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# ENDOTHELIAL GROWTH FACTORS AND EXTRACELLULAR MATRIX REGULATE DNA SYNTHESIS THROUGH MODULATION OF CELL AND NUCLEAR EXPANSION

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## SUMMARY

Studies were carried out to analyze the mechanism by which extracellular matrix (ECM) molecules and soluble growth factors interplay to control capillary endothelial cell growth. Bovine adrenal capillary endothelial cells attached to purified matrix components but spread poorly and exhibited low levels of DNA synthesis in the absence of exogenous growth factors or serum. Addition of cationic, heparin-binding growth factor purified from either human hepatoma cells or normal bovine pituitary (fibroblast growth factor) induced extensive cell spreading and up to eight fold increases in DNA synthetic rates relative to levels observed in cells on similar substrata in the absence of mitogen. However, the extent of this response differed depending upon the type of ECM molecule used for cell attachment (fold increase on type III collagen > gelatin > type IV collagen > fibronectin > type V collagen >>> laminin). Computerized morphometry demonstrated that endothelial cell DNA synthetic rates increased in an exponential fashion in direct relation to linear increases in cell and nuclear size (projected areas). Similarly sized cells always displayed the same level of DNA synthesis independent of the type of ECM molecule used for cell attachment or the presence of saturating amounts of growth factor. In all cases, DNA metabolism appeared to be coupled to physical expansion of the cell and nucleus rather than to a specific cell morphology (e.g. polygonal versus bipolar). These findings suggest that ECM may act locally as a "solid state" regulator of angiogenesis through its ability to selectively support or prohibit cell and nuclear extension in response to stimulation by soluble mitogens.

*Key words:* fibroblast growth factor; cell shape; nuclear shape; signal transduction; angiogenesis.

## INTRODUCTION

A great deal of progress has been recently achieved in the search for hormones that control angiogenesis. Soluble factors have now been purified from both normal and neoplastic tissues which can induce neovascularization in vivo and stimulate endothelial cell proliferation in vitro (3,27,42). However, the growth-promoting action of angiogenic factors is alone not sufficient to explain the generation of higher order tissue architecture. Repeated endothelial cell divisions would result in production of a disorganized pile of cells in the absence of additional regulatory controls. Indeed, development of branching capillaries and other specialized vascular structures is made possible through establishment of local differentials in endothelial cell proliferative rates in the midst of active angiogenesis (i.e., in the presence of high concentrations of soluble endothelial mitogens)(12). Thus, to understand the basis of angiogenic regulation, we must first dissect the mechanism by which endothelial growth factor action is selectively prohibited or supported locally.

Extracellular matrix (ECM) molecules are good candidates for local regulators of capillary development. In the embryo, tissue-tissue interactions that guide histogenesis are transduced over intervening ECM (17) and, in certain tissues, pattern formation appears to be linked to directed alterations of ECM turnover (1,45). Regional variations of ECM composition and integrity are observed during vascular development (13,23) and past studies have shown that purified ECM components can modulate the growth and organization of capillary endothelial cells in vitro (36). Yet, the mechanism by which ECM and soluble

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growth factors interact to control capillary endothelial cell growth remains unknown.

In the present study, bovine adrenal capillary endothelial (BCE) cells were plated on purified ECM components (laminin, fibronectin, types III, IV, & V collagens) or gelatin and exposed to purified human hepatoma-derived growth factor (HDGF) or basic fibroblast growth factor (FGF) isolated from normal bovine pituitary gland. HDGF and FGF are members of a newly described class of cationic, heparin-binding growth factors which share sequence homology, antigenic sites, and the ability to stimulate angiogenesis in vivo (7,29,32). This investigation was also carried out in the absence of serum so that we could determine effects that resulted solely from interactions between cells and distinct matrix molecules. We now show that the mechanism by which cationic angiogenic factors and ECM interact to regulate capillary endothelial cell growth depends upon their ability to modulate cell and nuclear form.

## Materials and Methods

*Cell Culture.* BCE cells were isolated from bovine adrenal glands as previously described (11) and passaged on gelatin-coated dishes in Dulbecco's modified Eagle's Medium (DMEM) supplemented with 10% calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 U/ml streptomycin (all from Gibco, Grand Island, ME), and 5 μl/ml retinal extract (14).

*Matrix-Coating Procedures.* Laminin, and types III, IV & V collagens were purified as previously described (33,35). Fibronectin (Cooper, Malvern, PA) and gelatin (Gibco; the same as used for cell passaging) were also used in these studies. All ECM components were diluted to 1 μg/ml in 0.1 M carbonate buffer, pH 9.4, plated at 100 μl (100 ng) per well of bacteriological plastic 96 well plates (Immunolon II; Dynatech Laboratories, Inc., Alexandria, VA), and allowed to adsorb by incubating overnight at 4° C. Amounts of matrix components bound were found to be in the range of 1 to 50 ng/cm² as determined using a published quantitative ELISA inhibition assay (36). These coating densities correspond to coverage of approximately 5 to 55% of the culture surface area (calculations were made based upon size and mass estimates that were determined by scanning transmission electron microscopy). Dishes prepared in this fashion supported optimal BCE cell attachment in the absence of serum.

*Endothelial Growth Factors.* HDGF was kindly supplied by Dr. M. Klagsbrun and Robert Sullivan (The Children's Hospital) who purified this endothelial mitogen from human SK hepatoma cells using a combination of ion-exchange, heparin-affinity, and reversed-phase high pressure liquid chromatography (29). FGF and EGF were obtained from Collaborative Research.

*Experimental System.* Newly confluent BCE cells were cultured for 2 days free of retinal extract in DMEM containing 2.5% calf serum. Endothelial cell layers were dissociated into single cells with trypsin-EDTA (Gibco) and transferred into DMEM containing 1% bovine serum albumin (Fraction V; Sigma, St. Louis, MO). To study the effects of growth factor stimulation, cell suspensions were supplemented with saturating amounts of HDGF (2 ng/ml), FGF (100 ng/ml), or EGF (100 ng/ml), plated at 1 × $10^4$ cells/well on ECM-coated substrata and cultured at 37° C. Similar results were obtained with cells plated at lower density.

*DNA Synthesis Assay.* $^3$H-methyl thymidine (New England Nuclear, Boston, MA) was added to BCE culture media after 1 hr of culture (final concentration, 5 μCi/ml). Incorporation of radiolabel was terminated after 18 hr by addition of 100 fold excess of non-radioactive thymidine. Attached cells were washed with phosphate-buffered saline, rinsed with methanol, and DNA was precipitated on the dish with cold trichloracetic acid. Acid-precipitates were dissolved in 0.3 N NaOH and counted in a Beckman LS 3801 scintillation counter. In order to analyze DNA synthesis on a per cell basis, the number of cells in parallel wells was quantitated using a published assay based on cellular acid phosphatase (6).

*Morphological Studies.* To analyze alterations of cell shape, BCE cells were fixed in 2% glutaraldehyde and stained in 1% toluidine blue. Cells were photographed within 96 well plates using a Nikon Diaphot inverted

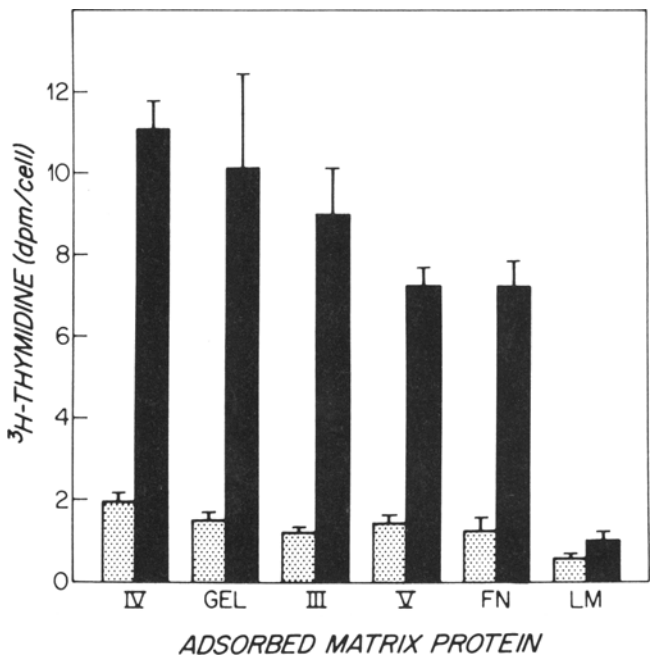


FIG. 1. Regulation of Capillary Endothelial Cell DNA synthesis by ECM and HDGF. Incorporation of $^3$H-thymidine by BCE cells was measured after 18 hrs on different matrix-coated dishes in serum-free medium alone (stippled bars) or in medium supplemented with HDGF (black bars; 2 ng/ml). IV, type IV collagen; GEL, gelatin; III, type III collagen; V, type V collagen; FN, fibronectin; LM, laminin. Data presented are the result of three separate experiments. Error bars indicate standard error of the mean.

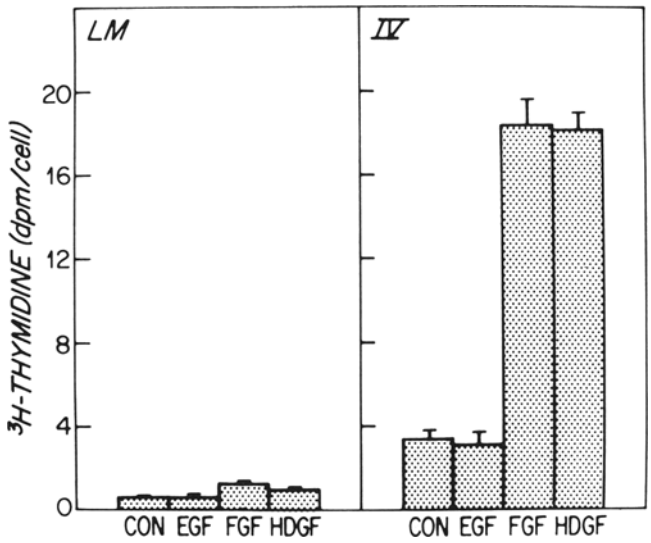


FIG. 2. Effects of Laminin and Type IV Collagen on Capillary Endothelial Cell Growth Responsiveness to Different Polypeptide Growth factors. Incorporation of $^3$H-thymidine by BCE cells was measured after 18 hrs in serum-free medium alone (CON) or supplemented with EGF (100 ng/ml), FGF (100 ng/ml), or HDGF (2 ng/ml). LM, laminin; IV, type IV collagen.

microscope with AGFA 25 film. Computerized morphometric analysis of stained cells was carried out using a Zeiss Interactive Digital Analysis System. Cells were randomly selected from three different regions of each culture well using an external grid system. Cell and nuclear circumferences were then outlined with a light cursor within images projected on a digitizing tablet (1,000× magnification; Zeiss Photomicroscope II). Mean cell and nuclear areas (± standard error of the mean) were determined using the area-perimeter function of the Zeiss computerized image analysis system. Data represent the result of scoring a total of 60 cells from three different experiments for each condition. We have previously shown that alterations of projected cell area correspond directly with changes in total cell volume (10).

## RESULTS

*Regulation of DNA Synthesis.* On all substrata, BCE cells exhibited low levels of DNA synthesis in the absence of growth factor. Yet, cell DNA synthetic rates differed depending upon the type of ECM used for cell attachment (Fig. 1). Cells incorporated the least amount of $^3$H-thymidine on laminin and the most on type IV collagen, type III collagen, and gelatin with cells on type V collagen and fibronectin exhibiting intermediate values.

In the presence of saturating amounts of HDGF, all capillary endothelial cells increased their DNA synthetic rates but again these increases varied with the type of attachment substratum (Fig. 1). Cells cultured on gelatin and types III and IV collagens exhibited the greatest stimulation of DNA synthesis in response to HDGF relative to cells on the same substrata in the absence of factor (6 to 8 fold increase on a per cell basis). Type V collagen and fibronectin supported 5 to 6 fold increases, while cells on laminin only doubled their DNA synthetic rates. Importantly, growth factor-stimulated cells on laminin synthesized less DNA than cells cultured on other substrata (e.g., type IV collagen) in the absence of growth factor. Similar results were obtained when FGF was substituted for HDGF although EGF had no effect (Fig.

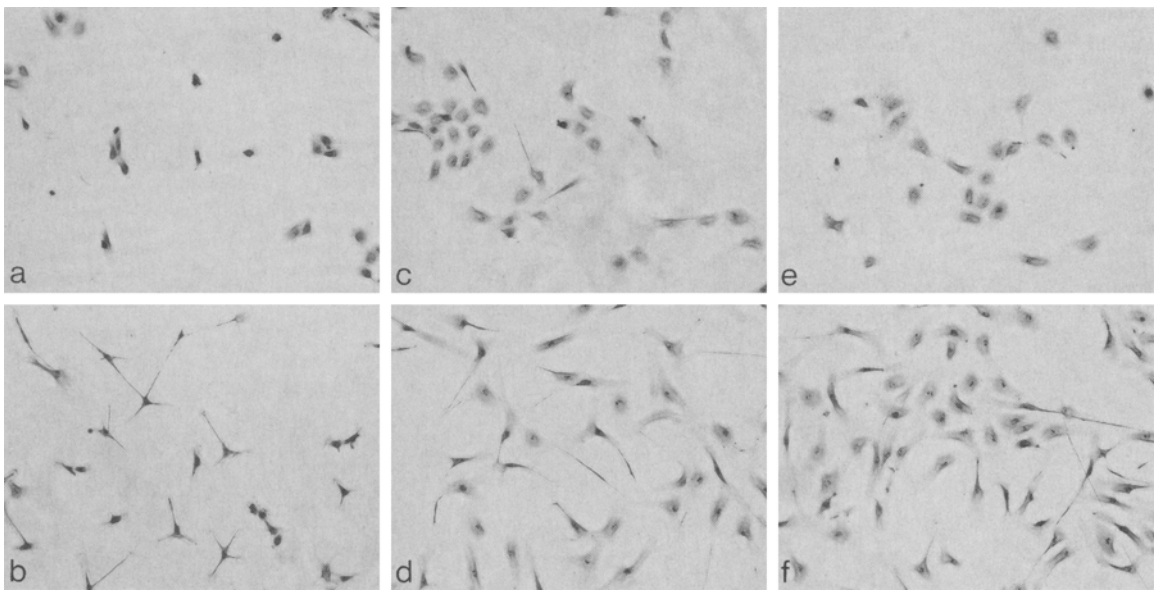


FIG. 3. Effects of ECM and HDGF on Capillary Endothelial Cell Shape. BCE cells were cultured on laminin (a,b), type V collagen (c,d), or type IV collagen (e,f) in the absence (a,c,e) or presence of HDGF (b,d,f). (×150).

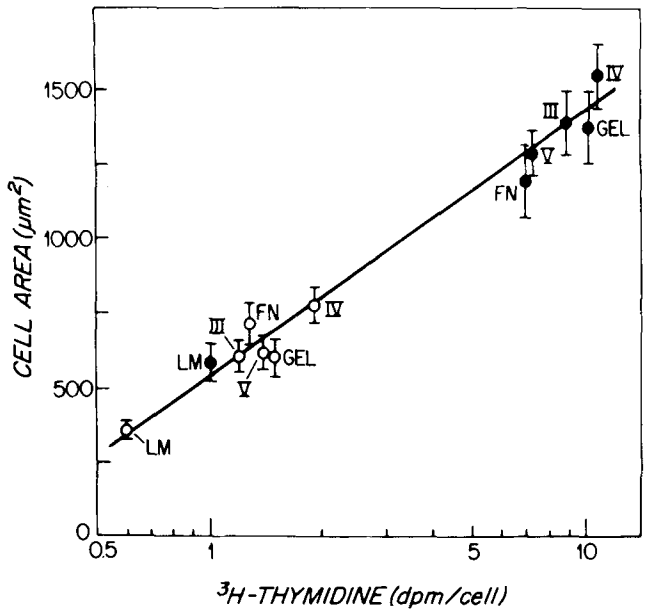


FIG. 4. Relation between Capillary Endothelial Cell Shape and DNA Synthesis. Projected cell areas were determined by computerized morphometric analysis as described in Methods. Data presented were obtained from cells cultured on different matrix components in the absence (open circles) and presence (closed circles) of HDGF. Note that $^3$H-thymidine incorporation is plotted on a logarithmic scale. Solid line represents an exponential regression curve best fit to the data points. LM, laminin; GEL, gelatin; FN, fibronectin; III, type III collagen; IV, type IV collagen; V, type V collagen.

2). EGF also fails to stimulate capillary endothelial cell proliferation in serum-containing medium (28). Autoradiographic studies confirmed that growth factor-induced alterations of DNA synthesis were due to increased percentages of cells that exhibited nuclear incorporation of $^3$H-thymidine (not shown).

*Modulation of Cell Form.* BCE cell shape varied enormously depending upon the ECM molecule used for cell attachment and whether endothelial growth factor was present (Fig. 3). In the absence of HDGF, capillary endothelial cells were smallest and most rounded on laminin, while cells on all other substrata appeared larger and had more polygonal profiles. The relative inability of laminin to support BCE cell spreading was not due to lack of portions of the laminin molecule that could support cell spreading since different types of cells (e.g., pancreatic acinar tumor cells) preferentially spread on our laminin coated dishes (22).

Addition of HDGF induced major changes in BCE cell size and shape. Cells on laminin responded to HDGF by elongating and occasionally forming extended multicellular networks, but still remained much smaller than cells on any of the other substrata. On type V collagen and fibronectin, BCE cells responded to growth factor primarily by extending long thin processes although some cells also expanded their central cytoplasmic regions. Cells on gelatin and types III and IV collagens exhibited the greatest expansion in size by both projecting long extensions and spreading out their cell bodies. Nuclei also appeared to be more extended and larger within better spread cells. FGF induced similar cell shape alterations although EGF had no effect.

*Dependence of Growth upon Cell and Nuclear Size.* Computerized morphometry revealed that projected BCE cell areas ranged from 350 to 800 $\mu m^2$ in the absence of growth factor (laminin < gelatin, type III collagen, type V collagen < fibronectin, type IV collagen) (Fig. 4). Morphometric studies also confirmed that addition of HDGF promoted cell expansion on all substrata with the most extensive cell spreading being observed on gelatin and types III and IV collagens (approximately 1400 to 1600 $\mu m^2$). Cells on type V collagen and fibronectin also exhibited large increases in projected cell area (1150 to 1350 $\mu m^2$). While cells on laminin elongated in response to HDGF, their projected areas remained extremely small (600 $\mu m^2$).

Comparison of results of morphometric studies with those from $^3$H-thymidine incorporation experiments clearly demonstrated that DNA synthetic rates were always tightly coupled to BCE cell size (Fig. 4). In general, DNA synthesis increased in an exponential fashion in direct relation to linear increases in projected cell area. For example, DNA synthesis increased approximately 8 fold as BCE cell area doubled from 600 to 1200 $\mu m^2$. In addition, similarly sized cells always

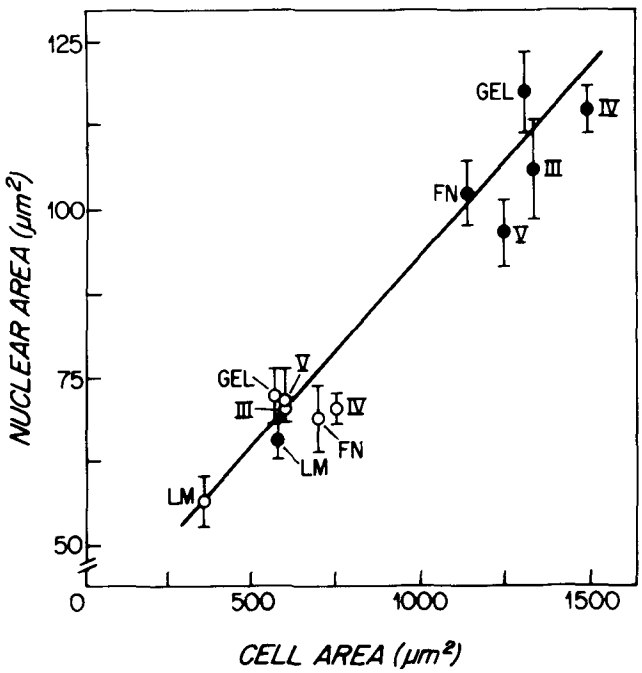


FIG. 5. Relation between Capillary Endothelial Cell and Nuclear Size. Note that projected cell and nuclear areas are both plotted on linear scales. Solid line represents a linear regression curve best fit to the data points. Open circles, absence of growth factor; closed circles, presence of HDGF; LM, laminin; GEL, gelatin; FN, fibronectin; III, type III collagen; IV, type IV collagen; V, type V collagen.

displayed the same level of DNA synthesis independent of the type of ECM component used for cell attachment or of the presence of growth factor (e.g., HDGF-stimulated cells on laminin exhibited the same DNA synthetic rates as the same sized, unstimulated cells on type III collagen). Furthermore, morphometric analysis confirmed that cell and nuclear size were linearly related altering in a coordinated fashion under all circumstances (Fig. 5). Thus, DNA synthetic alterations were also always linked to changes of nuclear geometry.

## Discussion

The present study was not designed to measure the relative growth-promoting activities of distinct types of ECM molecules. Rather, we set out to determine whether the mechanism by which soluble growth factors and ECM components interplay to control endothelial cell growth is related to their ability to modulate cell spreading. While most past studies have viewed changes in cell form as secondary effects of growth factor action, it is possible that cell shape may serve as a physiological control element in the regulation of anchorage-dependent cell growth. For example, we have previously shown that the growth of a variety of normal cells can be finely controlled in serum-containing medium by artificially holding cells in different shape configurations using culture surfaces coated with varying amounts of poly-hydroxyethyl methacrylate, a non-adhesive synthetic polymer (10). In general, cell shape appears to be permissive for entrance of normal cells into the synthetic phase of the cell cycle with the most spread cells exhibiting the highest levels of DNA synthesis (10,15,24). However, cell spreading is normally mediated by deposition of ECM molecules (34,43) or through adhesive interactions with exogenous matrix-containing substrata (15,22,39). Thus, the importance of the findings of the present study lies in the demonstration that the growth-modulating effects of physiological cell adhesion molecules (i.e., ECM components) relate directly to their ability to regulate capillary endothelial cell shape alterations in response to stimulation by soluble angiogenic factors. The growth stimulatory effects of a variety of different classes of hormones and chemical mediators (e.g., polypetides, steroids, cyclic nucleotides) similarly correlate with ECM-dependent alterations of cell shape (15,39) or general changes in cell size (8,10,30,31).

Growth factor-induced alterations in cell form appeared to be the result of specific adhesive interactions between cell surface molecules and distinct ECM components. Spontaneous cell attachment was never observed in the absence of absorbed ECM protein (even in the presence of growth factor) and dishes coated with hemoglobin or albumin similarly did not support cell attachment, spreading, or growth. FGF-like growth factors could stimulate expression of specific ECM receptors (e.g., type IV collagen receptor) or induce deposition of additional cell adhesion molecules (e.g., fibronectin binds type III collagen). FGF has been previously shown to stimulate matrix deposition by large vessel and corneal endothelial cells (16). Endothelial growth factor binding may also induce changes in the distribution of cell surface ECM-receptors and cytoskeletal elements since stimulated cells extended out in an elongated fashion rather than flattening out evenly. Capillary cells similarly elongate and grow in tandem during angiogenic factor-induced neovascularization in vivo (12).

Endothelial growth factors stimulated BCE cell extension and DNA synthesis in a coordinated fashion on all substrata although the extent of this response depended upon the type of ECM component used for attachment. For example, growth factor-stimulated cells on type IV collagen spread much more extensively and synthesized greater than 15 times more DNA on a per cell basis than similarly treated cells on laminin. Complex ECM substrata have been previously shown to alter corneal endothelial cell shape and proliferative response to FGF in a related fashion in serum-containing medium although the specific relationship between cell extension and cell growth responsiveness was not quantitated (15).

In the present study, linear increases in endothelial cell extension were associated with exponential increases in cellular DNA synthesis on all purified ECM components tested. All of the matrix molecules could even support low levels of DNA synthesis in the absence of soluble growth factor ostensibly based exclusively on their ability to promote cell and nuclear extension. These low DNA synthetic levels could be the result of capillary endothelial cells responding to autocrine growth factors (41) or to residual serum mitogens. However, even under these conditions, cellular responsiveness was clearly linked to matrix-dependent alterations of cell form. These observations suggest that the growth regulating effects of different matrix molecules are related directly to their structural role as mediators of cell attachment and spreading. This may explain why the growth-promoting effects of purified ECM components may differ in experiments utilizing serum (or conditioned medium which contain undefined attachment factors) as well as dishes coated with different densities of matrix molecules (13).

Observed matrix-dependent variations in BCE cell growth responsiveness could be due to modulation of growth factor-receptor number or affinity (37). Alternatively, distinct ECM molecules could bind growth factor directly and differ in their abiliy to present factor to adjacent cells. However, these possibilities are unlikely since we have been able to obtain similar results by pre-incubating suspended cells with HDGF and then plating the cells on ECM in the absence of growth factor (unpublished observation). The sensitivity of corneal and mammary epithelial cells to EGF similarly differs on different substrata although receptor binding is not affected (15,39). As previously suggested (15,39,40), receptor occupancy by polypeptide growth factors may not be sufficient for stimulation of DNA synthesis. While ECM components could also directly affect growth factor

action by influencing cell surface growth factor receptor clustering (2,40), transduction of this signal apparently must still be linked in some way to changes in cell and nuclear geometry.

It is also important to clarify that DNA synthesis was not linked to BCE cell shape per se in these studies, but rather to cell and nuclear size or extension. For example, HDGF-stimulated cells on laminin were thin and elongated while unstimulated cells on type III collagen appeared more regular and polygonal in form. Yet, these cell populations with very different "shapes" displayed similar projected cell and nuclear areas and incorporated nearly the same amount of $^3$H-thymidine on a per cell basis. These observations indicate that regulation of cell growth by "cell shape" may actually represent a process in which DNA metabolism is in some way coupled to physical expansion of the cell and/or nucleus rather than to a distinct cell morphology (e.g., polygonal versus bipolar).

Endothelial growth factor-induced changes in cell extension could alter intracellular molecular arrangements and cell metabolism in many ways. For instance, matrix-dependent alterations of cell form can produce changes in cytoskeletal structure which may affect the position, and hence function, of much of the cell's metabolic machinery (e.g., membrane receptors, transport proteins, mRNA, polysomes, nuclear pore complexes) (4,9,46,47). Alternatively, the significance of cell size changes could be based on mechanical coupling between cell and nuclear assemblies since nuclei are physically interconnected with cell surfaces by a continuity of cytoskeletal elements (9,25). For example, we have previously shown that inorganic cell models which similarly depend upon tensional continuity for their integrity (i.e., tensegrity models) can predict the coordination between cell spreading and nuclear extension observed in the present study (20). A cell may be considered as a tensegrity structure since it is comprised of a discontinuous array of compression-resistant struts (e.g., microtubules) that are interconnected by a continuous series of tensile elements (e.g., contractile microfilaments)(20,21).

Cell shape changes are commonly made possible by mechanical interactions in which tensile forces generated within the cytoskeleton are resisted by extracellular attachment sites (18,20). Studies with tensegrity cell models similarly show that the three dimensional form of a cell and its nucleus are determined through a dynamic balance between underlying tensile and compressive forces (20). It is possible that these structural forces could provide the regulatory information that is commonly thought to be conveyed by "cell shape" modulation. For example, if a tension-dependent architectural system is used by cells, then externally-modulated changes of cell geometry (e.g., through interference with cell anchorage as observed in the present study) could affect complementary force interactions between ECM, microfilaments, and microtubules and so result in local alterations of cytoskeletal filament assemly (20,26). [A thermodynamic basis for the effects of tension and compression on intracellular chemical reaction and cytoskeletal polymerization rates has been previously described (19,20).] Furthermore, cytoskeletal rearrangements that alter nuclear structure (e.g., induction of nuclear expansion) could change the arrangement and function of DNA regulatory proteins that are physically associated with the nuclear protein matrix (e.g., DNA polymerase, topoisomerase II) and thus directly promote DNA replication (38). Interestingly, physical swelling of isolated nuclei has been shown to similarly result in derepression (i.e., stimulation) of DNA synthesis in vitro (5).

Thus, we propose that expansion of the cell and/or nucleus may represent common structural intermediates in the physiological regulatory mechanism by which ECM and soluble growth factors interplay to control endothelial cell growth. Endothelial growth factors appear to function by promoting cell surface interactions with ECM components that stimulate cell extension. ECM molecules, in turn, may control growth factor action locally by either supporting or preventing changes of cell and nuclear structure. ECM may similarly serve as a local regulator of endothelial cell extension and growth factor action in vivo since induction of ECM dissolution during active neovascularization correlates with endothelial cell rounding (i.e., prohibition of spreading), inhibition of growth, and capillary regression (23). In this manner, angiogenesis may be triggered and sustained by soluble chemical stimuli acting over large distances (millimeters) whereas development of distinct tissue patterns would be directed locally (across nanometers) through a system of "solid-state" regulatory controls.

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## EDITOR'S STATEMENT

This manuscript describes the important relationship between growth factors and extracellular matrix components and nuclear and cytoplasmic structure. It further demonstrates that matrix molecules may play a regulatory role in angiogenesis by preventing endothelial cells from undergoing cytostructural reorganization in response to growth factors.

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