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Extracellular Matrix and Cell Shape: Potential Control Points for Inhibition of Angiogenesis

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Abstract Capillary endothelial (CE) cells require two extracellular signals in order to switch from quiescence to growth and back to differentiation during angiogenesis: soluble angiogenic factors and insoluble extracellular matrix (ECM) molecules. Soluble endothelial mitogens, such as basic fibroblast growth factor (FGF), act over large distances to trigger capillary growth, whereas ECM molecules act locally to modulate cell responsiveness to these soluble cues. Recent studies reveal that ECM molecules regulate CE cell growth and differentiation by modulating cell shape and by activating intracellular chemical signaling pathways inside the cell. Recognition of the importance of ECM and cell shape during capillary morphogenesis has led to the identification of a series of new angiogenesis inhibitors. Elucidation of the molecular mechanism of capillary regulation may result in development of even more potent angiogenesis modulators in the future.

Key words: fibronectin, integrins, signal transduction, growth factors, angiogenic factors, capillary differentiation, mechanical tension

Solid tumors require continual neovascularization for their sustained growth [1]. While tumor angiogenesis may be a potentially specific target for cancer chemotherapy in the future, development of new modes of therapeutic intervention is currently limited by an incomplete understanding of the mechanism of angiogenic regulation. Recent identification, purification, and cloning of multiple endothelial mitogens has resulted in better insight into the mechanism by which angiogenesis is initiated [2]. However, the growth-promoting action of angiogenic mitogens by itself is not sufficient to explain how functional capillary networks develop. For example, formation of branching capillary networks requires that local differentials of cell growth and differentiation must be established in a tissue microenvironment which is saturated with soluble mitogens. One CE cell responds to mitogenic stimulation by sprouting from a preexisting vessel, while its neighbors, only microns away, remain quiescent. Thus, to understand the basis of angiogenic regulation within a tissue microenvironment, we must first elucidate the mechanism by which CE cell growth is selectively supported or prohibited locally.

ECM AS A TARGET FOR ANGIOGENESIS INHIBITION IN VIVO

The first clue to this local mechanism of angiogenic control came from studies that were initiated to analyze the mechanism of capillary involution. In 1983, Dr. Judah Folkman reported that combination of heparin with steroids, such as hydrocortisone, inhibited embryonic angiogenesis in the chick chorioallantoic membrane and produced regression of a variety of solid tumors in mice [3]. He later defined an entire new class of "angiostatic" steroids that lack all known glucocorticoid, mineralocorticoid, and sex steroid activities, but retain the ability to inhibit capillary growth when administered with heparin [4]. Nothing was known about the mechanism by which steroid/heparin combinations inhibited angiogenesis when I joined the Folkman laboratory. However, we soon were able to demonstrate that steroid/heparin combinations produce capillary basement membrane (BM) dissolution as part of their anti-angiogenic action [5].

I focused on BM as a potential target for angiogenesis inhibitors based on past work which

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demonstrated a direct correlation between BM breakdown and epithelial involution in other tissues which undergo physiological regression, such as mammary epithelium [6] and Mullerian Duct [7]. In subsequent studies, we also were able to confirm that the anti-angiogenic activity of steroid/heparin combinations correlated directly with their ability to inhibit collagen accumulation using biochemical techniques [8]. Furthermore, we could demonstrate that ECM changes were the cause rather the result of capillary involution. For example, specific inhibitors of collagen deposition and processing (e.g., proline analogues, α,α-dipyridyl, β-aminopropionitrile, retinoids) were shown to be potent inhibitors of angiogenesis [8]. These findings have been recently confirmed by other laboratories [9,10].

SWITCHING BETWEEN GROWTH AND DIFFERENTIATION WITHIN THE LOCAL MICROENVIRONMENT

To more directly address the question of how ECM molecules regulate CE cell responsiveness to soluble mitogens, we have developed a simplified in vitro system in which cell-ECM contact formation can be varied in a controlled fashion in the presence of a constant, saturating amount of soluble FGF [11]. We have done this by coating bacteriological plastic petri dishes, which are otherwise non-adhesive, with defined densities of purified ECM molecules, such as fibronectin (FN). Bovine adrenal CE cells are then cultured on these dishes in chemically-defined medium composed of DMEM supplemented with transferrin (5 μg/ml), high density lipoprotein (10 μg/ml), FGF (2 ng/ml), and 1% bovine serum albumin to quench non-specific binding sites on the dish.

Using this system, we found that CE cells attached well, spread, formed many cell-cell contacts, and organized within monolayers on high FN densities (> 500 ng/cm^2), much as they do in routine cultures using tissue culture substrata and serum-containing medium. Cells also attached to dishes coated with low FN densities (< 100 ng/cm^2); however, they could not spread and thus remained round within multicellular aggregates. In contrast, when CE cells were plated on moderate FN coating densities (100–500 ng/cm^2), they first spread, formed multiple cell-cell contacts, and then began to physically retract over a period of hours. Multicellular retraction resulted in formation of multicellular cords raised above the surface of the culture dish which reorganized into networks composed of interlinked capillary tubes within 24 to 48 h after cell plating. Importantly, studies using tritiated-thymidine autoradiography revealed that cells within the retracted tubes became quiescent, whereas neighboring cells that remained adherent and spread on the culture dish continued to synthesize DNA [11]. Similar results were also obtained using dishes coated with different densities of other ECM molecules (e.g., type IV collagen).

To summarize, these in vitro studies demonstrated that changes in the adhesivity of ECM which promoted cell retraction were able to switch off growth and turn on tube formation in a microenvironment (the culture dish) that was saturated with soluble mitogens (FGF). The major alteration that we observed in these cultures was a change of cell shape. Growing cells extended long processes over the surface of the FN-coated dish, whereas cells within differentiated tubes appeared to physically retract and round. Moreover, analysis of a variety of in vitro angiogenesis models consistently demonstrated the same relation in which rigid ECM-coated surfaces inhibited capillary differentiation and supported proliferation, whereas substrata that promoted cell retraction (e.g., malleable ECM gels, poorly adhesive dishes) induced formation of quiescent capillary tubes [11].

GROWTH CONTROL BY ECM THROUGH MODULATION OF CE CELL SHAPE

To address the question of whether or not cell shape modulation was important for growth control by ECM, we utilized the same in vitro system that we had used for inducing tube formation, except that lower cell numbers were plated [12]. In this manner, we could focus exclusively on the role of cell-ECM contact formation in growth control, without complication by cell-cell interactions. Again, studies were carried out in the presence of a saturating amount of soluble FGF. Using this method for modulating cell-FN contact formation, we found that cell and nuclear spreading increased in a dose-dependent fashion as FN coating densities were raised. Also, by carrying out computerized image analysis (to quantitate projected cell areas), measuring incorporation of tritiated-thymidine into DNA, and counting the number of adherent

cells in parallel wells, we could demonstrate that DNA synthesis levels increased in an exponential fashion as FN coating densities were increased and cell spreading was promoted [12].

In addition, we found that cell binding to FN was not sufficient for growth; large-scale changes of cell shape were also required for CE cells to enter S phase [12]. For example, round cells would not grow in suspension when cultured in medium containing high concentrations of soluble FN or FN-coated microbeads (4.5 μm diameter) which can bind and cluster cell surface ECM receptors, but can not support cell extension. Addition of soluble RGD containing peptides, which bind to members of the integrin family of ECM receptors, also did not support CE cell spreading or DNA synthesis. In fact, they induced cell rounding and suppressed growth when added to cells cultured on FN-coated dishes.

Taken together, these findings strongly suggest that it is the extent to which a CE cell physically stretches that determines its ability to grow in response to soluble angiogenic mitogens. It is important to emphasize that growing CE cells commonly appear much larger and more extended in length than their quiescent neighbors during neovascularization [13] and that angiostatic steroid/heparin combinations that induce capillary regression produce CE cell rounding in vivo [5].

ECM MOLECULES ACTIVATE INTRACELLULAR CHEMICAL SIGNALING PATHWAYS

How could ECM alter cell growth or differentiation by modulating cell shape? Clearly, to answer this question cell "shape" must be translated into molecular terms. One explanation for the effects we observed could be that changing cell shape results in exposure of different numbers of FGF receptors on the cell surface. However, we found that this is not the case. FN-dependent changes of cell shape do not alter the number or affinity of cell surface FGF receptors.

Instead, we found that FN controls growth directly by altering the "set-point" of chemical signaling pathways inside the cell. Working with Drs. Martin Schwartz (Scripps Institute of Vascular Biology) and Claude Lechene (Harvard Medical School), we found that binding of ECM molecules to transmembrane integrin receptors activates the Na^+/H^+ antiporter on the cell surface [14–16]. This antiporter is also activated by virtually all known peptide growth factors within minutes after they bind to their own cell surface receptors [17]. When the antiporter is activated, protons are extruded in exchange for sodium, and, thus, a net increase in intracellular pH (pH_i) results. Microfluorimetry can be used in combination with pH-sensitive dyes to quantitate intracellular pH within individual cells and thereby measure effects on antiporter activation.

Using microfluorimetry, we found that pH_i increased by approximately 0.18 pH units in CE cells cultured in FGF-containing medium as FN coating densities were raised and cell spreading was promoted [14]. To determine whether or not this was a direct effect of the binding of FN to its cell surface receptors or if it resulted from indirect modulation of FGF-induced signaling events, we simply removed FGF from the chemically-defined medium and carried out the same experiment. When FGF was removed, pH_i was lowered by approximately 0.06 pH units in CE cells, regardless of the FN density. However, we still observed a similar dose dependent increase in pH_i of approximately 0.18 pH units as FN coating densities were raised from low to high. These results indicate that FGF and FN exert their effects on the Na^+/H^+ antiporter via separate signaling pathways and that these effects are additive.

To confirm that pH_i was linked to FN receptor occupancy, we measured changes in pH_i induced during the initial phases of CE cell attachment and spreading on FN-coated dishes [14]. Cytoplasmic pH increased in a time-dependent fashion as cell spreading was promoted, with a significant increase in pH_i being observed within 10 min after plating. Furthermore, we could show that this effect resulted from increased Na^+/H^+ exchange, since the alkalinization was rapidly reversed by addition of ethylisopropylamiloride (EIPA), a specific inhibitor of the Na^+/H^+ antiporter. Importantly, addition of EIPA also inhibited DNA synthesis in CE cells at a similar dose range to that which suppressed the rise of pH_i, demonstrating that activation of the antiporter by FN was required for growth.

Given these effects, we decided to carry out experiments to ask how the binding of FN to specific cell surface ECM receptors regulates Na^+/H^+ exchange and thereby controls CE cell growth. To determine whether or not changes of cell shape were necessary, we measured pH_i within cells that were allowed to adhere to the

microbeads I described earlier, which were coated with FN, other adhesive ligands, or specific anti-integrin receptor antibodies [15]. When suspended CE cells bound FN-coated microbeads, they remained round. Nevertheless, pH_i increased in a bead-dependent manner, with maximal alkalinization being observed within cells that bound greater than 9 beads/cell. No alkalinization was observed in cells that bound to beads coated with non-specific ligands, such as polylysine or acetylated-LDL, even though both bind well to CE cell surfaces.

Importantly, beads coated with monoclonal antibodies against either the α5 or β1 integrin chains were able to induce a similar increase of pH_i in round cells [15]. Additional studies using soluble integrin antibodies and Fab fragments revealed that integrin receptor occupancy is not sufficient for antiporter activation. While integrin clustering alone can transmit a transmembrane chemical signal (i.e., activate Na^+/H^+ exchange), this signal is rapidly terminated upon receptor internalization. Thus, to maintain a sustained growth signal, the ECM ligand must be immobilized on a surface that can both cluster integrins and physically resist their removal (by cytoskeletal tension) from the cell surface. In this manner, integrins differ considerably from growth factor receptors. This finding may in part explain why ECM molecules must be insoluble or immobilized in order to support cell proliferation.

It is important to reemphasize that while activation of chemical signaling pathways by ECM is required for growth, it is not sufficient. Binding of FN-coated microbeads increases pH_i in round CE cells; however, it does not stimulate DNA synthesis [12]. Thus, it appears that integrin clustering may be necessary to induce the cascade of intracellular signaling events that characterize the G_0/G_1 transition. However, large-scale changes of cell shape are also required for subsequent cell cycle progression and entry into S phase. Adhesion to ECM may therefore control cell cycle progression in CE cells by activating two integrated signaling pathways: 1) by stimulating release of chemical second messengers which are required for entry into the growth cycle from the resting state, and 2) by promoting cytoskeletal alterations that are necessary for large-scale changes of cell shape and hence, for entry into S phase (Fig. 1). Apparently, both pathways must be activated by ECM in order for soluble mitogens to exert their maximal effects on both second messenger systems and cell proliferation.

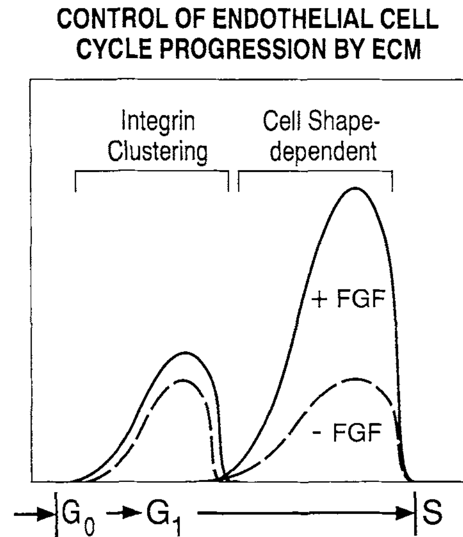

Fig. 1. Diagramatic representation of control of endothelial cell cycle progression by ECM. Integrin clustering alone is sufficient to activate chemical signaling pathways and growth-related genes that are associated with the G_0/G_1 transition. Soluble angiogenic factors (e.g., FGF) produce additive effects on chemical second messenger systems, such as Na^+/H^+ exchange. For cells to progress through G_1 and into S phase, large-scale ECM-dependent changes of cell shape are also required. Soluble growth factors synergize with these cell shape alterations and induce cell proliferation. Growth factors may also promote growth in part by stimulating additional cell spreading [29].

MECHANICAL SIGNALING ACROSS CELL SURFACE INTEGRIN RECEPTORS

The finding that cell shape changes are important for cell cycle progression suggests that cells also may be sensitive to mechanical signals that are transmitted across ECM. In fact, recent studies suggest that ECM and transmembrane integrin receptors may provide a molecular mechanism for transduction of mechanical forces into a biochemical response inside the cell [18]. The concept that ECM molecules may convey information in the form of mechanical forces is also based in part on studies using three dimensional "cell" models that are constructed out of sticks and elastic string [19,20]. These structures are known as "tensegrity" models because they are built according to the rules of an architectural system, first described by Buckminster Fuller, which depends on tensional integrity, rather than compressive forces for its stability. Tensegrity structures are constructed by interconnecting a *discontinuous* series of compres-

sion-resistant struts with a *continuous* series of tension elements.

Studies with tensegrity cell models predict that both cell and nuclear shape are determined through a dynamic balance of mechanical forces which are generated within contractile microfilaments and resisted by both internal cytoskeletal struts (e.g., microtubules and/or large actin bundles) and external adhesion sites (i.e., ECM) [19,20]. Tensegrity models take on round forms when free of attachment because this is the minimum energy form in a symmetric structure that generates internal tension. The same structure spreads out and takes on a flattened form when it is physically attached to a rigid foundation. Furthermore, it spontaneously pulls in and retracts when attached in a similar manner to a malleable foundation. These observations predict results obtained within living cells cultured on malleable ECM gels [19], silastic rubber substrata [21], or ECM coating densities that can not resist cell-generated forces [11]. Recently, PC 12 neurites have been shown to use a tensegrity arrangement for organization of their cytoskeleton [22]. Studies in my laboratory using both intact and membrane permeabilized cells confirm that CE cells also use this type of architectural system (Ingber and Karp, submitted).

The importance of the tensegrity models is that they suggest that ECM molecules may convey different regulatory signals depending on whether or not they can sustain or resist high levels of cytoskeletal tension. For example, CE cells [23] as well as many other cell types can be switched from growth to differentiation by plating them on malleable ECM gels (e.g., Matrigel) which promote cell retraction and rounding. However, malleable ECM gels lose their differentiation-inducing effects when they are made rigid [24]. Similarly, in our studies, we could switch CE cells from differentiation to growth by increasing FN coating densities. Recent studies confirm that this method also increases cell tension [25], probably by promoting focal adhesion formation and hence, increasing the cell's ability to tranfer tension between the cytoskeleton and the substratum [26].

Taken together, these results suggest that ECM molecules may control capillary morphogenesis based on a mechanochemical mechanism, rather than one which is purely chemical in nature. The regulatory signals that ECM molecules convey depend on their ability to both bind specific cell surface receptors (e.g., integrins) and physically support mechanical loads that are applied to these receptors. Cell responsiveness to soluble angiogenic mitogens may therefore be controlled in the local microenvironment by altering either the composition or mechanical integrity of the ECM.

GROWTH CONTROL BY ECM AND CELL SHAPE AS TARGETS FOR ANGIOGENESIS INHIBITION

As I described above, recognition of ECM as a potential regulatory element during angiogenesis led to the identification of series of new angiogenesis inhibitors that share an ability to modulate collagen metabolism [8]. While the concept of a biomechanical signaling mechanism based on cell shape changes may seem bizarre, this working hypothesis has also led to the discovery of a new type of angiogenesis inhibitor which we feel may be very important for clinical applications. Based on our work on the role of cell shape in growth control, I decided to culture (rather than discard) a fungal contaminant that developed spontaneously in one of my routine CE cell cultures and which appeared to induce a gradient of cell rounding [27]. Subsequent identification of the active rounding agent as fumagillin and synthesis of novel fumagillin analogues (working in collaboration with Dr. Judah Folkman and scientists at Takeda Chemical Industries, Ltd.) led to identification of a new class of angiogenesis inhibitors which we have called "angioinhibins." Angioinhibins inhibit the growth of cultured endothelial cells and prevent angiogenesis both in vitro and in vivo [27,28]. Their specificity is based on the fact that they only inhibit growing endothelial cells and thus, they are not toxic to quiescent cells within vessels in normal tissues. Angioinhibins are particularly exciting because they also suppress the growth of a wide variety of animal tumors without producing any significant side effects [27]. We expect that one of the angioinhibins (AGM-1470) will be entering Phase I clinical trials over the next year.

Angioinhibins may provide a glimpse of a new field of pharmacological modifiers that can inhibit neovascularization in response to all types of soluble stimuli. This type of therapy would be greatly preferred over specific growth factor antagonists, given the incredible redundancy of soluble stimulators that are involved in the neovascularization response [2]. It is likely that

the first targets for angiogenesis inhibitors will be solid tumors. However, non-toxic angiogenesis inhibitors also may be useful for treatment of other pathological conditions that exhibit uncontrolled capillary growth as a major clinical manifestation (e.g., rheumatoid arthritis, diabetic retinopathy, psoriasis, etc.). The series of compounds that were described in this article were discovered based on the recognition of the importance of ECM and cell shape control during angiogenesis. Further characterization of the molecular mechanisms involved in control of chemical and cytoskeletal signaling pathways by ECM may lead to identification of new and even more powerful angiogenesis inhibitors in the future.

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