Mechanisms of Signal Transduction by Hormones
and Growth Factors, pages 273-282

REGULATION OF ENDOTHELIAL GROWTH FACTOR ACTION: SOLID STATE CONTROL BY EXTRACELLULAR MATRIX

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Studies on cellular mechanisms of signal transduction usually focus on the process by which extracellular stimuli alter plasma membrane components and trigger release of intracellular chemical messengers. This presentation will focus on possible mechanisms by which neighboring cells within the same tissue can respond differently to the same soluble mitogens. Differential cell growth responsiveness is central to morphogenetic processes, such as angiogenesis, which involve coordinated alterations of tissue form. We will discuss a model of tissue regulation in which extracellular matrix (ECM) serves as a local "solid state" regulator of soluble growth factor action through its ability to modulate cell and nuclear structure.

REGULATION OF ANGIOGENESIS

Our laboratory is interested in the mechanism by which blood vessel growth is regulated. Soluble endothelial growth factors have now been purified which can stimulate capillary endothelial cell proliferation in vitro and induce neovascularization in vivo (Shing et al., 1984; Klagsbrun et al., 1986). However, the growth-stimulating action of angiogenic factors is not sufficient to explain generation of complex branching capillary networks. For instance, in the absence of additional regulatory controls, repeated rounds of mitogen-induced cell doublings would simply result in a disorganized pile of endothelial cells. To produce morphogenetic alterations of tissue form, local endothelial cell growth differentials must be established in the midst

of high concentrations of soluble endothelial mitogens. Thus, to understand the process of angiogenic regulation, we must first analyze the mechanism by which endothelial growth factor action is selectively prohibited or supported locally.

Regional variations in ECM integrity and composition could be involved in local regulation of angiogenesis. During development, maintenance of intact basement membrane stabilizes tissue morphology (Bernfield and Banerjee, 1978; Ingber et al., 1981; Ingber et al., 1986) and local alterations of matrix turnover appear to be required for changes in tissue form (Bernfield and Banerjee, 1978; Wicha et al., 1980). ECM breakdown is one of the earliest events in neovascularization and local variations in ECM integrity and composition are observed throughout capillary development (Folkman, 1982). Purified ECM molecules can also modulate the growth and organization of capillary endothelial cells in vitro (Madri and Williams, 1983). However, the mechanism by which the growth-promoting action of soluble endothelial mitogens could be fine-tuned locally by ECM remains unclear.

SOLID STATE REGULATION OF GROWTH FACTOR ACTION

We have previously proposed that the growth and development of an entire society of cells (e.g., capillary endothelium) could be controlled through structural alterations of ECM that produce associated changes in cell shape and associated physical force redistributions (Ingber et al., 1981; Ingber and Jamieson, 1985). This model is based on the following observations: 1) cell shape has been previously shown to be permissive for entrance of anchorage-dependent cells into the synthetic phase of the cell cycle (i.e., in the presence of serum) with the most spread cells exhibiting the highest levels of DNA synthesis (Folkman and Moscona, 1978; Gospodarowicz et al., 1978; Ben Ze'ev et al. 1980), 2) cell attachment and spreading are commonly mediated by deposition of ECM components (Madri and Stenn, 1982; Wicha et al., 1979) or through adhesion to exogenous matrix-containing substrata (Gospodarowicz et al., 1978; Salomon et al., 1981; Ingber et al., 1986), 3)cells exert tensile forces on culture substrata via their adhesion sites (Emerman and Pitelka, 1977; Harris et al., 1980; Ingber and Jamieson, 1985), and 4) inorganic cell models that are built according the rules of tensile architecture (i.e., "tensegrity") can be used to predict many alterations of cell form that are observed experimentally (Ingber and Jamieson, 1985; Joshi et al., 1985; Ingber et al., 1986). [A cell may be considered as a tensegrity system 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).]

These observations suggest that cell shape may actually represent a dynamic balance of structural forces which, in turn, could provide regulatory information. For example, if a tension-dependent system is used by cells, then externally modulated changes of cell geometry (e.g., through interference with cell anchorage) could affect complementary force interactions between ECM, actin filaments, and microtubules and so result in local alterations of cytoskeletal filament assembly (Ingber and Jamieson, 1985; Joshi et al., 1985). A thermodynamic basis for the effects of tension and compression on polymerization of individual microtubules and actin filaments has been described (Hill and Kirschner, 1982). Indeed, both attachment to exogenous ECM components (Sugrue and Hay, 1981; Ingber et al., 1986) and external application of mechanical forces at the cell surface (White et al., 1983; Bray, 1984) can induce cytoskeletal rearrangements.

ECM-dependent alterations of cell geometry that affect cytoskeletal polymerization could alter cell responsiveness to soluble mitogens by a variety of mechanisms. The cytoskeleton appears to serve as an intracellular orienting scaffolding that can control the position, and possibly function, of much of the cell's metabolic machinery (e.g., mRNA, polysomes, nuclear protein matrix) (Wolosewick and Porter, 1979; Cervera et al. 1981). For example, effects of cell shape on cell metabolism could be mediated by associated alterations of membrane transport proteins or growth factor receptors since the distribution, mobility, and function of plasma membrane proteins may be regulated by the cytoskeleton (Yahara and Edelman, 1975; Weatherbee, 1982). Externally-modulated changes in cytoskeletal organization could also affect nuclear metabolism directly. Intermediate filaments within the cytoskeleton appear to physically link the nucleus to points of cell-cell and cell-substratum contact (Fey et al., 1984; Jones et al., 1985)

suggesting that mechanical forces may be rapidly distributed between extracellular, cytoplasmic, and nuclear structural proteins. Packing of DNA within the nuclear protein matrix, and so possibly regulation of its replication and transcription, may also be explained in largely mechanical terms (Luchnik et al., 1982; Pienta and Coffey, 1984; Villeponteau et al., 1984).

Thus, mechanical stimuli that are distributed over ECM attachment sites and cytoskeletal filaments could be transduced into chemical information via changes in thermodynamic parameters (e.g., Gibbs Free Energy) that promote local alterations in the form, arrangement, and function of distinct macromolecular complexes (Ingber and Jamieson, 1985). [Architectural stresses can be translated into pressure and volume changes through the use of stress tensors.] Changes of nuclear structure, such as nuclear expansion, could increase access to enzymatic sites, promote DNA unfolding, and facilitate its replication; topoisomerase II and DNA replication forks appear to be physically associated with the nuclear protein matrix (Pardoll et al., 1980; Villeponteau et al., 1984). Interestingly, physical swelling of isolated nuclei results in derepression of DNA synthesis in vitro (Coffey et al., 1974).

In context of the regulation of blood vessel development, this mechanochemical model would predict that while endothelial cell growth may be stimulated by angiogenic factors acting over large distances, distinct growth patterns could be controlled locally by alterations of capillary ECM integrity or composition that lead to redistributions of structural forces and associated changes of capillary endothelial cell and nuclear shape.

EXPERIMENTAL RESULTS

We recently began to test these hypotheses in context of the problem of angiogenic regulation. We have developed an in vitro system to explore the possiblity that the growth-promoting action of cationic, heparin-binding angiogenic factors may be regulated locally by ECM through its ability to modulate capillary endothelial cell form (Ingber, D.E., Madri, J.A., & Folkman, J.- submitted to PNAS). These studies were carried out in the absence of serum so that we could determine effects that resulted

solely from interactions between bovine capillary endothelial (BCE) cells, purified endothelial growth factors, and distinct ECM components.

Purified ECM molecules including laminin, fibronectin, types III, IV, & V collagens (obtained through a collaboration with Dr. Joseph Madri, Yale University School of Medicine) and gelatin were adsorbed to non-adhesive microtiter plates (100 ng/ cm^2) and tested for their ability to support BCE cell spreading and DNA synthesis. Capillary endothelial cells could not attach to these dishes in the absence of adsorbed matrix components, even in the presence of growth factor. BCE cells were exposed for 18 hr to saturating amounts of human hepatoma-derived growth factor (HDGF, 2 ng/ml; kindly provided by Dr. Michael Klagsbrun, Harvard Medical School) or normal pituitary basic fibroblast growth factor (FGF, 100 ng/ml; Collaborative Research) in Dulbecco's Modified Eagles Medium containing 1% bovine serum albumin. HDGF and FGF are members of a class of cationic, heparin-binding endothelial growth factors that share sequence homology, antigenic sites, and the ability to induce neovascularization in vivo (Klagsbrun et al., 1986; Lobb et al., 1986). Effects on DNA synthesis were measured by analyzing incorporation of ^{3}H-thymidine into TCA-precipitable material.

Computerized morphometric analysis was carried out using a Zeiss Interactive Digital Analysis System to quantitate alterations in BCE cell size. Cell and nuclear circumferences were outlined with a light cursor within images projected on a digitizing tablet (1,000x magnification). Mean cell and nuclear areas were determined by the computer. We have previously shown that alterations of projected cell area correspond directly with changes in total cell volume (Folkman and Moscona, 1978).

Results of these studies revealed that the action of purified endothelial growth factors differed depending on the matrix molecule used for cell attachment (Fig 1). In the presence of saturating amounts of HDGF, BCE cells on gelatin and types III and IV collagens increased their DNA synthetic levels 6 to 8 fold. While cells on fibronectin and type V collagen also exhibited large increases in DNA synthesis (5 to 6 fold), cells on laminin only doubled their synthetic rates.

Capillary endothelial cell spreading also varied depending upon the presence of soluble growth factors and the type of ECM component used for cell attachment (Fig. 2). In the absence of growth factor, BCE cells were smallest and most rounded on laminin (350 μm^2) while cells on all other substrata were larger and more polygonal in form (600 to 800 μm^2). Addition of saturating amounts of HDGF induced extensive cell spreading on gelatin and types III and IV collagens (approximately 1400 to 1600 μm^2). Growth factor-stimulated cells on type V collagen and fibronectin similarly displayed large increases in projected cell areas (1150 to 1350 μm^2). While cells on laminin responded to HDGF by increasing in size, they still remained extremely small (600 μm^2).

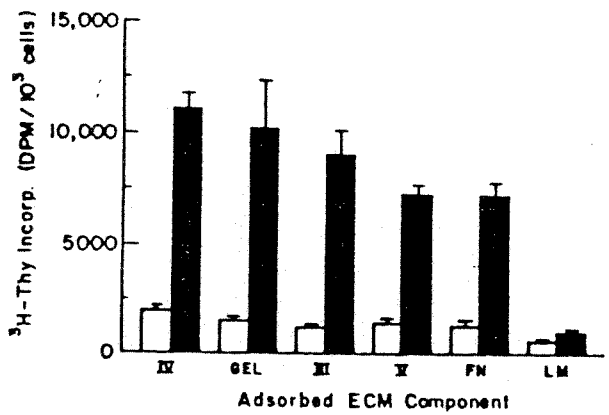

Fig. 1. Regulation of BCE Cell DNA Synthesis by ECM and HDGF. Open bars, serum-free medium alone; Closed bars, medium supplemented with HDGF; IV, type IV collagen; GEL, gelatin; III, type III collagen; V, type V collagen; FN, fibronectin; LM, laminin. Error bars indicated standard error of the mean.

When alterations of cell spreading were contrasted with observed effects on DNA synthesis, we found that DNA synthetic rates altered in an exponential fashion directly in relation to changes in cell size regardless of the presence of growth factor or of the molecule used for cell attachment (Fig. 2). For example, BCE cells responded to

growth factors by spreading on laminin, but only covered an area similar to that exhibited by unstimulated cells on other substrata (e.g., type III collagen). Similarly sized cells in both situations displayed similar levels of DNA synthesis. Interestingly, coordinated alterations in cell and nuclear form were also observed in these cells. Thus, linear increases in projected nuclear areas were associated with exponential increases in DNA synthesis (Fig. 3).

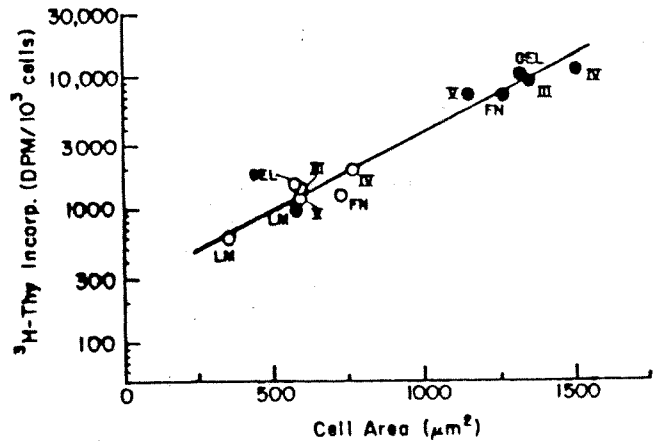

Fig. 2. Relation Between BCE Cell Size and DNA Synthesis. Open circles, serum-free medium alone; Closed circles, medium supplemented with HDGF. Note that ^{3}H-thymidine incorporation is plotted on a logarithmic scale. Solid line represents an exponential regression curve best fit to the data points.

Observed variations in BCE cell growth responsiveness could have been due to ECM-dependent modulation of growth factor membrane receptors with cell shape changes representing only a secondary effect. Similarly, adsorbed ECM molecules could bind growth factor and differ in their ability to present factor to adjacent cells. However, preliminary studies suggest that these possibilities are unlikely. For example, a similar dependence of growth factor action upon ECM-related alterations of cell size could be demonstrated by pre-incubating suspended BCE cells

with HDGF (3 hr at 37°C) and then plating the cells on ECM-coated plates in the absence of growth factor (not shown). The sensitivity of corneal and mammary epithelial cells to EGF similarly differs on different substrata although receptor binding is not affected (Gospodarowicz et al., 1978; Salomon et al., 1981). These findings suggest that the action of polypeptide growth factors may require structural alterations that are secondary to growth factor receptor binding, rather than being solely the result of receptor occupancy. Receptor clustering and associated cytoskeletal alterations may be involved in this process (Schreiber et al., 1982; Weatherbee, 1982).

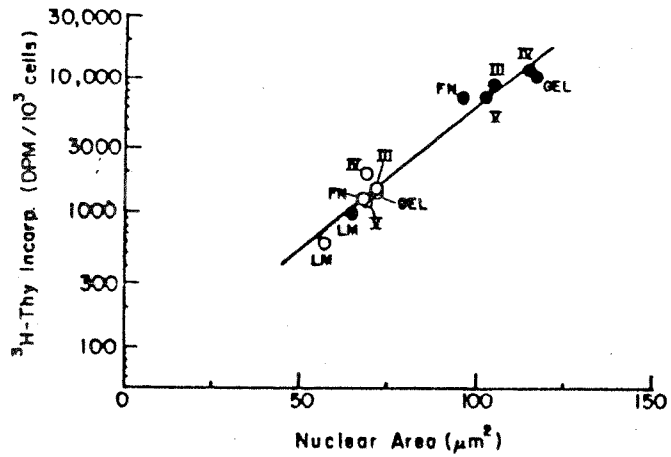

Fig. 3. Relation Between BCE Nuclear Size and DNA Synthesis. Open circles, absence of HDGF; Closed circles, presence of HDGF. Solid line represents an exponential regression curve best fit to the data points.

CONCLUSIONS

These findings demonstrate that endothelial growth factors apparently act by promoting capillary endothelial cell extension. ECM in turn seems to control the growth-promoting action of angiogenic factors by selectively prohibiting or supporting changes of cell and nuclear structure. The possibility that ECM-dependent alterations of cell shape serve to regulate angiogenesis in vivo is also

supported by our recent finding that compounds that induce capillary regression (e.g., angiostatic steroids and heparin; Crum et al., 1985) produce capillary basement membrane dissolution, loss of cell anchorage, and associated endothelial cell rounding (Ingber et al., 1986a). [Endothelial cells similarly can not survive in a round, unattached state in vitro (Folkman and Moscona, 1978).]

In this manner, capillary growth may be triggered and sustained by soluble factors acting over large distances (millimeters) whereas development of distinct tissue patterns may be directly locally (across nanometers) through structural changes of ECM that alter endothelial cell form. In this "solid state" system of regulatory controls, ECM-dependent alterations of cytoskeletal and nuclear structure would act in a permissive fashion to modulate the effects of soluble mitogens on intracellular biochemical transduction cascades and DNA metabolism. Studies are currently underway to analyze the molecular mechanisms by which alterations of ECM, cell shape, and physical force distributions can modulate cytoskeletal arrangements, nuclear matrix organization, and release of intracellular chemical messengers in response to growth factor stimulation.

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