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Fibronectin controls capillary endothelial cell growth by modulating cell shape

(integrin/fibroblast growth factor/extracellular matrix/angiogenesis/RGD peptides)

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ABSTRACT **An *in vitro* system has been developed to study the mechanism by which fibronectin (FN) regulates capillary endothelial cell growth in the presence of soluble angiogenic mitogens. Endothelial cells were cultured in chemically defined medium containing a constant, saturating amount of basic fibroblast growth factor. Formation of cell–FN contacts was then varied in a controlled fashion by three different techniques: (*i*) nonadhesive, bacteriological dishes were precoated with increasing densities of FN; (*ii*) soluble RGD peptides were used to progressively inhibit binding of cell-surface integrin receptors to adsorbed FN; and (*iii*) FN-coated surfaces were covered with increasingly thick layers of polyhydroxyethylmethacrylate (a nonadhesive polymer) to physically restrict cell access to FN binding sites. Endothelial cells became more extended and proliferated more rapidly as FN coating concentrations were raised from ≈250 to ≈10,000 FN molecules per μm^2. Computerized morphometric analysis confirmed that cell shape (projected cell areas) was determined by the density of FN contacts and that DNA synthetic levels were tightly coupled to the extent of cell spreading, regardless of the method used to perturb cell adhesion. In contrast, neither soluble FN nor cell-surface binding of FN-coated microbeads (diameter, 4.5 μm) had any effect on growth when cells were grown in suspension and cell spreading was prohibited. These results suggest that FN controls capillary endothelial cell proliferation based on its ability to support tension-dependent alterations of cell shape—i.e., both by binding to cell-surface integrins and by resisting mechanical loads that are applied to these receptors.**

A central problem in capillary morphogenesis is the question of how local endothelial cell growth differentials are established. During initiation of angiogenesis, one capillary endothelial cell grows in response to soluble mitogens, such as basic fibroblast growth factor (FGF), while neighboring cells remain quiescent (1). The regulatory signals that control endothelial cell sensitivity to FGF appear to be provided by extracellular matrix (ECM) molecules within the local tissue microenvironment (2, 3); however, the mechanism is unknown.

One possibility is that ECM molecules alter capillary endothelial cell growth based on their ability to mediate attachment and thus alter cell shape. Most anchorage-dependent cells proliferate more rapidly in response to serum mitogens as they become more extended, whereas growth and viability are commonly reduced when cells are maintained in rounded forms or in suspension (2–6). Cell shape and growth also appear to be coupled *in vivo*. For example, during initiation of capillary development, endothelial cells respond to angiogenic mitogens by remodeling underlying ECM, extending long cell processes, and increasing their proliferative rates (1). This process is reversed during capillary regression: breakdown of basement membrane results in endothelial retraction, cell rounding, and tissue involution (7). FGF-stimulated capillary cells can be similarly switched between growth, differentiation, and involution modes *in vitro* by altering the density of ECM attachment sites and thereby changing cell shape (3).

Nevertheless, an essential question remains unanswered that is crucial for determining the role of cell shape in growth control in general and during regulation of angiogenesis in particular: do ECM molecules alter cell sensitivity to soluble growth factors by promoting cell spreading, or do cells change their ECM binding properties and take on different shapes as a result of altered proliferative rates? I address this question in the present study by analyzing the mechanism by which a single type of ECM molecule, fibronectin (FN), modulates capillary endothelial cell growth in chemically defined medium supplemented with a saturating amount of soluble FGF. The relation between cell shape and growth is then studied using a variety of methods to specifically modulate formation of cell surface–FN contacts from outside of the cell.

MATERIALS AND METHODS

Experimental System. Capillary endothelial cells isolated from bovine adrenal cortex were maintained in culture as described (3). Quiescent confluent monolayers were dissociated into single cells by brief exposure (1–2 min) to trypsin/EDTA (GIBCO) and washed in Dulbecco's modified Eagle's medium (DMEM) containing 1% bovine serum albumin (BSA; fraction V; Armour Pharmaceuticals). Cell aliquots were pelleted and resuspended in defined medium consisting of DMEM supplemented with transferrin (5 μg/ml) (Collaborative Research), high density lipoprotein (10 μg/ml) (Bionetics Research Institute), 1% BSA, and recombinant basic FGF (2 ng/ml) (kindly supplied by Takeda Chemical Industries, Osaka).

To promote cell–FN contact formation, various amounts of human serum FN (Cappell Laboratories) were dissolved in 0.1 M carbonate buffer (pH 9.4), added to bacteriological 96-well plastic dishes (100 μl per well; Immunolon II; Dynatech) or 8-well multichamber slides (400 μl per well; Lab-Tek glass culture slides; Miles), and allowed to adsorb for 24 hr at 4°C. Dishes were washed with phosphate-buffered saline, DMEM, and DMEM containing 1% BSA immediately prior to use. Trace amounts of ^{125}I-labeled FN (1.31 μCi/μg; 1 Ci = 37 GBq; ICN) were included in the coating solution in experiments designed to quantitate FN adsorption. The total amount of FN adsorbed per well (in 96-well plates) was determined based on the percentage efficiency of adsorption of radiolabeled FN (i.e., counts removed from coated dishes using boiling 10% SDS). Molecular coating densities were

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Abbreviations: FGF, basic fibroblast growth factor; ECM, extracellular matrix; FN, fibronectin; BSA, bovine serum albumin; poly(HEMA), polyhydroxyethylmethacrylate.

calculated by dividing the total number of molecules of adsorbed FN (nonradioactive plus radioactive) by the total exposed area of the culture well and side walls that was covered by the carbonate solution.

For cell proliferation studies, cells were plated onto FN-coated 96-well plates in defined medium (7.5×10^3 cells per well at the lowest FN density; 5×10^3 cells per well at the higher concentrations). Every day attached cells were washed once with phosphate-buffered saline and then were released by treatment with trypsin. Suspended cells from 4 wells of each condition were pooled and counted in a Coulter counter.

For analysis of effects on cell shape and DNA synthesis, endothelial cells were plated on FN-coated 96-well plates (7.5×10^3 cells per well). In experiments analyzing the role of FN–integrin interactions, soluble GRGDSP (Gly-Arg-Gly-Asp-Ser-Pro) or GRGESP (Gly-Arg-Gly-Glu-Ser-Pro) hexapeptides (Peninsula Laboratories) were included in the medium from the time of plating. Polyhydroxyethylmethacrylate [poly(HEMA)] coatings were applied to FN-coated 96-well plates (500 ng of FN per well) by adapting a previously described technique (4). The FN-coated plates were air dried at 37°C, overlaid with prewarmed solutions of either 95% ethanol (EtOH) or various dilutions of poly(HEMA) (Hydron, type NCC; Interferon Sciences, New Brunswick, NJ) in EtOH, placed on an antivibration table, and allowed to dry for 2 days at 37°C. Dilutions of 10^{-2}, 10^{-3}, and 10^{-4} represent solutions of 0.12%, 0.012%, and 0.0012% poly(HEMA) in EtOH, respectively.

To analyze the effects of FN receptor occupancy in the absence of cell shape changes, cells were grown in suspension in defined medium that was supplemented with a high amount of soluble FN (500 ng per well; 2.5 μg/ml). The uncoated 96-well plates used in this study were precoated with a 1.2% poly(HEMA) solution to ensure that no binding interaction between cells and the planar substratum could take place [FN will not adsorb to poly(HEMA)]. For comparison, cells were grown on dishes coated with an equivalent amount of insoluble FN (500 ng per well) in defined medium without soluble FN. In another study, suspended cells were cultured in the presence of 4.5-μm-diameter microbeads (five beads per cell; tosyl-activated Dynabeads M-450; Robbins Scientific, Mountain View, CA) that were precoated with a high FN concentration (100 μg/ml) by the carbonate buffer method.

DNA synthesis was measured within cells after 18 hr of exposure to [^{3}H]thymidine (5 μCi/ml) by quantitating its incorporation into trichloroacetic acid-precipitable material. The number of attached cells was measured in parallel wells as described (2) to determine DNA synthesis on a "per cell" basis (i.e., normalized for cell number). Thus, all DNA synthesis data result from samplings of large populations of cells and changes of DNA synthesis correspond to differences in the percentage of cells that enter S phase rather than to changes in the "rate" of DNA synthesis within individual cells.

Morphometric Analysis. Projected areas of adherent cells were determined by a computerized image analysis system (Zeiss interactive digital analysis system) as described (2). A total of >80 cells within at least six randomly selected areas from three different culture wells were measured for each cell area determination. Photomicrographs of cells grown on different FN concentrations were taken on a Zeiss inverted photomicroscope using Nomarksi optics. Suspended cells attached to microbeads were photographed by phase-contrast optics on a Nikon inverted microscope.

RESULTS

In the first method used to modulate endothelial cell–FN interactions, adhesive contact formation was promoted by adsorbing increasing numbers of FN molecules onto bacteriological dishes—i.e., by progressively increasing the number of potential attachment points. FN coating concentrations were translated into molecular coating densities based on quantitation of adsorbed ^{125}I-labeled FN (Table 1). Capillary cells cannot attach to these bacteriological plastic dishes in the absence of preadsorbed ECM proteins when serum-free medium is used. Thus, variation of FN coating concentrations both promoted cell attachment and permitted a wide range of cell shape control (Fig. 1). As the FN coating density was increased, capillary cells became more flattened and extended cell processes of increasing length. Dishes coated with more than ≈9500 molecules per μm^2 promoted maximal extension. As demonstrated (2), nuclei also underwent drastic structural alterations and extended in a coordinated fashion within spreading cells (Fig. 1).

Increasing FN molecular densities resulted in enhanced sensitivity of capillary endothelial cells to the proliferative effects of FGF (Fig. 2). Capillary cells increased in number when cultured on high FN coating densities (100 and 500 ng per well). However, cells on low coating concentrations (≤10 ng per well) did not grow and actually lost viability over time. Cells on moderately adhesive dishes (25 ng per well) exhibited intermediate growth rates. Effects on cell proliferation correlated directly with alterations of [^{3}H]thymidine incorporation; DNA synthesis increased in a dose-dependent fashion as FN coating densities were raised from 10 to 500 ng per well (Fig. 3*A*). In contrast, effects of FN on cell attachment saturated at ≈100 ng per well, indicating that DNA synthesis could be varied over a wide range without significantly altering cell plating densities. This analysis revealed that half-maximal stimulation of DNA synthesis required approximately a 10-fold higher FN coating concentration than similar induction of cell attachment (2500 versus 250 FN molecules per μm^2).

In the second method of cell shape control, soluble GRGDSP peptides were used to inhibit the binding of FN-coated plastic dishes to cell-surface receptors (Fig. 3*B*). This peptide contains the RGD sequence that is found within FN and is responsible for its binding to transmembrane integrin receptors (8). As noted by previous investigators (9), soluble GRGDSP produced cell detachment from FN-coated dishes when added at concentrations greater than ≈10 μg/ml (17 μM) despite the continued presence of soluble growth factors (Fig. 3*B*). Addition of a control GRGESP hexapeptide containing a single amino acid change (replacement of aspartate by glutamate) at identical doses showed no effect on cell attachment (Fig. 3*B*).

Importantly, low concentrations of soluble GRGDSP peptides inhibited DNA synthesis in a dose-dependent fashion. Of note, >50% inhibition was observed before any significant detachment or change of cell plating density was induced (Fig. 3*B*). In contrast, only minor inhibition of DNA synthesis was observed with high concentrations of the control GRGESP hexapeptide.

The third method for cell shape modulation involved physical interference with cell binding to FN using the nonadhesive polymer, poly(HEMA). Poly(HEMA) has been previously used to alter the adhesivity of standard tissue culture dishes in conjunction with serum-containing medium (4, 6). In the present study, cell spreading was progressively inhibited in serum-free medium by overlaying FN-coated

Table 1. FN molecular coating densities

FN added, ng per well	FN adsorbed, ng per well	Molecular density, FN molecules per μm^2
10	9.2	235
25	20.9	547
50	39.6	1,036
100	78.2	2,045
500	362.0	9,466
1000	664.0	17,354

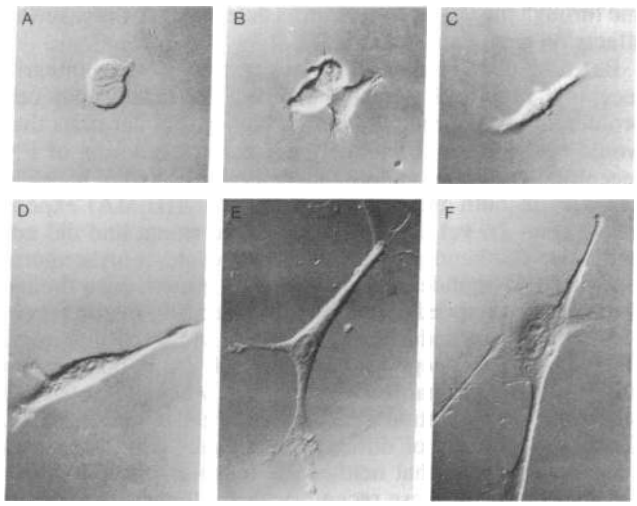

FIG. 1. Control of capillary endothelial cell shape by varying FN coating densities. Cells were grown on dishes coated with approximately 250 (*A*), 550 (*B*), 1000 (*C*), 2000 (*D*), 5000 (*E*), and 9500 (*F*) molecules of FN per μm^2. (×255.)

bacteriological dishes (500 ng per well) with poly(HEMA) coats of various thicknesses. Cell–substratum contacts that do form when this technique is used occur within defects in the polymer layer that are large enough to permit binding of multiple cell-surface receptors (10). Thus, in contrast to the previous two techniques, poly(HEMA) should not significantly alter the effective FN coating density that is exposed to the cell surface within individual adhesion sites. When this method was used to physically restrict cell spreading, FGF-stimulated DNA synthesis was again progressively suppressed before significant inhibition of cell attachment or changes of cell density were observed (Fig. 3*C*).

To determine whether effects on cell shape could be separated from effects on DNA synthesis, computerized morphometry was carried out with cells in parallel wells. Cell spreading data (i.e., projected cell areas) were pooled from all three experiments and compared with effects on DNA synthesis (Fig. 4). This analysis revealed that thymidine incorporation increased in an exponential fashion in direct relation to linear increases in projected cell area, independent of the method of extracellular modulation. For example, cells that covered 750 μm^2 only incorporated ≈3 dpm of [^{3}H]thymidine per cell, whereas cells that were allowed to spread to twice this size increased their DNA synthetic rates by almost 10-fold (i.e., regardless of the technique used).

In contrast, FGF-stimulated capillary endothelial cells did not enter S phase when grown in suspension in the presence

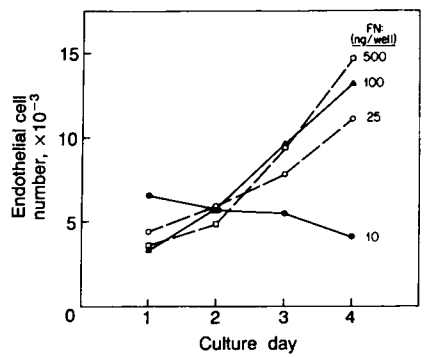

FIG. 2. Effects of varying FN coating densities on capillary endothelial cell proliferation. Each point was the result of triplicate cell number determinations (total of 12 wells); SEM was consistently <10%.

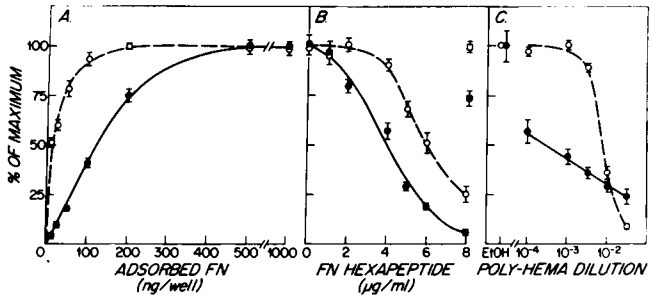

FIG. 3. Effects of extracellular modulation of cell–FN contact formation on cell attachment and DNA synthesis. ○, Cell attachment data; ●, DNA synthesis data. (*A*) Cells grown on various FN coating densities. (*B*) Cells grown on high FN density (500 ng per well) in the presence of various concentrations of soluble RGD-containing hexapeptide (GRGDSP) (○ and ●) or control GRGESP hexapeptide (8 μg/ml) (□ and ■). (*C*) Cells grown on FN-coated dishes (500 ng per well) that were overlaid with poly(HEMA) coatings of various thicknesses. Error bars indicate SEM.

of high concentrations of soluble FN (Fig. 5). Suspended cells can bind soluble FN; however, they may exhibit a lower binding affinity for soluble FN than for FN-coated surfaces. Alternatively, soluble FN may not produce local clustering of FN receptors—i.e., a cell-surface event that has been recently shown to be required for signal transduction in fibroblasts (11).

Thus, to determine the importance of local clustering of FN receptors in capillary growth control, a fifth experimental method was devised. Suspended endothelial cells were again used; however, they were cultured in the presence of FN-coated microbeads (diameter, 4.5 μm). Almost all of the suspended endothelial cells bound tightly to at least one FN-coated microbead (Fig. 6), whereas under identical conditions <1% of the cells bound to beads coated with the nonspecific ligand BSA (data not shown). Also, formation of bead clusters was observed whenever individual cells bound more than one microbead, providing visual evidence of local "capping" of FN receptors (Fig. 6). Using this system, it was found that binding of FN-coated microbeads to cell-surface receptors did not induce cell spreading (Fig. 6) or alter DNA synthesis (Fig. 5) in suspended endothelial cells. These results suggest that neither FN receptor occupancy nor integrin clustering is sufficient to support capillary endothelial cell growth in response to FGF.

DISCUSSION

The goal of this study was to devise a controlled experimental system that could be used to determine whether ECM molecules regulate capillary endothelial cell growth based on

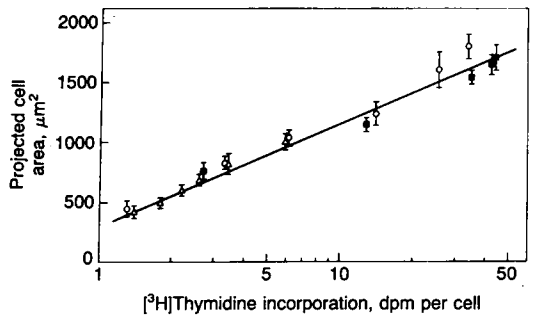

FIG. 4. Relation between capillary endothelial cell spreading and DNA synthesis. Cell spreading (projected cell areas) and DNA synthesis data were pooled from all three experiments described in Fig. 3. ○, Increasing FN coating densities; ■, GRGDSP experiment; △, the poly(HEMA) study; —, an exponential regression line best fit to the data points.

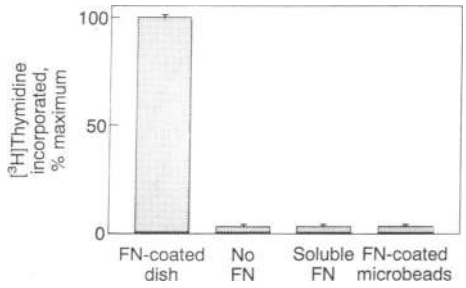

FIG. 5. Effects of soluble FN and different FN-coated substrates on DNA synthesis in FGF-stimulated endothelial cells. Adherent cells were cultured on plastic dishes coated with a high FN density (500 ng per well). Suspended cells were cultured in the presence or absence of soluble FN (500 ng per well) or FN-coated microbeads (five beads per cell).

their ability to modulate cell shape. Most past studies of the role of ECM and cell shape in growth control utilized poorly defined substrata (e.g., standard tissue culture substrata, complex ECM substrata) as well as serum-containing medium (4–6, 12–14). Serum contains a variety of different soluble growth factors and attachment molecules in various amounts. Thus, in these past experiments, it was not possible to determine whether shape changes directly promoted growth, whether the cells became sensitive to different classes of serum growth factors when substratum adhesivity was altered, or whether cell growth was regulated indirectly as a result of *de novo* deposition of ECM molecules beneath the cells. For these reasons, in the present study chemically defined medium was used that contained a constant, saturating amount of FGF as well as bacteriological dishes that did not support cell attachment or ECM deposition in the absence of preadsorbed matrix proteins. Consequently, only cell–FN contact formation was varied.

The results of the present study show that capillary endothelial cell growth can be controlled locally by modulating formation of cell–FN contacts at the cell surface and demonstrate that the presence of soluble FGF is not sufficient to stimulate endothelial cell proliferation. The successful use of low concentrations of RGD-containing peptides to alter capillary cell growth strongly suggests that FN exerts its regulatory actions by binding to integrins (8) on the cell surface. Cell-surface contact with FN-coated dishes also promotes reentry of suspended, quiescent fibroblasts into the cell cycle

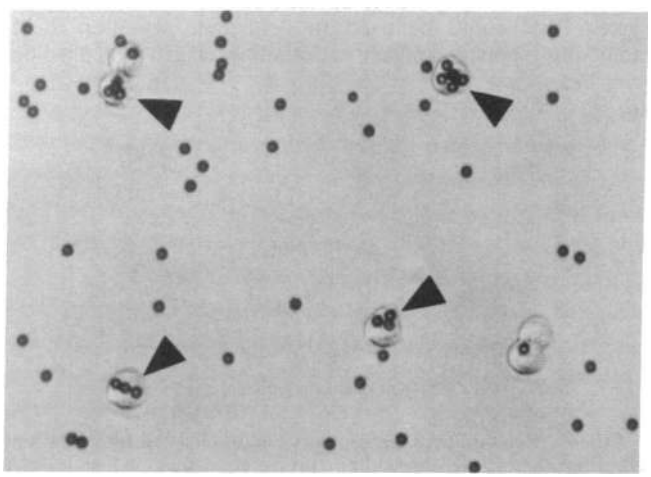

FIG. 6. Effects of FN-coated microbeads on endothelial cell shape. Arrowheads indicate cells that exhibit clustering of FN-coated microbeads. (×320.)

and through the G_0/G_1 transition as determined by measuring effects on gene expression (15).

But how does binding of FN to transmembrane integrin receptors alter cell growth? Clearly, the most rapid cell proliferation was promoted by the highest FN densities that would be expected to induce the greatest clustering of FN receptors. However, endothelial cells also bound to high FN densities in both the microbead and poly(HEMA) experiments (Fig. 7), yet these cells remained round and did not enter the synthetic phase of the cell cycle. Furthermore, soluble RGD peptides did not stimulate growth even though they clearly occupied endothelial cell surface integrin receptors; in fact, they induced cell rounding and were inhibitory. In contrast, RGD peptides stimulate cell proliferation when linked to a rigid planar foundation—i.e., when presented in a structural configuration that supports cell extension (16). Thus, by a variety of different techniques (Fig. 7), it was consistently found that neither FN receptor occupancy nor local clustering of these receptors was sufficient to promote DNA synthesis if the cells were prevented from spreading.

Importantly, growth was stimulated by FN in direct proportion to its ability to promote cell extension (Fig. 4). This finding is in direct contrast with the effects of FN on expression of differentiation-specific genes, which can be controlled independently of cell shape and are induced by either integrin occupancy alone (17) or clustering of integrins (11), depending on the cell type studied. Binding of FN to cell-surface integrins may similarly induce expression of subsets of genes in round endothelial cells. Nevertheless, our results show that cell extension (i.e., an increase in projected cell area) must also take place before all of the genes that are required for cell-cycle progression are activated.

These data, in conjunction with those from past studies (2–6, 12–14), strongly suggest that it is the extent to which an anchorage-dependent cell spreads that determines its ability to enter the synthetic phase of the cell cycle. This requirement for cell extension also may explain why ECM molecules such as FN usually only alter cell growth when provided in an insoluble form—i.e., adsorbed to a rigid substrate or organized within a complex ECM. However, it is important to note that cell spreading does not cause cells to proliferate, it only governs progression from G_0 to S phase. For example, migrating cells and cells blocked in G_2 can spread on FN-coated dishes without growing (12, 14); however, cell shape is coupled to growth when cells become nonmotile and form focal adhesions during G_1 phase (ref. 14; reviewed in ref. 18).

The signals that control cell shape and regulate cell-cycle progression appear to be tightly coupled in capillary endothelial cells. Thus, we may gain some insight into growth control by analyzing how cell shape is determined. In the present study, capillary endothelial cells only spread on FN if it was adsorbed to a rigid planar substratum and extended cells spontaneously retracted as FN contacts were progres-

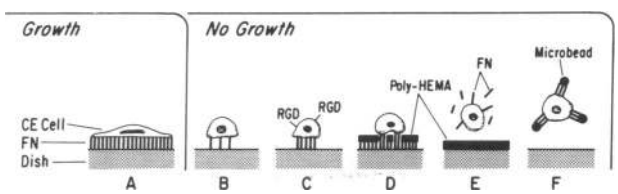

FIG. 7. Diagram comparing the different methods used to modulate cell–FN contact formation. Endothelial cells were plated on rigid, planar dishes coated with a high FN density in the absence (*A*) or presence of soluble RGD peptides (*C*) or poly(HEMA) overlays (*D*). Cells were also grown on dishes coated with a low FN density (*B*) or maintained in suspension in the presence of either soluble FN (*E*) or FN-coated microbeads (*F*). Occupancy and clustering of FN receptors did not promote growth when cell spreading was prevented.

sively dislodged by soluble GRGDSP peptides. These results suggest that endothelial cell shape is determined through a dynamic balance of structural forces. In fact, anchorage-dependent cells commonly exist in a state of isometric tension; they exert tensile forces on their attachment points and spontaneously take on different forms depending on the structural integrity of their attachment foundation (19–21). Also in support of the importance of mechanical forces is the observation that similar substratum-dependent control of cell shape can be obtained by using inorganic tension-dependent (tensegrity) "cell" models that are constructed out of sticks and elastic string (18, 21). Thus, FN may regulate cell shape and growth based on its ability to resist cell-generated mechanical loads.

What is the mechanism by which changing cell shape or increasing intracellular tension enhances endothelial cell sensitivity to the proliferative effects of FGF? A simple explanation might be that physical extension of the cell membrane modulates FGF–receptor interactions. However, this does not appear to be the case; FN exerts its growth-modulating effects independently of FGF (22). Alternatively, FN may alter growth by reorganizing the cytoskeleton since transmembrane integrin receptors interconnect with microfilaments through binding interactions with actin-associated molecules, such as talin (23). Also, preliminary studies suggest that capillary endothelial cells assemble actin bundles of increasing number and length as the FN coating density is raised (24). Furthermore, the importance of the ability of FN to resist cell-generated tensile forces may be explained in part by related mechanochemical effects on cytoskeletal filament assembly. Microfilament polymerization, microtubule assembly, and DNA synthesis are all sensitive to external application of mechanical forces (25–29).

FN-dependent changes of microfilament organization may provide a mechanism for integrating signals that control cell shape and growth because much of the cell's metabolic machinery appears to be physically associated with the cytoskeletal lattice (reviewed in ref. 18). For example, changes of cytoskeletal polymerization might alter the distribution and function of transmembrane proteins or "stretch-activated" ion channels (30–32) that are involved in the early steps of growth regulation. Signal integration also may be accomplished by activating intracellular chemical signaling pathways that are normally utilized by soluble growth factors. In this context, it is important to note that spreading on FN activates a cell-surface Na^+/H^+ antiporter in capillary endothelial cells and that addition of ethylisopropylamiloride (a specific inhibitor of the antiporter) results in suppression of growth (22).

Cytoskeletal assembly also may facilitate growth by promoting nuclear extension since enlargement of the nucleus appears to be a prerequisite for entrance into S phase (2, 33). Nuclear expansion, as observed within spreading endothelial cells in the present study (Fig. 1), may promote DNA synthesis by releasing mechanical restraints to DNA unfolding (34, 35), changing nucleocytoplasmic transport rates (36), or altering the distribution and function of DNA regulatory proteins that are associated with the nuclear protein matrix (35). In this manner, cytoskeletal interconnections that physically link the cell surface to the nuclear protein matrix (e.g., intermediate filaments; ref. 37) may transduce mitogenic signals by mechanically coupling cell and nuclear shape changes (18, 21).

In summary, these results suggest that control of capillary endothelial cell growth by FN is based on a mechanochemical mechanism. FN conveys growth-regulatory information to the cell by both binding cell-surface integrins and physically resisting cytoskeletal tension that is applied to these receptors.

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