile apparatus and distinct membrane-associated molecules that mediate cell–cell mechanochemical coupling (e.g., junctional proteins, cell adhesion molecules (Pitelka and Taggart, 1983; Edelman, 1984).

Time-lapse studies demonstrated that the filamentous ECM web was a dynamic structure; it served as a major conduit for cell traffic and underwent elastic transitions of form. In this manner, the filament network functioned as a three-dimensional template that assured maintenance of pattern integrity. The web also served as a true construction scaffold in that it was removed once the tubular endothelium became organized. We have previously shown that dissolution of these fibrillar materials results in formation of a hollow capillary containing a central lumen (Folkman and Haudenschild, 1980). Tubes formed by cultured endothelial cells from bovine aorta and human umbilical vein also contain ECM materials in their lumina that appear to progressively dissolve (Maciag et al., 1982; Feder et al., 1983). Thus, these findings in conjunction with results of analysis in vivo (Auspink et al., 1981) confirm that accumulation of a continuous lamina densa along the capillary periphery (as detected by EM) is not required for organization of tubular networks. Rather, intact basal lamina may serve to formalize or add structural stability once the hollow capillary is formed.

A Mechanochemical Switching System

Capillary endothelial cells appear to walk a fine line of structural stability with extensive cell spreading and growth at one extreme and complete loss of anchorage resulting in rounding and cell death (i.e., “involution”) at the other. Endothelial cell elongation required a sufficient number of attachment points and an underlying attachment foundation that could physically resist cell-generated tensile forces. Capillary cells experienced a “resting tension” when present within an adherent confluent monolayer and spontaneously retracted the elastic ECM web. Endothelial cells also pulled themselves off poorly adhesive dishes although substrata that were highly adhesive and rigid promoted extensive cell spreading. Capillary endothelial cells must be able to spread to grow in response to stimulation by FGF (Ingber et al., 1987) and rapidly lose viability when completely detached (Folkman and Moscona, 1978).

Cell-generated tensile forces also produced mechanical changes in larger endothelial cell aggregates that appeared to trigger capillary organization. Capillary tube formation was greatly facilitated in growth factor-containing medium by culturing capillary cells on dishes with beveled sidewalls, adding microcarrier beads to the upper surfaces of endothelial monolayers, and continuously refeeding endothelial monolayers until they spontaneously detached. In each of these systems, tube formation was tightly coupled to multicellular retraction. Furthermore, we were able to trigger artificially both retraction and tube formation in a parallel fashion using our new model of in vitro angiogenesis; i.e., solely by modulating ECM coating densities. Angiogenesis was only observed on low to moderate adhesive FN coating concentrations that permitted cell retraction and partial release from contact with the rigid dish. Key to the capillary organization process was the ability of endothelial cells to arrange themselves in tandem along their own malleable adhesive scaffolding in vitro as they do in vivo (Folkman, 1982; Coffin and Poole, 1988). This apparently resulted in amplification of tension and multicellular retraction in a coordinated fashion in a manner analogous to that observed during muscle contraction.

Multicellular retraction might trigger capillary differentiation by a variety of different mechanisms. Retraction results in increased contacts between neighboring cells, decreased adhesion to the rigid substratum, cell rounding, as well as a reduction of tensile stresses. Any one of these alterations could serve to induce tubular reorganization. For example, cell–cell interactions are known to play a central role during tissue development. However, it is important to point out that endothelial cell densities and thus the extent of cell–cell contact varied from one model system to the other. Tubes also did not form in serum-containing medium if confluent endothelial monolayers remained adherent or in defined medium when cells were cultured on highly adhesive fibronectin coatings that strongly resisted cell tractional forces. Yet, a high degree of cell–cell contact formation occurred in both of these systems. The mechanical resistance of the substratum therefore apparently could overcome signals derived from cell–cell contact formation that might, in part, promote capillary differentiation. Furthermore, plating endothelial cells in high numbers on low ECM coating densities resulted in formation of large, fully-retracted cellular aggregates that exhibited multiple cell–cell contacts; however, these cell
clusters did not display tubular organization or lumina formation. Thus, cell–cell contact formation is not sufficient to trigger capillary organization, although increased cell–cell interactions clearly facilitate this process.

One property that was common to all of the systems that induced tube formation was suspension of multicellular cords within the culture medium with commensurate loss of cell contact with the rigid planar dish. Suspension within liquid medium is clearly not a prerequisite for capillary differentiation since tubes form rapidly when endothelial cells are cultured within three-dimensional ECM gels (Montesano et al., 1983; Kubota et al., 1988). However, loss of contact with a rigid foundation may be a critical event. This possibility is supported by our observation that the inhibition of capillary tube formation imparted by high fibronectin coating densities could be suppressed by using higher cell plating numbers. This procedure resulted in formation of cell multilayers. In this manner, cell–cell mechanochemical coupling was facilitated and cell-generated contractile forces amplified, while the mechanical resistance of the adhesive substratum remained constant. These studies also demonstrate that a specific ECM coating "density" or degree of substratum adhesiveness is not the only trigger for tube formation.

Multicellular retraction and release from contact with the rigid substratum resulted in endothelial cell shortening. A decrease of cell extension alone is not sufficient to induce capillary differentiation; isolated cells do not form tubes, regardless of their size. Yet, changes of endothelial cell shape could produce intracellular metabolic alterations that might be required for capillary differentiation (i.e., if multiple similarly shaped endothelial cells were allowed to contact one another). Cell rounding promotes expression of the differentiated phenotype in a variety of cells (Emerman and Pitelka, 1977; Glowacki et al., 1983; Lee et al., 1984; Ben Ze'ev et al., 1988). However, endothelial cells do not form capillary tubes when completely round. Rather, they aggregate into disorganized clusters. The sensitivity of endothelial cells to cell shape perturbation, at least in terms of differentiation events, may be limited to a very narrow range of cell size or extension relative to other types of specialized cells.

In conclusion, capillary tube formation is not exclusively the result of either cell–cell interactions, culture on a conducive "type" of ECM component, maintenance of a defined "cell shape," or addition of a specific angiogenic molecule. Rather, capillary organization results from mechanochemical interplay between all of these factors.

**Regulation of FGF Multifunctionality: Local Control by ECM**

The importance of peptide growth factors in development is based upon their multifunctionality; the same soluble mitogen can stimulate growth in one microenvironment and promote differentiation in another. It has been suggested that the action of peptide growth factors depends upon the context of other chemicals or soluble regulatory molecules present (Sporn and Roberts, 1988). Data from the present study, in which constant amounts of soluble FGF were present under all culture conditions, suggest that growth factor action may also depend upon the mechanical context in which it acts. The stimulus for capillary differentiation was not a specific ECM component or a particular matrix coating density; tube formation was promoted by moderately adhesive substrata regardless of whether fibronectin, type IV collagen, or gelatin was used for attachment. Furthermore, endothelial cell binding to distinct "chemical addresses" in these ECM molecules clearly was not sufficient to trigger capillary differentiation since tubes did not form on dishes coated with high fibronectin concentrations when cells were plated at moderate cell densities. Rather matrix-coated substrata that induced capillary tube formation acted in a permissive fashion, they allowed FGF-stimulated cells to retract into more favored minimum energy configurations (i.e., tubular arrays).

ECM configurations that favored capillary differentiation also suppressed cell growth. Differentiating endothelial cells retracted, rounded, and ceased synthesizing DNA even though they were cultured in the presence of saturating amounts of soluble FGF. At the same time, extended cells that remained adherent only microns away continued to progress through the cell cycle. Capillary endothelial cells decrease their growth rates in an exponential fashion in direct relation to linear decreases in cell extension (Ingber et al., 1987). This growth inhibition occurs in the absence of any change of FGF receptor number or affinity; yet intracellular pH, a common chemical second messenger system used by many peptide mitogens, is greatly altered (Ingber et al., submitted for publication). Thus, the simultaneous switching between capillary growth and differentiation may result from tension-dependent alterations of endothelial cell shape that in turn influence intracellular signaling pathways and thereby alter cell responsiveness to soluble factors. Interestingly, malleable substrata that permit substratum retraction, promote cell shortening, and induce differentiation also often inhibit cell growth (Emerman and Pitelka, 1977; Li et al., 1987; Ben Ze'ev et al., 1988; for review, see Kleinman et al., 1987). Furthermore, Clark and Clark (1938) noted that growing sprouts became functional capillary tubes when the perivascular matrix "changed to a tissue substance resembling a soft gel" in a camera lucida study of physiological neovascularization published over fifty years ago.

These results suggest that one of the most important properties of complex ECM substrata (e.g., intact basal lamina, native collagen gels, laminin gels), at least in terms of controlling cell growth and differentiation, may be their inherent malleability. This hypothesis is in contrast to explanations that solely ascribe the differentiating properties of these matrices to distinct chemical constituents. Our model system allowed us to modulate multicellular retraction in a controlled fashion under defined medium and substrate conditions and thus it may also be useful for study of other cell types that can undergo differentiation in vitro.

**Theoretical Implications: Mechanical Forces as Biological Regulators**

How could mechanical perturbation and associated changes of cell shape alter endothelial cell responsiveness to soluble FGF? We and others have shown that a variety of nuclear functions including DNA and RNA synthesis and expression of differentiation-specific genes can be controlled by perturbing cell shape (Folkman and Moscona, 1978; Ben Ze'ev et al., 1980; Bissell et al., 1982; Lee et al., 1984; Ingber et al., 1987; Ben Ze'ev et al., 1988). However, the molecular mechanism by which tension-dependent changes of cell shape might convey regulatory information remains unclear.

To understand the biological significance of cell "shape,"
this phenomenological entity must be redefined in terms of chemical and physical determinants that can be studied at the molecular level. Actin-containing, contractile microfilaments are responsible for force transduction in cells (Korn, 1978), and thus they play a central role in cell shape determination. ECM molecules such as fibronectin appear to be structurally interconnected with microfilaments via a continuous series of noncovalent binding interactions involving actin-associated proteins (e.g., talin) and transmembrane integrin receptors (Hynes and Destree, 1978; Horowitz et al., 1986). Microfilaments interact with microtubules and intermediate filaments within the cytoskeleton and thus form an integrated system of structural organization (Bissell et al., 1982; Ingber and Jamieson, 1985; Ingber and Folkman, 1989).

Cell shape also represents a visual manifestation of underlying physical force distributions since cells spontaneously change their shape and take on new minimum energy forms when the adhesivity (i.e., mechanical integrity) of their attachment substratum is varied. This was demonstrated in the present and past studies (Harris et al., 1980; Emerman and Pitelka, 1977; Ingber and Jamieson, 1985) as well as with three-dimensional tension-dependent ("tensegrity") cell models that are built using sticks and string to represent microtubules and microfilaments, respectively (Ingber and Jamieson, 1985; Ingber and Folkman, 1989). Studies with the inorganic cell models suggest that if cytoskeletal filaments also form a continuum within living cells, then complementary force interactions between ECM, cell surface matrix receptors, contractile microfilaments, and microtubules could serve to control cytoskeletal filament assembly (Ingber and Jamieson, 1985; Joshi et al., 1985). This mechanochemical relation (i.e., dependence of cytoskeletal polymerization upon tension and compression) has a strong thermodynamic basis (Hill and Kirschner, 1982; Ingber and Jamieson, 1985; Buxbaum and Heidemann, 1988) as well as excellent supporting data from experiments with living cells (Bray, 1984; Joshi et al., 1985; Dennerll et al., 1988). Alterations of cytoskeletal polymerization may in turn affect cell metabolism in many ways. For example, transmembrane proteins, protein kinases, polyribosomes, mRNA, mitochondria, and glycolytic enzymes all appear to be physically associated with elements of the cytoskeletal lattice (Gall and Edelman, 1981; Wolosewick and Porter, 1979; Fulton et al., 1980; Cervera et al., 1981; Browne et al., 1982; Masters, 1984). Mechanical perturbation of the endothelial cell surface or cortical cytoskeleton may also have direct effects on the function of stretch-activated ion channels in the plasma membrane (Lansman et al., 1987). Interestingly, recent studies with fibroblasts (Schwartz et al., 1989) and with our capillary endothelial cells (Ingber et al., submitted for publication) suggest that ECM-dependent changes of cell shape may in part alter cell growth by modulating a Na⁺/H⁺ antiport on the cell surface.

Another possibility is that tension-dependent changes of cell shape and cytoskeletal organization might alter a structural system for signal transduction within capillary cells. For example, extracellular alterations of physical force distributions may be translated directly into changes of nuclear structure via transmission of mechanical forces across intermediate filaments that physically link the plasma membrane to the nuclear envelope (Fey et al., 1984; Georgatos and Blobel, 1987). In normal cells, nuclear enlargement appears to be a prerequisite for entrance into the synthetic phase of the cell cycle (Nicolini et al., 1986; Ingber et al., 1987). DNA synthesis also can be activated within isolated nuclei by artificially inducing nuclear swelling (Coffey et al., 1974). Thus, the mechanical restraints of a compact nucleus within a shortened cell may physically limit large scale biophysical alterations that are required for initiation of DNA synthesis, such as DNA unwinding and unfolding (Pienta and Coffey, 1984; Roberts and D'Urso, 1988), and thereby suppress DNA replication. For this reason, endothelial cell retraction during capillary differentiation may induce quiescence.

Alterations of torsional strain within DNA also can have potent effects on gene transcription (Luchnik et al., 1982). Thus, this is one way in which cell retraction and nuclear rounding (i.e., local mechanical perturbations) could switch on differentiation-specific genes as well as tissue-specific patterns of histodifferentiation. Interestingly, both experiments with cultured capillary cells (Ingber et al., 1987) and studies with "nucleated" tensegrity cell models (Ingber and Jamieson, 1985) confirm that cell and nuclear shape alter in a coordinated fashion as cells progress from round to spread suggesting that biophysical interactions may play a central role in this process.

Control of Angiogenesis: a Solid-State Regulatory System

Soluble FGF can act over large distances to initiate angiogenesis. However, our results suggest that capillary growth, differentiation and involution may be controlled locally through modulation of ECM adhesivity or structural integrity. This possibility is supported by the finding that capillary involution can be induced in vivo using compounds that produce capillary basement membrane breakdown, inhibit collagen accumulation, or interfere with collagen cross-link formation and alter its tensile strength (Ingber et al., 1986; Ingber and Folkman, 1988). Regression of mammary and Mullerian Duct epithelium is similarly associated with loss of basement membrane integrity (Wicha et al., 1980; Trelstad et al., 1982). Furthermore, Bernfield and coworkers have previously suggested that local patterns of tissue organization may be established during epithelial morphogenesis based upon controlled alterations of ECM turnover at selective sites (Bernfield and Banerjee, 1978).

However, the most novel finding of the present study is that local control of FGF action is based upon a pivotal "switch" that is mechanochemical rather than purely chemical in nature. This switching mechanism acts like a photomultiplier in that it is triggered as a result of summation or amplification of many smaller signals, in this case, multiple individual cell contractions or changes in cell extension. In this hypothetical solid-state regulatory system, ECM molecules would transmit growth and pattern-regulating signals as a result of mechanical interactions with specific cell surface receptors; i.e. by physically resisting cell-generated tensile forces applied by those receptors. In this manner, the composition, polymerization chemistry, and turnover rates of complex extracellular matrices would in part serve to convey regulatory information by determining their physical properties.

We would like to thank L. Jennings and S. Delehans for their expert technical assistance, Drs. S. Brahmuth and C. Blood for their critical review of this manuscript, and C. Pavlos for her editorial comments.
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