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Angiogenesis: A unifying model for the proliferative and differentiation phases of capillary formation.

Corey Keith Goldman

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ANGIOGENESIS: A UNIFYING MODEL FOR THE PROLIFERATIVE AND DIFFERENTIATION PHASES OF CAPILLARY FORMATION

by

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A DISSERTATION

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Blood vessels are required in multicellular organisms to transport nutrients and growth factors to cells and remove toxic waste metabolites. Endothelial cells comprise the lining of all blood vessels and are responsible for the formation of capillaries. Simply stated, capillaries are endothelial cells positioned in an array that form a tube-like conduit for the passage of blood. The growth of capillaries is a complex, albeit ubiquitous process in both physiogenic and pathogenic conditions.

This thesis addresses the sequence of molecular events that result in capillary formation. The studies resolve a paradox in understanding the behavior of endothelial cells during angiogenesis. The growth of capillaries requires the proliferation of endothelial cells; however, capillary tube formation is associated with suppressed endothelial cell proliferation.

The results of these experiments suggest a paradigm whereby the endothelial cell is stimulated by the direct angiogenic growth factor, VEGF to proliferate and produce proteases. The proteases can then act locally on surrounding extracellular matrix to liberate active fragment molecules. This research demonstrates that protease degradation of the extracellular matrix protein fibronectin results in formation of fragments that will suppress endothelial cell proliferation and induce formation of endothelial cell sprouts, a precursor to capillaries. These results provide a comprehensive model explaining the neovascularity of tumors and present several mechanistic targets for the blockade or enhancement of angiogenesis.

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I gratefully thank my mentor, Dr. G. Yancey Gillespie, for his guidance, assistance, support, inspiration, and persistence without whom this work could never have been realized.

I wish to acknowledge all who have spent their lives in science with the goal of enhancing the quality of life for others.

I am grateful to God, who has given me life and the inspiration to pursue this research.

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LIST OF ABBREVIATIONS

Con-A	Concanavalin A
DNA	Deoxyribonucleic acid
DVT	Deep venous thrombosis
EGF	Epidermal growth factor
EGFr	Epidermal growth factor receptor
EC	Endothelial cell
ECM	Extracellular matrix
FN	Fibronectin
GF	Fibroblast growth factor
GBM	Glioblastoma —ultiforme
HBGF	Heparin binding growth factor
HPLC	High pressure liquid chromatography
mRNA	messenger ribonucleic acid
PD-ECGF	Platelet-derived endothelial cell growth factor
PDGF	Platelet-derived growth factor
RT	Room temperature
TGF-α	Transforming growth factor–alpha
TGF-β	Transforming growth factor–beta
TNF-α	Tumor necrosis factor–alpha
VEGF	Vascular endothelial growth factor

INTRODUCTION

Angiogenesis, or the formation of new capillaries, is a complex biological phenomenon that follows a highly ordered sequence of molecular interactions among parenchymal cells, endothelial cells and the intervening interstitial extracellular matrix with cytokines and enzymes providing important soluble signals [1–7]. Under physiological conditions, angiogenesis is coincident with tissue growth (i.e., organogenesis), or repair of tissue damage (i.e., wound healing). Angiogenesis is also an integral part of many disease states such as malignancy [8, 9] and arthritis [10, 11]. In some disease states, growth of new blood vessels is central to the morbidity of the disease process. Examples of these processes include proliferative retinopathy [12], moya-moya disease and von Hippel-Lindau disease [13].

Anti-angiogenesis therapy is an attractive concept, but rational design of therapeutic agents has not been advanced because the individual molecules responsible for capillary formation have not been identified. To identify individual molecules directly involved in angiogenesis, I have focused on *glioblastoma multiforme* tumors because of the intense neovascularity characteristic of this tumor.

Angiogenesis: A Process, not an Event

In 1972, Folkman [2] hypothesized that angiogenesis is mediated by release of substances that support growth of blood vessels. Since then, several angiogenic molecules have been isolated, and include fibroblast growth factor (FGF) [14–16], platelet derived-endothelial cell growth factor (PD-ECGF) [17], transforming growth factor-α (TGF-α) [18], transforming growth factor-β (TGF-β) [19], tumor necrosis factor-α (TNF-α) [20], angiogenin [21] and vascular endothelial growth factor (VEGF) [22, 23]. The concept of

an angiogenic molecule was advanced by Folkman [3] and includes any substance capable of inciting neovascularization in vivo. With this definition, the list of angiogenic molecules grows enormously, including many that may not be relevant to physiologic states. Mechanistically, angiogenic factors can be broadly characterized as either direct or indirect angiogenic factors. A direct angiogenic factor is a substance that stimulates endothelial cell proliferation in vitro and induces neovascularization in vivo. FGF, VEGF and PD-ECGF are examples of direct angiogenic factors. It is important to point out that endothelial cell proliferation alone does not constitute angiogenesis. A specific example of this is the clinical entity known as an endothelioma [24] that presents as a slowly growing mass of endothelial cells. This demonstrates that endothelial cell proliferation in the absence of morphological differentiation does not result in neovascularization. An indirect angiogenic factor is a substance that induces capillary formation and neovascularization in vivo but acts through mechanisms other than endothelial cell proliferation. These molecules may alter the responsiveness of the endothelial cell to factors already present in the cellular milieu. Examples of such factors include TGF-β [19], which suppresses endothelial cell proliferation, TNF-α [20], which has variable effects on cell proliferation [9], and angiogenin [21], which probably has no direct action on endothelial cells. Until the mechanism underlying angiogenesis is better understood, concepts such as direct and indirect angiogenic factors provide useful operational definitions.

The formation of endothelial cell tubes in vitro provides an attractive model for dissection of underlying mechanisms of angiogenesis [6, 14, 25]. Tube formation can be induced by diverse methods, such as culture of endothelial cells on particular extracellular matrix proteins [15, 20, 26] or under reduced serum concentrations [25]. Regardless of the method employed, endothelial cells that display tube formation have decreased cell proliferation and diminished DNA synthesis. This finding highlights a potential paradox in angiogenesis definitions; namely, proliferating endothelial cells

obviously exhibit increased levels of DNA synthesis, while those undergoing tube formation have decreased rates of DNA synthesis and cell proliferation [3, 25]. A major question remains: how is it possible that angiogenesis is associated with endothelial cell proliferation and inhibition of proliferation? To resolve this paradox, it is important to regard angiogenesis not as a single event but as a well-orchestrated process that occurs as a series of events. At present there is scant data linking the apparently mutually exclusive phenomena of endothelial cell proliferation and tube formation [3, 4]. Data to be presented in this dissertation suggest a working framework to link these disparate phenomena and provide a paradigm with which multiple hypotheses can be tested.

Clinical Manifestations of Vascular Pathology in Malignant Neoplasms

Characteristic histologic features of aggressive tumors include extensive neovascularization, focal areas of necrosis and rapid cellular proliferation. Clinically, patients with tumors experience clinical consequences ranging from tumor edema secondary to leaky blood vessels. The most frequent and most malignant primary, intracranial neoplasm is glioblastoma multiforme (GBM), which manifests significant brain edema. The increased vascular permeability that produces peritumoral brain edema is the root cause of a life-threatening situation that can be partially controlled by progressively increasing dosages of glucocorticoids such as dexamethasone. Patients with breast cancer also experience clinical sequelae from vascular permeability, such as debilitating pleural effusions. Similarly, ovarian cancer spreads throughout the peritoneal cavity and is associated with severe abdominal ascites. Hypercoagulability, an increased propensity to clot formation, is a common manifestation of malignancy and results from pathological activation of the clotting cascade. Clinically, this manifests as an increased incidence of thrombophlebitis, deep vein thrombosis (DVT) and pulmonary emboli (PE). These phenomenon represent perturbations in the vascular endothelium, the chief role of which is to provide a non-thrombogenic surface for the passage of blood. Moreover,

occult malignancies may present initially as an acute thromboembolic event, which represents enhanced activation of the coagulation cascade, presumably initiated by the effects of the tumor on the endothelium.

Glioblastoma Multiforme: Malignancy-driven Angiogenesis

Glioblastoma multiforme is a highly vascularized and rapidly fatal malignancy in humans. It originates from transformation of the primary support cell in the brain, the astroglia, and constitutes the most frequent and malignant primary brain tumor with the poorest prognosis of all brain tumors [26]. Endothelial cell proliferation is a prominent histopathologic characteristic of this tumor and can be identified by light microscopy using standard histochemical techniques. A rich capillary network can be visualized angiographically in almost all patients with GBM.

VEFG: a Potent and Specific Angiogenic Growth Factor

The combination of biological activities attributable to VEGF is unique among protein cytokines closely paralleling the vascular pathology of GBM and likely accounts for several features of malignant tumors and metastasis. VEGF is highly specific for endothelial cells and can induce a broad range of phenotypic changes including endothelial cell proliferation and angiogenesis; enhanced endothelial cell calcium release that leads to increased vascular permeability [10]; and activation of the coagulation-related proteins, von Willebrand Factor and endothelial cell thromboplastin [27]. The clinical correlates for these biological activities are neovascularity, leaky vasculature and hypercoagulability, and are prominent features of malignancy. While myofibroblast invasion and fibrosis are prominent features in some types of healing wounds and may result from FGF-induced neovascularity, these are not common features of GBM tumors. Endothelial cell proliferation in the absence of myofibroblast proliferation strongly implicates vascular endothelial growth factor [23], rather than FGF [29], as the more

likely central mediator of angiogenesis in GBM. Furthermore, the brain edema surrounding GBM tumors occurs in "normal" brain, suggesting liberation of a vascular permeability factor i.e. VEGF, by the tumor [28].

In situ hybridization of human GBM tumor sections has revealed extraordinarily high levels of VEGF mRNA when compared to normal brain or benign intracranial tumors (Napoleon Ferrara: personal communication). These findings suggest that VEGF/VPF is the mediator of angiogenesis in malignant GBM.

In the tumor angiogenesis model proposed in this dissertation, VEGF is operationally and functionally defined as a direct-acting angiogenic factor capable of initiating the 'proliferative phase' of angiogenesis by stimulating the division of endothelial cells and the secretion of proteolytic enzymes. The presence and relevance of VEGF in GBM are discussed in the third paper of this dissertation, "Epidermal Growth Factor Stimulates Vascular Endothelial Growth Factor Production by Human Malignant Glioma Cells: A Model of Glioblastoma Multiforme Pathophysiology."

For purposes of defining the angiogenesis paradigm, the second phase of angiogenesis has been operationally labeled the "transitional phase." This phase involves generation of active molecules that regulate the early stages of capillary formation and results from secretion of proteases in the proliferative phase. The phenotypic changes that take place in the transitional phase include, suppression of thymidine uptake; ,formation of sprouts, and production of molecules mediating formation of capillary tubes. Proteases and extracellular matrix molecules, both products of the endothelial cell, interact in the transitional phase and result in the liberation of biologically active protein fragments. The second paper in this dissertation, "Extracellular Determinants of Sprout Formation in the Rabbit Endothelial Cell Line, REVC," addresses the transitional phase of angiogenesis.

Molecules generated during the transitional phase allow endothelial cells to develop sprouts that eventually become capillary tubes. This morphologic change represents the

final and "differentiation" phase of angiogenesis and is a topic that this dissertation puts forth as future research.

Angiogenesis related proteases: an undefined role. VEGF, like FGF, enhances production of the proteases, collagenase and plasminogen activator, by endothelial cells [30]. The substrate for these endothelial cell proteases is the surrounding extracellular matrix [31, 32]. Ultrastructural studies of budding capillaries have indicated that new capillaries originate from preexisting capillaries or postcapillary venules [33]. Additional microscopic studies have suggested the presence of a contiguous basement membrane at the basal border of venular endothelium [33]. The presence of a contiguous basement membrane has lead to speculation that its focal dissolution is a prerequisite for angiogenesis [21]. Hence, this speculation directly supported the hypothesis that the role for proteolytic enzymes in angiogenesis is to allow endothelial cells to degrade basement membrane and invade perivenular stroma [7]. This assumption has gone largely unchallenged and subsequent hypotheses have been tested accepting this as fact [34]. Lymphocytes and macrophages frequently cross endothelial basement membranes in transit to a target tissue and it is questionable whether proteases are significant to this process. Evidence is lacking that the sine qua non for endothelial cell-generated proteases is to "break" through the basement membrane. In this dissertation, I present evidence supporting the concept that the function of proteases in angiogenesis process is to generate active molecules by cleavage of extracellular matrix protein. These bioactive proteolytic fragments bind to the endothelial cell and induce cytoskeletal rearrangements resulting in sprout formation. Specifically, I hypothesize that transition from an endothelial cell monolayer to endothelial cell tubes is dependent upon local generation of fibronectin (FN) fragments by enzymes. It is these fragments that provide a signal for endothelial cells to initiate actin cytoskeleton rearrangements that are requisite to formation of capillary sprouts. This proposed model is consistent with the observation that angiogenesis may manifest as alternating cycles of morphologic differentiation (tube

formation) and cell proliferation. Thus, subsequent tube formation in angiogenesis will depend on these early cytoskeletal changes and the local generation of additional molecules for the process of angiogenesis to proceed to completion.

Extracellular Matrix: Modulation of Cell Function and Morphology

With advances in mammalian tissue culture over the past 20 years, it has become clear that relatively insoluble extracellular matrix factors play a pivotal role in cell functions. Endothelial cells in particular are difficult to culture without the presence of an underlying insoluble adhesive surface or substratum [16, 35]. Furthermore, the composition of the extracellular matrix will alter growth rate and differentiation of most cell types. Endothelial cells grow most rapidly on collagen I and slowly on FN. Proliferation rates on laminin and collagen IV are moderate [16].

Matrigel is a commercially available tumor extract derived from Engelbreth-Holm-Swarm mouse tumor implants and contains numerous extracellular matrix proteins commonly found in the basement membrane of endothelium and epithelium [6, 14]. When used as a substratum in tissue culture, Matrigel profoundly alters morphology and antigen expression of many cell types. Specifically, endothelial cells will elongate and form capillary-like tubes on Matrigel substratum [6, 11, 14, 25]. Studies examining differentiation of neurons [16] and endothelial cells [6, 23] implicated laminin as a critical basement membrane component required in final differentiation steps. Kubota et al. [6] have demonstrated that anti-laminin antibodies inhibit formation of endothelial cell tubes in vitro. In vivo, laminin is a component of endothelial cell basement membrane [32] and can be found at an increased density in basement membrane of tumor capillaries [36]. However, in vitro [37] and in situ [32] immunohistochemical studies examining neovascularization have indicated that initiation of capillary tube formation precedes

appearance of laminin in the basement membrane, which suggests that other signals, possibly FN fragments may be present that initiate tube formation.

Fibronectin: A Multidomain Molecule

Fibronectin is a major glycoprotein component of extracellular matrix derived from mesenchymal cells, and a chief component of endothelial and epithelial cell basement membranes [33, 38]. Early work with FN focused on its role in enhancing cellular adhesion [33]. However, it has since become apparent that FN may be involved in diverse cellular functions including chemotaxis [1, 39], cell spreading [40], enhancement of cellular proliferation [41], suppression of cellular proliferation [42–44], regulation of protein synthesis [39] and cytoskeletal changes [40]. Fibronectin is highly susceptible to spontaneous and proteolytic degradation that results in the generation of biologically active fibronectin domains. Particular cleavage patterns for individual enzymes have been identified and these cleavage sites have been mapped on the FN molecule [33, 38] (fig. 1). Specific biophysical characteristics are retained after limited proteolytic cleavage of FN and provide the basis for operational designation of unique intramolecular domains. Fibronectin is a homodimeric glycoprotein with each monomer consisting of two heparin binding regions, one gelatin or collagen binding region, two fibrin binding sites and a cell binding region. The size of each region fragment is determined by the enzyme used for degradation and can alter the properties of each fragment [33, 38]. In the second paper in this dissertation, trypsin-generated fibronectin fragments that induce sprout formation lose activity after prolonged trypsin cleavage, suggesting that the sprout inducing activity of fibronectin fragments may be regulated by duration of exposure to proteases.

Interestingly, several functional attributes have been attributed to FN fragments that are latent when FN is in its native conformation. These include alternations in cytoskeleton, neutrophil chemotaxis and alterations in cellular proliferation rate [39, 41–45]. This suggests that FN fragments convey different information to the cell than does

native FN. While at least seven cell surface receptors have been idenfitied for native FN [46], identification of receptors that bind unique FN fragments [1] is a forthcoming field for investigation. Several clinical studies have detected certain FN fragments in the serum of patients with disease states including diabetes, autoimmune disease and burns [39, 47]. This has lead to speculation that FN fragments have a functional role in the pathogenesis of these disease processes. For instance, Skrha et al. [48] found elevated levels of free N-termianl 30-kDa FN domain in the plasma of diabetic patients with diabetic angiopathy copared with that in plasma of diabetics without microangiopathy. This particular heparin-binding fragment inhibited endothelial cell proliferation [42, 43] and enhanced fibroblast proliferation [41], both of which are features in the differentiative phase of angiogenesis.

Austria and Couchman [49] demonstrated that FN and various combinations of FN fragments stimulated production of laminin by the PFHR-9 endodermal cell line. Together, these data indicated that combinations of fibronecting fragments induced secretion of laminin and suppressed endothelial cell proliferation, two events that are coincident with formation of endothelial cell tubes. The results to be presented in this dissertation establish that fibronectin fragments are a link between the proliferative phse of angiogenesis mediated by VEGF and the differentiation phase of angiogenesis, mediated in part by laminin.

Endothelial Tube Formation is Preprogrammed

In vitro studies have established that endothelial cell tubes will form spontaneously in tissue culture after prolonged incubation for 1–45 days [50], depending on the origin of the endothelial cell. This observation implied that progression from endothelial cell monolayers to tubes is driven by cellular signals generated by the endothelial cell itself and not dependent on signals from other cells [2]. My hypothesis that tube formation is

initiated by protease activity on fibronectin (both of which are secreted by the endothelial cell) is consistent with the concept that endothelial tube formation is an autocrine process.

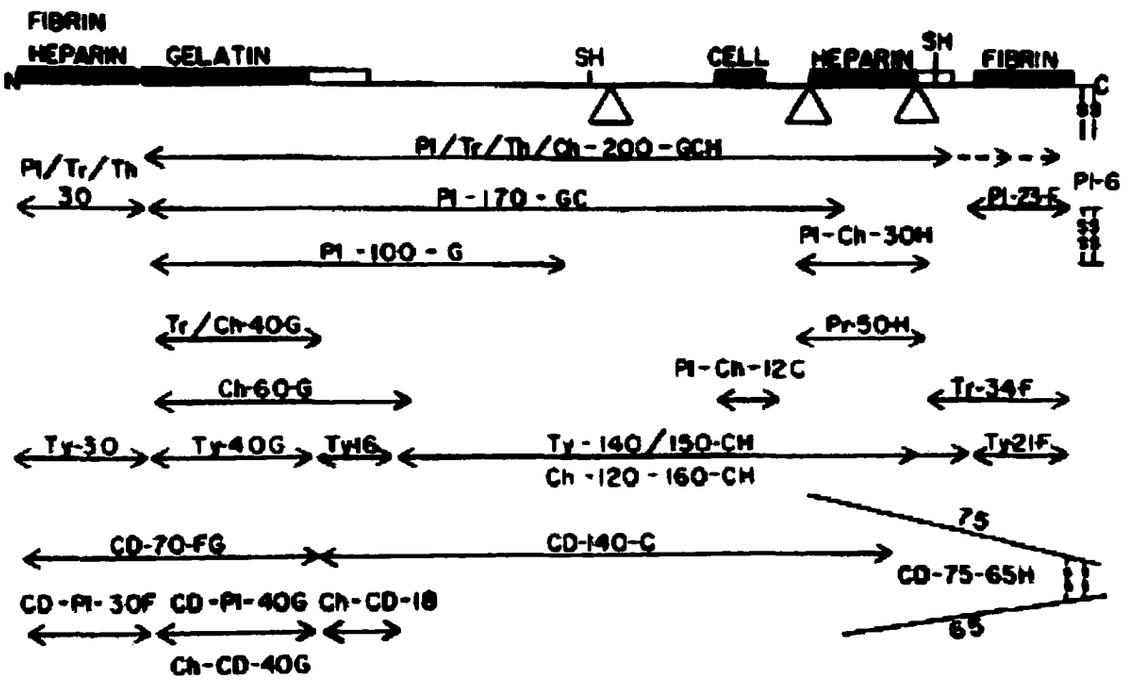

Fig. 1. The cleavage pattern of Fibronectin by several proteases. Enzymes- Pl:Plasmin, Tr:Trypsin, Th:Thrombin, Ch:Chymotrypsin, Ty:Thermolysin, Pr: Pronase, CD:Cathepsin D (Reproduced with permission from Fibronectins (1990, Springer-Verlag). Fibronectin is cleaved into unique and distinct fragments after proteolysis. While the native dimeric Fibronectin molecule has well-characterized properties, proteolytic fragmentation generates latent molecules, some of which are involved in endothelial cell sprout formation.

Abstract

Endothelial cells (EC) are fastidious in their growth requirements in vitro and will not survive extensive passages. We have partially characterized a continuous cell line (>40 passages) established in culture from New Zealand White rabbit vena cava endothelium (REVC). REVC cells resemble typical EC, but remain hardy when grown on uncoated plastic in DMEM/F12 + 10% FBS. REVC cells have typical cobblestone appearance, are contact-inhibited in monolayers and express factor VIII-related antigen. Weibel-Palade bodies were not seen by electron microscopy. REVC cells grown in 2% FBS on plastic demonstrate dose-dependent increases in [^{3}H]thymidine uptake in response to acidic FGF (10–100 ng/ml), basic FGF (3–100 ng/ml), EGF (10–50 ng/ml), and ECGS (10–100 μg/ml). Heparin (5–100 μg/ml) potentiates proliferation induced by aFGF and lowered the ED_{50} for aFGF. REVC cells did not show an increased proliferative rate in response to vascular endothelial growth factor. Transforming growth factor β_1 and β_2 profoundly inhibited thymidine uptake at doses as low as 100 pg/mL. When grown on a collagen I substratum, REVC cells became larger, more polygonal and assumed a sheet-like appearance upon reaching confluence. REVC cells plated on fibronectin, laminin or poly-*L*-lysine demonstrated increases in pericellular granularity and pronounced spreading, especially on fibronectin. Phorbol myristate acetate produced profound morphological changes characterized by swirling whorls of bipolar cells surrounding patches of polygonal cells and multilayered overgrowth. When plated on EHS (Engelbreth-Holm-Swarm) tumor extracellular matrix (Matrigel), REVC cells became quiescent and underwent morphological changes reminiscent of differentiation with elongated cytoplasmic extensions. Chromosomal examination of REVC cells revealed a normal diploid karyotype (2n = 44). This continuous cell line is undergoing further characterization and may be quite useful in investigating many aspects of endothelial cell biology in vitro.

Introduction

In vitro research of vasculature endothelium has been hindered by a paucity of continuous endothelial cell (EC) lines that manifest characteristics commonly associated with normal, primary-cultured EC. Furthermore, most EC types freshly derived from a variety of different blood vessels have complex and specific growth requirements and grow slowly. Primary EC cultures usually will not survive in the absence of a biologic substratum, growth factor-supplemented medium, high serum concentrations, tumor cell-conditioned medium or exogenous metabolic stimulators such as dibutyryl cAMP and xanthine derivatives [1–5]. Moreover, EC in vitro also exhibit cellular senescence to a variable degree. SV_{40} transformation of normal EC has been explored in an attempt to overcome some of these obstacles but virally-transformed cell lines frequently lose distinguishing properties (e.g., factor VIII positivity) [6] and may exhibit other, less obvious and undesirable, changes [7].

In this paper, we describe a continuous rabbit EC cell line that has no additional substratum requirements other than standard tissue-culture grade plastic or glass, maintains robust viability in complete culture medium supplemented to 6–10% with fetal bovine serum (FBS) and retains many of the important physical, biological and biochemical properties that characterize primary cultures of EC. Importantly, we will present several observations which suggest that this cell line may be very useful in experimental studies of vascular endothelium.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium mixed 1:1 with Ham's Nutrient Mixture F-12 (DMEM/F12; Sigma Chemical Co., Inc., St. Louis, Mo., USA) was used in all experiments and was supplemented to 2 m*M* *L*-glutamine (Sigma). FBS and calf serum (Hyclone, Inc., Logan, Utah, USA) were heat-inactivated (56°C, 45 min). Acidic

with medium changes every third day. REVC cells used in this study were harvested 1–7 days postconfluence using brief incubation (room temperature, 10 min) in 0.05% trypsin/0.53 m*M* EDTA solution (GIBCO Inc., Grand Island, N.Y., USA). Bovine calf serum (2.5 ml; Hyclone Inc.) was added to the flask to inactivate the trypsin and adherent cells were detached via trituration using a 10-ml sterile pipette. The cells were pelleted by centrifugation (200 *g*, 8 min, 22°C), resuspended in 10 ml of fresh serumless DMEM/F12 and viable cells were enumerated in the presence of 0.04% trypan blue solution by microscopic counting using a hemacytometer.

Cell Counting Studies

To determine the growth requirements of REVC cells for serum proteins, five replicate 24-well Falcon microculture plates containing REVC cells (40,000/ml) were established in DMEM/F12 with 2, 6 or 10% FBS. Duplicate wells were harvested on days 1, 2, 4, 6 and 8 for cell counting as described above.

Tritiated Thymidine Uptake Studies

To assess the effect of cell density and serum concentration on REVC cell [^{3}H]thymidine uptake, 500 – 4,000 cells were plated in 96-well microculture plates in the presence of 0–15% FBS in 190 µl of medium. Triplicate wells were examined for all studies unless otherwise noted. Tritiated thymidine (1 µCi) in a volume of 10 µl was added for a final 24-hour pulse on days 1, 2, 3 or 4 postplating. Following a [3H]thymidine pulse, cells were harvested for β-particle scintillation counting. Cell harvesting was facilitated by the addition of 20 µl of a cell release solution (CRS; 0.1 % trypsin – 2.3 m*M* EDTA – 0.4 *M* sucrose) and a 10-min incubation at room temperature. Detached REVC cells were harvested with an automated cell harvester (Skatron, Inc, Sterling, Va., USA) and collected on filter paper. The filter paper was baked at 45°C until

dry and individual disks were transferred into scintillation vials. One milliliter of scintillation solvent (Budget-Solve; Research Product International Corp., Mount Prospect, Ill., USA) was added and individual vials were counted in a β-particle counter (LKB-Wallac 1211 scintillation counter) for 60 s.

To examine the effect of growth factors and cytokines on REVC cell thymidine uptake, 2,000 REVC cells were plated in the presence of 2% FBS and incubated for 24 h. At that point, individual growth factors, cytokines or solvent controls were added to each well at various concentrations in a volume of 10 µl. After an incubation for 24 h, tritiated thymidine (1 µCi/10 µl) was added for the final 24 h of this 72-hour assay. REVC cells were harvested as described and incorporated radioactivity was determined. In experiments with heparin, heparin or PBS control was added in a volume of 10 µl just prior to the addition of aFGF.

Extracellular Matrix Studies

Native collagen I was prepared for thymidine uptake studies by mixing together 1 vol of cold 10 x PBS, 1 vol of 0.1 *N* NaCl and 8 vol of cold collagen solution (Collagen Corp., Palo Alto, Calif., USA) and the pH was adjusted to 7.5. The initial collagen I concentration was 2.35 mg/ml. Collagen I solution (50 µl) was pipetted into 96-well plates and incubated (37°C, 4 h). The plates were dried in a laminar flow tissue culture hood and rinsed twice with sterile PBS. A similar preparation was made by coating 96-well plates with 50 µl of ice-cold full-strength Matrigel (Collaborative Research). These plates were not allowed to dry but stored aseptically in a moist incubator at 37°C until used. REVC cells (2,000/well) were plated onto uncoated plastic or coated (collagen I or Matrigel) microculture wells in the presence of 2, 4, 6, 8, or 10% FBS, incubated (37°C, 48 h), then pulsed with tritiated thymidine for a final 24 h. Cells were detached with 20

μl of dispase (Collaborative Research) and 20 μl of CRS and harvested for scintillation counting.

To examine the effect of reconstituted extracellular matrix on REVC cell morphology, 1 x 10^5 REVC cells were plated in 6-well culture dishes precoated (Collaborative Research) with laminin, collagen I, poly-*L*-lysine or fibronectin. The cells were plated in 2, 6, or 10% FBS. Changes in viable cell morphology were monitored using phase-contrast inverted microscopy. On day 4–5, the cells were fixed with methanol, stained with tetrachrome stain (Diagnostic Systems Inc., Gibbstown, N.J., USA) and representative photomicrographs were taken.

Immunoreactivity of Cultured REVC Cells

Immunoperoxidase staining of REVC cells for factor VIII-related antigen (VIII-R:Ag) was performed on semiconfluent cell cultures fixed (30 min, 22°C) in neutral-buffered formalin. Fixed REVC monolayers were washed with PBS and incubated (30 min, 22°C) with polyclonal rabbit anti-factor VIII-R:Ag (BioGenex Laboratories, San Ramon, Calif., USA; anti-factor VIII-R:Ag streptavidin kit). Monolayers were washed 3 times with PBS then incubated with biotinylated anti-rabbit antibody. Excess second antibody was removed by 3 washes of PBS and streptavidin-horseradish peroxidase complex was added to the slides with an incubation time of 20 min at 22°C. The monolayers were washed 3 times with PBS and incubated in freshly prepared AEC-H_2O_2 solution until a visible precipitate was observed. Immunoperoxidase-stained cells were washed and counterstained with Mayer's Hematoxylin (Sigma). Slides were coverslipped with polyvinyl alcohol (PVA) mounting medium (10% PVA in 25% glycerol, 0.05*M* Tris, pH 8.5) and representative photomicrographs were taken.

To exclude the possibility that the REVC cells may have been transformed by SV_{40} virus, neutral-buffered formalin-fixed cells in semiconfluent monolayers were

permeabilized with cold ethanol:acetone (1:5) and then reacted with either of two mouse monoclonal IgG antibodies specific for SV_{40} large T antigen or an antifluorescein control mouse IgG (kindly supplied by Dr. Elizabeth Gurney, University of Utah). Monolayers were incubated (1 h, 37°C) with the anti-T antigen antibodies, then rinsed 3 times with PBS and reacted (1 h, 37°C) with FITC-labeled goat anti-mouse antibody (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md., USA). Monolayers were rinsed with PBS 3 times and cov- .slipped using PVA mounting medium. Negative controls were similarly processed using a mouse monoclonal antifluorescein IgG as first antibody. Positive confirmation of anti-SV_{40} reactivity was obtained by staining a SV_{40}-transformed 3T3 fibroblast cell line [9] in a manner similar to that used for REVC cells. As a further experimental control, a similar procedure was employed using mouse IgG anti-p53 (also supplied by Dr. Elizabeth Gurney) as a first antibody.

Immunohistochemical staining for fibronecting was performed on methanol-fixed subconfluent REVC cells. REVC cells grown on glass slides (8-place Lab-Tek culture chambers) were immersed in ice-cold 100% methanol for 30 s and allowed to dry. Slides were incubated in 3% BSA/PSA (room-temperature, 30 min) to block nonspecific binding and excess blocking solution was replaced bya primary antibody solution in 3% BSA/PSA containing either 1:2,000 dilution of P_3NP/PFN (antifibronectin) or 1:2,000 dilution of 15E2E2 control antibody (anti-S-100). REVC cell monlayers were incubated 2 h in a humid atmosphere at room temperature and then extensively rinsed with PBS. For flurescence staining, the slides wer incubated (room temperature, 1 h) with rabbit FITC-conjugated anti-mouse IgG (Southern Biotechnology, Birmingham, Ala., USA) at 10 µl/ml in 3% BSA/PBS, then rinsed 3 times with PBS. All slides were coverslipped with 10% PVA.

Results

Morphological Findings

The cells used in this study were derived by long-term culture of EC obtained from New Zealand White Rabbit vena cava and exhibited a typical cobblestone morphology when grown to confluence on an uncoated plastic culture dish (fig. 1A). REVC cells did not grow outside of the monolayer and appeared to be contact-inhibited even with maximal serum stimulation. Mitotic figures were occasionally seen; however, numerous binucleate, presumably dividing, cells were very abundant in subconfluent REVC cell cultures. Confluent cell layers contained few binucleate cells.

There was little evidence of apoptosis in REVC cells even in the absence of medium changes for a period of one month. REVC cells deprived of serum did not shed apoptotic bodies but instead either detached or degraded leaving behind cytoskeletal debris.

When examined by electron microscopy, REVC cells appeared flat and elongated with a prominent nucleus. The cytoplasm contained scattered vacuolar structures, rough endoplasmic reticulum and occasional mitochondria. There were no obvious Weibel-Palade bodies (fig. 1B).

These cells exhibit factor VIIIR:Ag immunoreactivity as demonstrated by immunoperoxidase staining (fig. 2A) when compared to control (fig 2B). The most intense factor VIIIR:Ag reactivity was observed in cells that were confluent or semiconfluent. In contrast, sparsely plated cells exhibited pale or no visible factor VIII-R:Ag immunoreactivity.

Immunofluorescence staining for SV_{40} large T antigen was negative as compared to that seen for SV_{40}-3T3, a SV_{40}-transformed fibroblast cell line (not shown). REVC cells also lacked anti-p53 immunoreactivity.

Serum Requirements

The effect of varying FBS concentration on REVC cell proliferation can be seen in figure 3. After an initial loss of plated cells which is a reflection of plating efficiency, REVC cells proliferate in a dose-dependent fashion with increasing concentrations of FBS between 2 and 6%. The REVC cells plated in 6 and 10% FBS appeared to proliferate at similar rates as demonstrated by cell number. The cells grown in 10% FBS, however, did appear to reach confluence earlier. Maximal cell proliferation, as revealed by the rate of growth (slope), was observed between days 2 and 4 postplating for all concentrations of FBS employed.

The effect of increasing FBS concentration on [^{3}H]thymidine uptake by REVC cells is demonstrated in figure 4. Similar to the trends seen with cell-counting data, there was a dose-dependent increase in REVC [^{3}H]thymidine uptake which occurred with increasing serum concentrations. Maximum [^{3}H]thymidine uptake in a given 24-hour period was observed between the second and third days after plating. At no time did there appear to be any crowding of the cells when plated at an initial density of 2,000 cells/well (0.28 cm^2) as judged visually by phase-contrast microscopy. In contrast, when REVC cells were plated at an initial density of 4,000 cells/well, cell monolayers were apparently confluent by day 3 (data not shown).

REVC cells also exhibited significant serum dependence. When REVC cells were assayed in serum-free medium, there was no significant [^{3}H]thymidine uptake at any time. Moreover, when plated in the absence of serum, REVC cells did not appear to spread out and attach to the plastic surfaces of the microwells.

Mitogenic Growth Factors

The mitogenic effect of several growth factors was examined in REVC cells by measuring changes in [^{3}H]thymidine uptake at varying growth factor concentrations. Most assays were performed in a medium containing 2% FBS. Conducting the assay in

this suboptimal FBS concentration facilitated detection of enhancement as well as suppression of [^{3}H]thymidine uptake. The cells were harvested on the third day post plating as described above.

As shown in figures 5 and 6, bFGF and aFGF both enhanced [^{3}H]thymidine uptake, with bFGF appearing to be slightly more potent than aFGF. Effective dose for 50% of maximal stimulation (ED_{50}) was 8 vs. 30 ng/ml, respectively. Maximal stimulation by bFGF occurred with 50 ng/ml of growth factor whereas maximal stimulation by aFGF was in excess of 100 ng/ml. While aFGF alone enhanced REVC cell [^{3}H]thymidine uptake when compared to control, heparin profoundly enhanced the mitogenic effect of aFGF when added several minutes prior to addition of 0.5–100 ng/ml aFGF. This potentiation was seen at heparin concentrations ranging from 5 to 100 μg/ml. Figure 6 also demonstrates that heparin alone had no effect on REVC cell [3H]thymidine uptake. The interaction between heparin and bFGF was not tested.

ECGS also potentiated REVC cell 3H-thymidine uptake at concentrations ranging from 10 to 100 μg/ml (fig. 7). ED_{50} for ECGS was 20 μg/ml, with maximal stimulation of REVC cells occurring between 50 and 100 μg/ml. Increases of thymidine uptake seen with maximal ECGS stimulation were comparable to those of maximal FBS stimulation. EGF was also a potent mitogenic stimulus for REVC cells, enhancing [^{3}H]thymidine uptake in a dose-dependent fashion between 5 and 125 ng/mL (fig. 8). Maximal [^{3}H]thymidine uptake induced by EGF was comparable to maximal FBS stimulation.

Vascular endothelial growth factor (VEGF), while shown to be a potent mitogen for certain endothelial cells, did not stimulate proliferation of REVC cells when tested over a broad concentration range (0.1–500 ng/ml). ED_{50} for human umbilical vein endothelial cells for this VEGF preparation was >1 ng/ml.

Suppressive Growth Factors

TGF-β_1 and TGF-β_2 both profoundly inhibited [^{3}H]thymidine uptake in REVC cells (fig. 9). At TGF-β concentrations of 1 ng/ml, both TGF-β_1 and TGF-β_2 induced a 80% suppression of REVC cell [3H]thymidine uptake. Preliminary findings suggest that REVC cells were more responsive to TGF-β_2 than to TGF-β_1. These observations are the subject of separate manuscript in preparation.

Extracellular Matrix Effects

The effect of collagen I and Matrigel on REVC cell [^{3}H]thymidine uptake is shown in figure 10. Matrigel, when compared to collagen I and plastic, had a suppressive effect on REVC cell [^{3}H]thymidine uptake. This effect could not be overcome by increasing the FBS concentrations. Cells plated on this matrix rapidly took on a differentiated appearance with dendritic-like processes. Lumen formation could not be clearly identified (fig. 11B).

In contrast, collagen I did not alter [^{3}H]thymidine uptake when it was used as a REVC cell substratum. The cells grown on this substratum were larger with a more uniform polygonal shape and assumed a sheet-like appearance upon reaching confluence (fig. 11A).

Distinguishing features of REVC cells plated on fibronectin, laminin and poly-*L*-lysine (figs. 11C–E) included an increase in pericellular granularity and a pronounced spreading. Cells plated on fibronectin, in particular, appeared flattened and epithelioid. These cells phenotypically assumed the appearance of malignant cells, characterized by swirling whorls of bipolar cells surrounding patches of polygonal cells and multilayered overgrowth as a result of loss of contact inhibition.

Histochemistry

REVC cells were immunoreactive for fibronectin as demonstrated in figure 12a by immunohistoflourescence. The distribution of fibronectin immunoreactivity in the REVC cell cultures was fine and wispy and consistent with previous reports demonstrating fibronectin immunoreactivity in EC cultures [23]. REVC cell lysates generated by detergent solubilzation were immunoreactive with antifibronectin antibody P_3NP/PFN in Western blots (fig. 12B). Calf serum was not immunoreactive with P_3NP/PFN in Western blots and demonstrates that REVC cell fibronectin does not form as a result of absorption or endocytosis of calf serum fibronectin. REVC cells also demonstrated a prominent intranuclear anti-p53 immunoreactivity. Immunofluorescence staining for SV_{40} large T antigen was negative as compared to that seen for SV_{40}-3T3, a SV_{40}-transformed fibroblast cell line (not shown).

Karyotypic Analysis

Representative chromosomal content of a REVC cell arrested in metaphase with colcemid was examined under light microscopy (not shown). In many cells, there were 21 pairs of autosomes and a pair of XX sex chromosomes, 2n = 44. This is the expected number of a normal laboratory rabbit, *Oryctolagus cuniculus* [10]. There were several cells with 45 chromosomes suggesting trisomy of a single chromosome, however the most frequent triploid chromosome was not identified. Karyotypic distribution of 30 cells yielded a range of 43–45 chromosomes/metaphase.

Discussion

At present, there are few established, continuous endothelial cell lines that retain most of the physiologic characteristics of primary cultures of EC. In light of this, most investigators use freshly harvested endothelium as a source for biologic and biochemical studies of EC. Unfortunately, most primary cultures of EC undergo senescence,

characterized by decreased growth rate, morphological abnormalities and loss of differentiating antigens such as factor VIII-R:Ag. In vitro, EC are fastidious, and depending on the type, require high serum concentrations [1, 6, 11], coated matrices [1, 4, 5, 11, 12], or complex media [2, 4, 5, 12]. These characteristics significantly encumber investigators' capacity to dissect the biology and biochemistry of EC in vitro.

There have been several attempts to establish continuous EC lines with varying degrees of success. Gimbrone and Fareed [6] demonstrated that human umbilical vein endothelium could be transfected and transformed with SV_{40} DNA. SV_{40}-transfected cells had increased longevity, reduced serum requirements and proliferated rapidly. However, these SV_{40}-transformed cells also lost important characteristics typical of EC, including loss of factor VIII positivity and loss of contact inhibition. Furthermore, these cells did eventually undergo a culture 'crisis' in which bizarre morphological shapes were exhibited with an increased number of giant cells. Viral (SV_{40}) transformation of mouse EC has been reported by O'Connell and Edidin [13]. Their studies demonstrated that mouse EC retained factor VIII-R:Ag positivity and maintained morphologic characteristics typical of EC.

An alternate approach at establishing a continuous, less fastidious cell line was taken by Suggs et al. [14]. In their studies, they have immortalized human EC via fusion with a less differentiated HAT-sensitive cell line. These hybridized EC sustained basal prostacyclin release, exhibited stimulated prostacyclin release and retained other characteristics of EC [14,15].

The spontaneous conversion of cultured rabbit EC to a continuous cell line is not an unprecedented phenomenon in mammalian cell culture. Spontaneous transformation of large vessel EC have been observed in human and bovine EC. Recently, Takahashi et al. [16] reported isolation of a spontaneously transformed, human umbilical vein cell line, ECV304, that has remained viable for greater than 180 passages. However, unlike the REVC cells described here, ECV304 proliferation was unaffected by bFGF, ECGS, or

EGF. Furthermore, ECV304 do not express factor VIII-R:Ag. A spontaneously transformed bovine EC line was described in 1984 by Gorman et al. [17]. That cell line maintained many of the characteristics of EC, but eventually underwent 'crisis' [17] and became senescent after 600 passages.

In contrast to primary EC which require an enriched culture environment, REVC grow well without a substrata, and maintain good viability in low serum concentrations. The hardiness of REVC would suggest that a transformation has taken place although the tumorigenic potential of these cells has not yet been examined. SV_{40} large T antigen could not be demonstrated in REVC using two monoclonal antibodies that bind to distinct epitopes [18]. This observation, however, does not preclude the possibility that REVC have been transformed by a virus other than SV_{40}. As an example, Smiley, et al. [19] have demonstrated that viral-induced transformation of EC could be induced by transfection with cytomegalovirus. However, the enhanced p53 immunoreactivity in the nuclei of REVC cells may provide indirect evidence that transforming viral proteins, which usually form stable complexes with p53, were not present. Additionally we are currently investigating the possibility that REVC immortilization is due to overexpression of the bcl-2 gene and oncoprotein, which would account for the minimal apoptotic body formation observed in REVC culture. This possibility seems highly plausible given the predilection for apoptotic body formation typical in other primary EC cultures and the lack of apoptotic bodies formation by REVC cells [20].

The presence of factor VIII-R:Ag and cobblestone morphology are characteristics of REVC that are considered to be hallmarks of EC cultures. Furthermore, as is typical of most EC, we observed the formation of tube-like structures [21], but without discernible lumens, when REVC cells were plated on Matrigel. The application of 'pure' matrices (e.g., laminin, fibronectin or collagen) as a means of differentiating EC from other cell types is less clear. Similarly, the morphological changes observed after plating REVC on pure matrices were less dramatic. Matrix-associated (or -induced) modulation of EC

differentiation is thought to be a integral component of EC responses [22]. It is unclear how this modulation has been altered in REVC cells, given that these cells grow so well on plastic. We have demonstrated that REVC secrete fibronectin which may then serve as a substrate for the cells' adhesion to plastic. However, other EC have been demonstrated to secrete fibronectin [23] both in vitro and in vivo thus, in itself fibronectin secretion is not sufficient for cell attachment to plastic.

Weibel-Palade bodies were not detected by electron microscopy in REVC. This is curious since WEibel-Palade bodies have been detected in situ in porcine vena cava [30]. To our knowledge, there are no published studies examining the distribution of Weibel-Palade bodies in the rabbit vascular tree. However, these structures, which contain factor VIII and gp140 [15], have not been reliably found in all primary EC cultures [3, 4, 6, 24].

In this paper we demonstrated that REVC proliferated rapidly in the presence of 6–10% FBS. This proliferation is paralleled by a rise in basal thymidine uptake. When maintained in 2% FBS, REVC cells proliferate at a much slower rate. This decreased proliferation rate is similarly paralleled by a decreased uptake of thymidine. Given this correlation made in the present study, we believe that [^{3}H]thymidine uptake can be used as a reliable measure for REVC proliferation.

We have performed all of our growth factor assays using 2,000 REVC cells/well in 2% FBS. As stated above, increasing the cell density led to a crowding of cells when maximally stimulated. We also found 2% FBS to be the optimal serum concentration to detect enhancement or suppression of [^{3}H]thymidine uptake for the growth factors tested. At this concentration, we have consistently observed an increased [^{3}H]thymidine uptake after administration of aFGF, aFGF + heparin, bFGF, EGF and ECGS, but not after exposure to VEGF. Contrariwise, TGF-β_1 and TGF-β_2 consistently inhibited basal thymidine uptake in a dose-dependent fashion (data not shown). Effective concentrations of growth factors used in this study are comparable to the those reported in other studies examining EC proliferation [2, 7, 25, 26].

In this paper we have introduced a previously uncharacterized EC line. This cell line has proved to be hardy and should prove to useful in studying various aspects of endothelial cell biology including thrombogenicity, growth factor interactions, angiogenesis, matrix interactions and lymphocyte-endothelial cell interactions. Clearly, there is a significant degree of heterogeneity among EC with respect to surface antigens, secreted products and response to cytokines [27–29]. While these REVC cells originate from vena cava and exhibit 'classical' endothelial cell characteristics, it remains unclear what changes have taken place to make REVC cells so robust, and to what significant extent they have been altered from normal rabbit vena cava.

Acknowledgements

We wish to express our appreciation to Dr. Fred C. Jensen for the gift of the REVC cell line and we thank Dr. Elizabeth Gurney for her gift of the mouse monoclonal antibodies. Pab 108 and Pab 101 (both SV_{40} large T-antigen-specific, Pab 122 (p53-specific) and 44-Voss (fluorescein isothiocyanate-specific). We gratefully acknowledge the assistance of Ms. Patricia St. John in preparing the electron photomicrographs of REVC. This research was supported in part by USPHS Research Grant NS31096, USPHS training grant NS07335 and a fellowship from the American Brain Tumor Association (C.K.G.).

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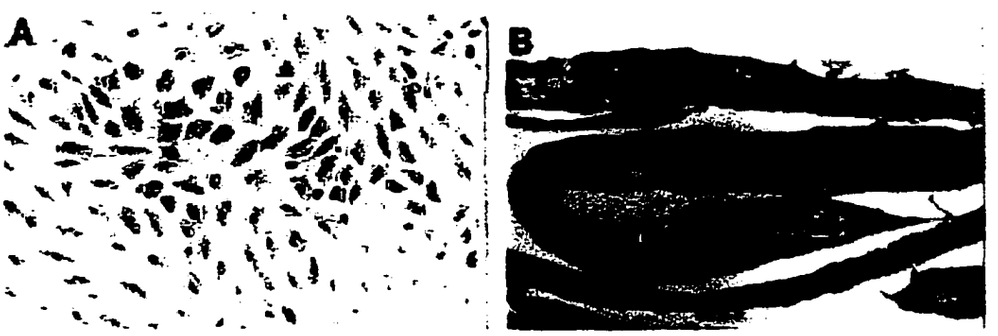

Fig. 1. A Photomicrograph (x 275) of confluent REVC plated on an uncoated plastic Petri dish in complete medium with 2% FBS. The morphological appearance is a cobblestone pattern commonly reported for cultured EC from various sources. **B** Transmission electron micrograph (x 9,000) of REVC cells that had been grown to confluence on a plastic tissue culture surface, fixed in osmium tetroxide, harvested by scraping and embedded in Epon. Notable features include a prominent nucleus, cytoplasmic inclusions and well-defined rough endoplasmic reticulum. Weibel-Palade bodies could not be identified and there was no evidence of lumen formation.

Fig. 2. A Formalin-fixed REVC cells staining positive for factor VIIIR:Ag using immunoperoxidase cytochemistry. For comparison, B shows lack of staining when nonimmune rabbit serum was used as a first antibody. x 275.

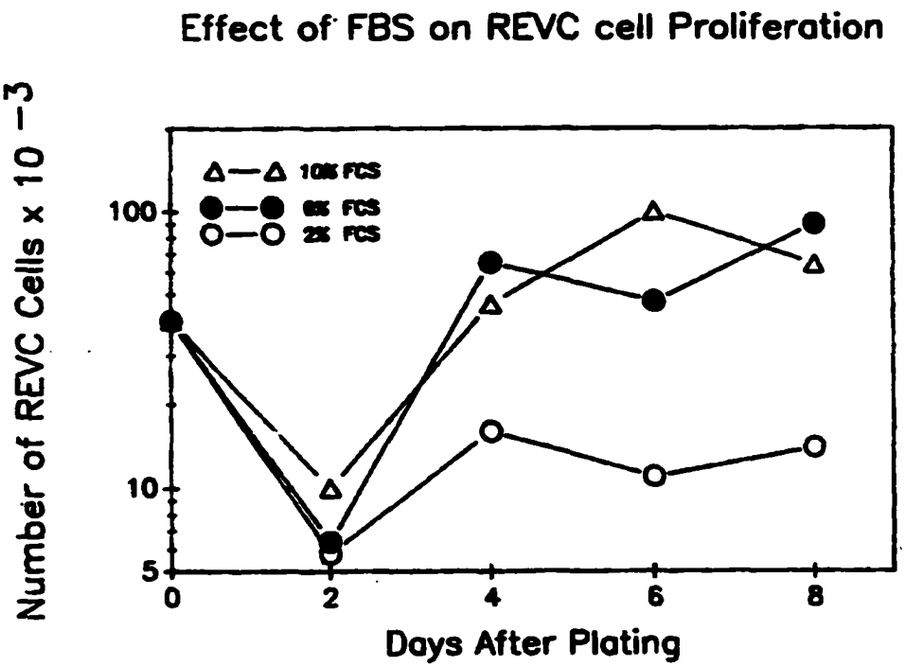

Fig. 3. Proliferation of REVC cells increased as a function of increasing FBS supplementation. After an initial decline in cell number as a reflection of plating efficiency, REVC proliferated most rapidly between days 2 and 4 for all serum concentrations with the largest increase in cell number occurring in the presence of 10 % FBS.

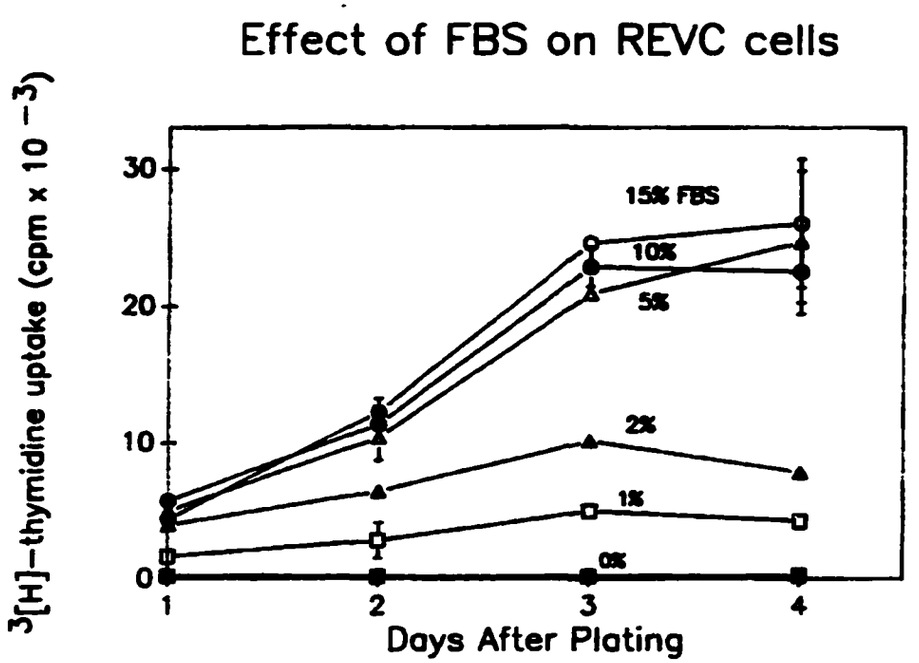

Fig. 4. Tritiated thymidine uptake by REVC also increased as a function of increasing FBS concentration in a dose-dependent fashion between 1 and 15% FBS. No [^{3}H]thymidine uptake was observed in cells that were maintained in a serumless (0%) media.

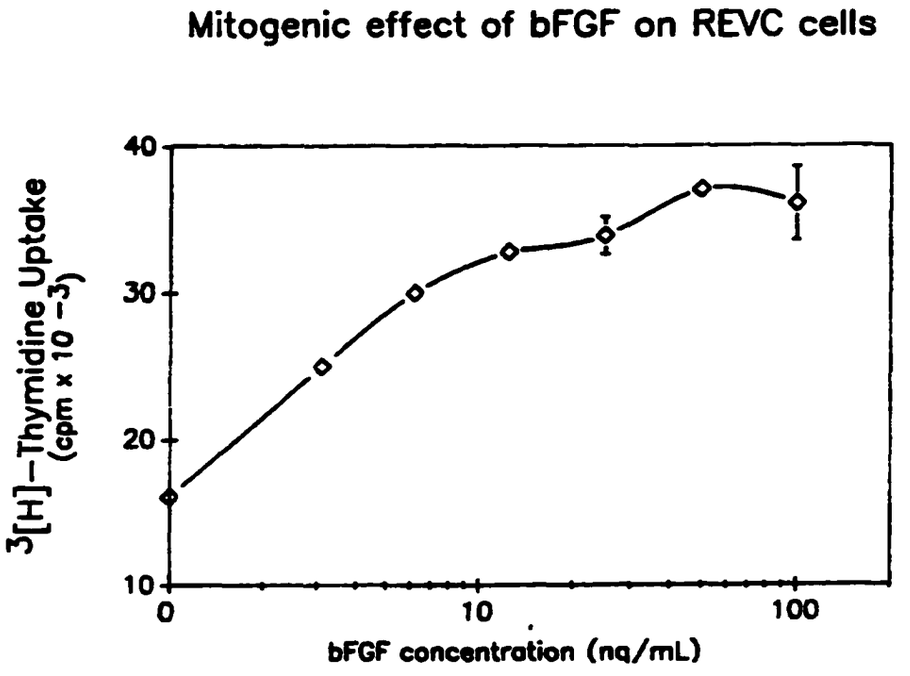

Fig. 5. bFGF stimulated [^{3}H]thymidine uptake by REVC cells in a dose-dependent fashion. ED_{50} is estimated to be 8 ng/ml.

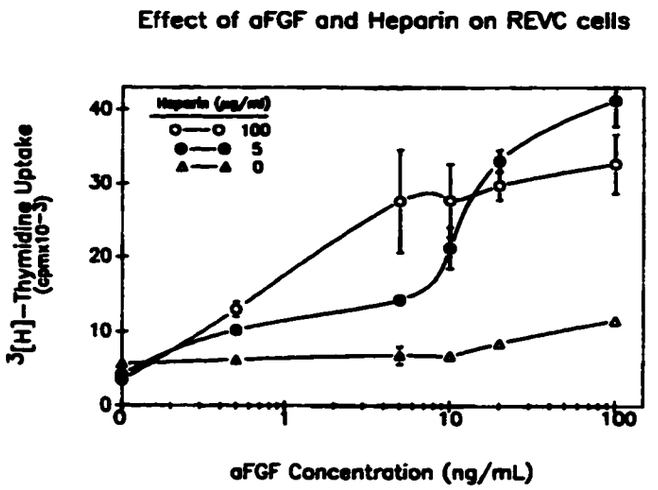

Fig. 6. Heparin (ng/ml) potentiates the mitogenic effect of acidic FGF in REVC cells.

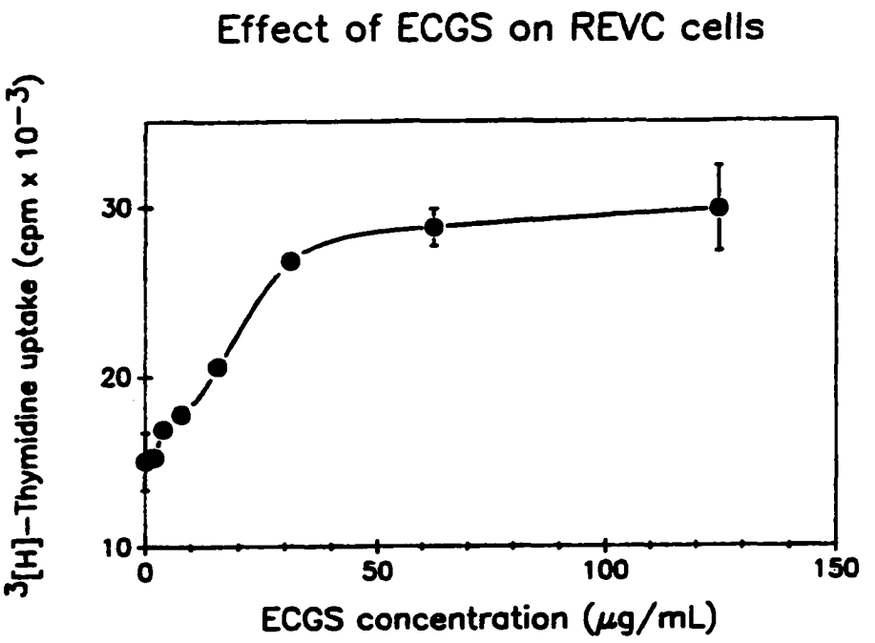

Fig. 7. ECGS enhanced [^{3}H]thymidine uptake by REVC cells in a dose-dependent manner. ED_{50} was estimated to be 20 μg/ml.

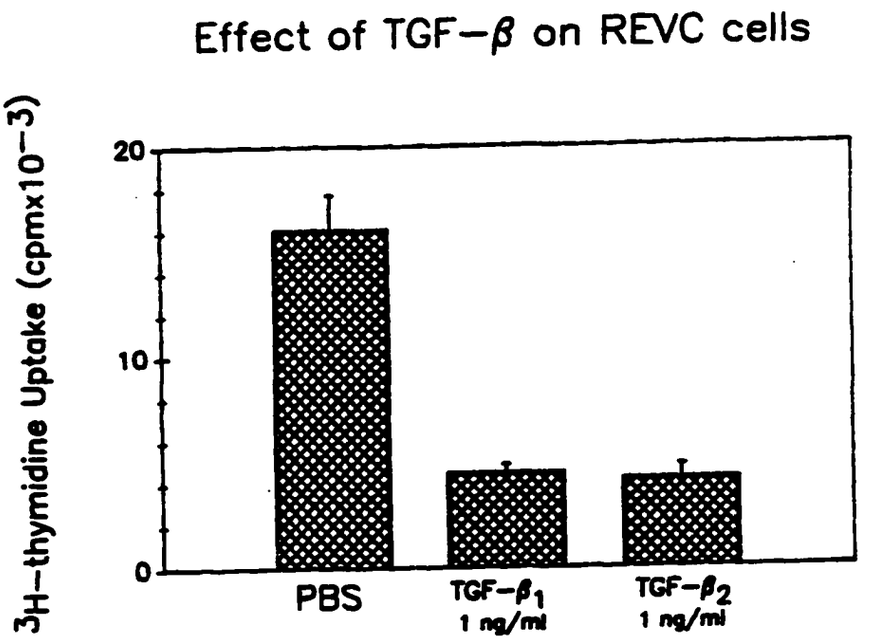

Fig. 9. TGF-β_1 and TGF-β_2 both reduced [^{3}H]thymidine uptake by REVC cells. TGF-β2 was more potent (ID_{50} = 8 pg/ml) than TGF-β_1 (ID_{50} = 32 pg/ml) in suppressing REVC [^{3}H]thymidine uptake.

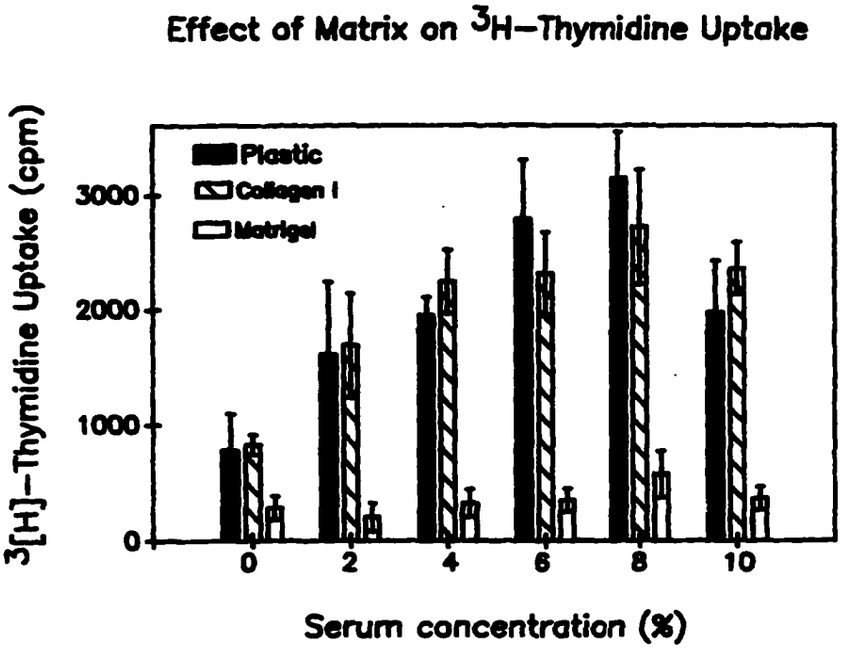

Fig. 10. REVC cells plated on Matrigel exhibited a marked reduction in [^{3}H]thymidine uptake at all serum concentrations tested when compared to REVC cells plated on either collagen I or plastic.

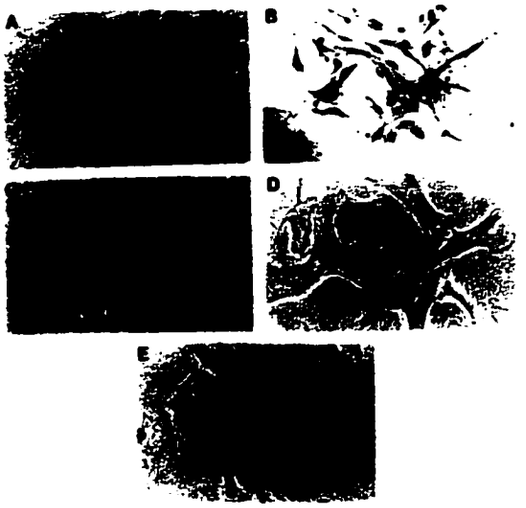

Fig. 11. Phenotypic changes observed for REVC cells that were plated on various substrata: (**A**) collagen I (x 275); (**B**) Matrigel (x 275); (**C**) fibronectin (x 550); (**D**) laminin (x 550), or (**E**) poly-*L*-lysine (x 550).

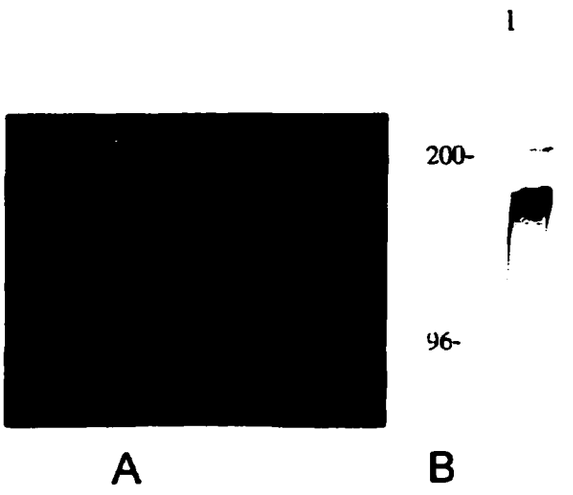

Fig. 12. A REVC cells secrete fibronectin on their surface as demonstrated by immunoflourescence. **B** In Western blots, monoclonal antibody P3NP/PFN recognized two major proteolytic fragments of REVC fibronectin with approximate molecular weights of 200 and 180 kD, lane 1, but was nonreactive with calf serum, lane 2.

EXTRACELLULAR DETERMINANTS OF SPROUT FORMATION IN THE RABBIT ENDOTHELIAL CELL LINE, REVC

by

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Abstract

The development of elongated cytoplasmic processes, called endothelial cell (EC) sprouts, is a critical early step in formation of capillaries by EC. Sprouting, produced in vitro by culturing EC from a wide variety of sources on Matrigel, characteristically leads to cessation of DNA synthesis. We explored the role of fibronectin in sprout formation and observed that either plasmin- or trypsin-generated fragments induced sprouts in the rabbit endothelial cell line, REVC. Sprouts could also be formed by REVC cells in response to addition of soluble ProNectin F, a recombinant protein which contains 13 fibronectin-derived RGD-containing domains, but not by FNfn 7–10, a monomeric recombinant fragment of human fibronectin containing the cell binding domain or by echistatin, a potent competitive antagonist of RGD-dependent integrin-ECM. Serendipitously, we determined that the bivalent lectin concanavalin A (Con A) induced formation of EC sprouts and suppressed DNA synthesis in subconfluent cultures of REVC cells in a manner similar to that seen for Matrigel. Sprout induction by Con A appeared species-specific for REVC cells, as neither human or bovine primary endothelial cells were affected by this lectin. The Con A effect occurred within a narrow concentration range, was reversible and could be blocked by increasing serum concentration. Two mouse monoclonal antibodies (mAbs), prepared against Con A-binding proteins from REVC cell lysates, reacted specifically with rabbit plasma fibronectin and each blocked both induction of sprouts and inhibition of DNA synthesis by Con A. We propose that various alterations in cell-binding activity of fibronectin can initiate fundamental changes in REVC cells that lead to formation of sprouts. The simplicity and reproducibility of using Con A to induce sprouts in REVC cells should facilitate delineation of molecular signals that regulate early stages of angiogenesis.

Introduction

The process of capillary formation, called angiogenesis, involves a complex and coordinated interaction among extracellular matrix (ECM) [1–5] proteins, growth factors and metalloproteinases. As studied in vitro, this process is initiated by the formation of elongated cytoplasmic processes, referred to as EC sprouts. Sprout formation, which is specific for ECs and is absent in other mesothelial cells, ultimately leads to the formation of elongated, endothelial-lined tubes that have a central lumen and which structurally mimic capillaries formed in vivo. Studying this process in vitro has generally required the use of synthetic extracellular matrix preparations. The most commonly used of these, Matrigel, is a highly complex extract prepared from the murine EHS sarcoma, and contains variable amounts of collagen IV, laminin, fibronectin, heparan sulfate proteoglycan, entactin, and transforming growth factor-β. While useful in generating both EC sprouts and capillary tubes, the complexity of Matrigel confounds identification of individual components that contribute to each stage in angiogenesis.

Angiogenesis proceeds in a fibronectin-rich ECM [6]. In vitro, fibronectin supports EC proliferation and can be used as a substrate to grow ECs in tissue culture. Numerous studies examining the activity of fibronectin in other cell types indicate that fibronectin is a multidomain molecule that can bind to other extracellular matrix proteins as well as to cells [7–14]. The latter is at least partially mediated through arginine-glycine-aspartate (RGD) sequences which bind to integrins on the cell surface. Fibronectin exhibits a broad spectrum of biological activities. These include cytoskeletal rearrangement, cellular adhesion, and chemotaxis [15]. Specific fibronectin domains may be responsible for some of these biological effects. For example, heparin-binding proteolytic fragments of fibronectin inhibit EC proliferation, stimulate fibroblast proliferation, and alter the fibroblast cytoskeleton [16, 17]. The precise role of fibronectin in angiogenesis, however, remains uncertain.

In this paper, we demonstrate that a variety of agents that altered either the structure of fibronectin or its availability for cellular interaction induced sprout formation in REVC cells. REVC is a continuous diploid rabbit EC line that has minimal substrate requirements, yet retains most morphologic and functional response characteristics of Ecs [18]. Our data are consistent with a model in which EC differentiation is triggered by loss of adhesion to substrate-bound fibronectin. Our data also provide a mechanism to explain how mitogenic stimulation by endothelial growth factors ultimately leads to cessation of DNA synthesis and morphologic differentiation. In examining the effect of various mitogens on REVC proliferation, we fortuitously discovered that the jack bean lectin, concanavalin A (Con A) inhibited cell proliferation and produced elongated EC processes and organized sprout-like networks — effects identical to those seen with Matrigel. In pursuing this, we obtained evidence to suggest that the sprout-inducing effect of Con A is mediated through fibronectin. As demonstrated in this and in a subsequent study [19], this effect of Con A appears to be restricted uniquely to the REVC system yet provides a useful methodology for studying the early stages in the process of capillary formation in vitro.

Materials and Methods

Materials

The following materials were supplied by the listed vendors: avidin-alkaline phosphatase kit, biotinylated rabbit anti-goat antibody, FITC and TRITC Con A from Vector, Inc. (Burlingame, Calif., USA); Centriprep concentrators from Amicon (Beverly, Mass., USA); colorimetric protein assay from Bio-Rad Laboratories (Oakland, Calif., USA); echistatin from Bachem (Philadelphia, Penn., USA); fetal bovine serum from Intergen (Purchase, N.Y., USA); FITC goat anti-mouse IgG, purified mouse IgG and purified mouse IgM from Southern Biotechnologies, Inc. (Birmingham, Ala., USA); Goat anti-rabbit fibronectin antibody IgG fraction) from Binding Site, Inc. (Birmingham,

U.K.); human serum fibronectin from The New York Blood Center (New York, N.Y., USA); human serum plasmin, PMSF, Con A, Sepharose 4B, and Con A-Sepharose 4B from Sigma Chemical (St. Louis, Mo., USA); Hunter's Titer-Max from Vaxel (Norcross, Ga., USA); Lab-Tek chamber slides from Fisher Scientific (St. Louis, Mo.); Matrigel from Collaborative Biomedicine (Bedford, Mass.); media for cell culture from Mediatech (Washington, D.C., USA); monoclonal antibodies 3E1 and 3E3 against human fibronectin from GibcoBRL (Grand Island, N.Y., USA); ProNectinF from Stratagene (La Jolla, Calif., USA); Tetrachrome from EM Diagnostic Systems, Inc. (Gibbstown, N.J., USA); and tritiated thymidine from Amersham (Arlington Heights, Ill.).

Purification of rabbit plasma fibronectin was performed as described by Ruoshlati et al. [20] FNfn 7–10, a recombinant fragment of human fibronectin, was kindly supplied by Dr. Harold P. Erickson, Duke University.

Cell Culture

REVC, a continuous rabbit EC line derived from rabbit vena cava, has been characterized as described in a previous study [18]. Cell cultures were grown to confluence in DMEM/F12 plus 10% FBS and harvested as previously described [21]. BALB 3T3 cells, clone A31, were cultured and prepared for tritiated thymidine uptake studies as previously described [22, 23]. HUVECs were isolated from fresh human umbilical veins [24] and were used at passage 2–3 . Bovine primary ECs were isolated from aorta and were a gift of Dr. T. Brock (University of Alabama at Birmingham).

Cell Viability

The concentration of viable REVC cells was measured by use of Alamar Blue, as described [25]. Separate studies have demonstrated that cell number and Alamar Blue absorbance for REVC cells are linearly related (data not shown).

Tritiated Thymidine Uptake Studies

The effect of Con A or fibronectin fragments on DNA synthesis was assessed by measuring changes in [^{3}H]thymidine uptake. REVC cells (2 x 10^5) or BALB 3T3 cells (4 x 10^3) were plated in 96-well microtiter plates in 190 ml of DMEM/F12 containing 2% FBS. After incubation for 24 h at 37°C and 5% CO_2, varying concentrations of sterile Con A in 10 ml of Dulbecco's phosphate-buffered saline (D-PBS), 10 ml of fibronectin fragments (see below), or 10 ml of D-PBS (as a control) were added to each well. After 24 h of additional incubation, 0.1 mCi of [^{3}H]thymidine (48 Ci/mmol) was added to each well in a volume of 10 ml. After 24 h of incubation, radiolabeled cells were detached from the well bottoms with a cell release solution (CRS; 0.1% trypsin, 2.3 mM EDTA, 0.4 M sucrose) and harvested onto fiberglass microfilter discs using a Skatron Harvester. [^{3}H]thymidine incorporation was measured using a LKB RackBeta 1210 liquid scintillation counter.

Cell Morphology

Morphologic changes induced by fibronectin fragments or Con A were evaluated in REVC or BALB 3T3 cells plated at an initial density of 7.5 x 10^4 cells/well into 6-well culture dishes in the presence of 2 mL of DMEM/F12 + 2% FBS. Fibronectin fragment fractions (50 ml) were added to 500 mL of medium. Alternatively, Con A was added 24 hours after plating in a volume of 1–10 ml to a final concentration of 2–20 μg/ml. In both cases, cells were incubated at 37°C for 3–4 days. Culture medium was removed and the cells were fixed with ice-cold methanol for 60 s. Cells were stained with tetrachrome stain and representative photomicrographs were taken.

Effects of Matrigel on cell morphology were examined in REVC cells (2.5 x 10^4/well) as well as in HUVECs and BALBc 3T3 cells plated in 24-well culture dishes coated with 200 ml of undiluted Matrigel in the presence of medium containing 0-10%

Concanavalin A Binding Proteins

Approximately 10^7–10^8 REVC cells were plated in DMEM/F12 + 2% FBS and incubated for 1–3 days until semiconfluent. The cells were rinsed three times with PBS and lysed by exposure (30 min, 4°C) to an extraction buffer (NP-40, 0.5% w/v, 10 m*M* Tris, 0.15M NaCl, 1 mM PMSF, 0.02% NaN3, pH 7.4). The REVC lysate was clarified by centrifugation at 20,000 x *g*, and concentrated and dialyzed in Centriprep-10 micro-concentrators against chromatography buffer (0.9% NaCl, 10 mM Tris HCl, 1 m*M* $CaCl_2$, 1 m*M* MgCl2 pH 7.4). The sample was applied to a 10 x 0.5 cm column of Con A-Sepharose 4B by recycling 20 ml at 3 ml/h for 18 h at 4°C. The column was then extensively rinsed with chromatography buffer until the effluent contained no detectable protein as monitored by absorbance at 280 nm. Con A binding proteins were eluted with 400 m*M* a-methylmannoside in chromatography buffer. Total protein in the eluant was 5–25 mg, as determined by a colorimetric protein assay. The eluted Con A binding proteins were concentrated to a volume of 250 ml and applied to a 250 x 10 mm Superose-12 FPLC column. Two major pools that fractionated with apparent molecular weights > 400 kDa (designated HMW) and 10-400 kDa (designated LMW) were used separately to raise monoclonal antibodies.

Production of Monoclonal Antibodies

HMW and LMW fractions were emulsified in Hunter's Titer-Max according to manufacturer's instructions and two groups of BALB/c mice were immunized by intramuscular thigh injection of 50 ml of either one or the other fraction followed by intraperitoneal injections at 3-week intervals. After 3 booster injections, candidate mice were sacrificed and hybridomas were generated by polyethylene glycol-mediated fusion of P3X63Ag.8.653 myeloma cells and splenocytes from the immunized mice, as previously described [26]. Colonies growing out were initially screened for mouse Ig production by ELISA; Ig positive colonies were then screened by ELISA for binding to

HMW or LMW fractions immobilized to Immulon II (Dynatech) plates. Hybridomas found to secrete reactive antibodies were recloned, isotyped and grown in culture or in ascites to generate sufficient reagents to use for cell culture and Western blotting.

Generation of Fibronectin Fragments

Five milligrams of purified human plasma fibronectin were incubated (30 min, 37°C) with 10 mg trypsin in 1 ml of sterile PBS. PMSF was added to a final concentration of 2 mM to stop the reaction. The sample was dialyzed against a 1,000-fold volume excess of sterile water using Centriprep-3 concentrators (MWCO = 3 kDa). The mixture was sterilized by passage through a 0.22 micron filter and fractionated in running buffer (3 mM CHAPS, 5% isopropanol, PBS) by HPLC using a 250 x 10 mm Superose 12 sizing column. One milliliter fractions were collected, lyophilized, and resuspended in 200 ml of sterile water. The fractions were tested for their effects on REVC thymidine uptake and morphology as described above.

Generation of Plasmin-Treated REVC Supernates

REVC cells (1.2–2.0 x 10^6) were plated in 75 mm^2 tissue culture dishes in DMEM/F12 + 2% FBS for 12–24 h to allow for adhesion, rinsed with serum-free DMEM/F12, and incubated (72 h, 37°C) in 10 ml of serum-free DMEM/F12 containing plasmin (1.8 μg/ml). Supernates from 2 dishes were pooled, sterilized by passage through a 0.22 micron filter, and loaded on a preparative reversed-phase C_{18} Vydac HPLC column using 0.1% Trifloroacetic acid as a buffer. A linear 60 min gradient (0–100% acetonitrile + 0.1% trifloroacetic acid) was used to elute bound components, which were collected in 1 ml fractions. As a control, a sample containing similarly incubated plasmin at 1.8 μg/ml in DMEM/F12 was loaded on the C_{18} column to determine its elution position. Column fractions were lyophilized and reconstituted in 200 ml of sterile water. To determine the

cleavage of human fibronectin generated at least five major bands, ranging in molecular weight from 140 to 160 kD. Fractionation of tryptic-cleaved fibronectin by molecular sieve chromatography resulted in fractions with variable effects on EC morphology and thymidine uptake (fig. 2). REVC cells normally displayed a cobblestone morphology in vitro (fig. 2, lower left panel). Sprouts similar in morphology to those induced by Matrigel were observed with low molecular weight fractions (lower right panel). In these fractions, REVC uptake of [^{3}H]thymidine was also diminished.

Morphologic Changes Induced by Plasmin

REVC cultures treated with 1–3 mg/ml of plasmin developed sprouts identical in morphology to those seen with Matrigel (fig. 3, Plasmin). Treating cells with identical concentrations of heat-inactivated plasmin was without effect. In order to determine whether the formation of sprouts was due to plasmin-catalyzed cleavage of a particular protein in REVC-conditioned media, plasmin-treated REVC-conditioned media were fractionated using reversed-phase HPLC and tested for sprout-inducing activity. We found that only fractions 47–49 could induce elongated sprouts in subconfluent cultures of REVC cells (fig. 4). Plasmin eluted significantly earlier than did sprout-inducing fractions (fractions 29–31), while undigested rabbit fibronectin eluted much later (fractions 80–82). Sprout-forming activity coincided with the major peak of fibronectin immunoreactivity by ELISA. Western blotting of the sprout-forming fractions with an anti-fibronectin antibody revealed a series of bands in the 160–180 kD range which were not present in native fibronectin, nor in fractions that were devoid of sprout-forming activity.

Human serum fibronectin was fragmented with plasmin and subjected to fractionation on reversed-phase HPLC. Fractions were assayed as above for sprout-inducing activity on REVC cells. In addition, fractions were assayed by ELISA, using

two commercially available anti-human fibronectin monoclonal antibodies: 3E1, directed against the carboxy-terminal, heparin binding domain; and 3E3, directed against the RGD-containing, cell binding domain. Sprout formation was only observed for fractions 53–57, which contained both 3E1 and 3E3 immunoreactivity. While ELISA of fractions using the 3E3 mAb demonstrated immunoreactivity over a broad range of fractions (solid line), the most prominent peak of immunoreactivity associated with sprout-inducing fractions was observed with the 3E1 mAb (dotted line).

The loss of extracellular fibronectin could be demonstrated by immunofluorescence when plasmin was used at concentrations effective in inducing sprout formation (fig. 5). Fibronectin staining in untreated cells showed a reticulated pattern, with staining limited largely to the extracellular space. Several cells were observed to be outlined by the fibronectin staining. By contrast, cells treated with plasmin showed typical sprouts, with loss of the extracellular and most of the cell-associated fibronectin.

Morphologic Changes Induced by Synthetic, RGD-Containing Proteins

A simple explanation for the sprout-inducing effects of plasmin and of trypsin-generated fibronectin fragments is that sprout formation results from loss of contact between REVC cells and substrate-bound fibronectin. This would presumably depend on competition between substrate-bound fibronectin and soluble, proteolytic fragments for binding to REVC integrins. Since several integrins bind to the sequence arginine-glycine-aspartate (RGD) found in several ECM proteins, we tested this hypothesis by examining the effects of several RGD-containing proteins on REVC morphology. ProNectin F is a recombinant protein which contains 13 fibronectin-derived RGD containing domains. When ProNectin F was coated onto tissue culture wells at least 24 h prior to plating, the REVC cells assumed a typical flattened, cobblestone appearance which persisted after 72 h of culture (data not shown). When an identical quantity of ProNectin F (2.5 μg/ml) was added directly to the medium immediately or shortly after plating, the REVC cells instead

concentrations in the 8–12 mcg/ml range could be reversed by increasing the serum concentration to 10%. Con A is a tetramer and contains two carbohydrate binding sites. By contrast, succinyl Con A is dimeric, containing only one carbohydrate binding site. In order to determine the importance of lectin valency (and cross-linking potential) in the process of sprout formation, we examined the effect of succinyl Con A on REVC cells. As figure 3 shows, succinyl Con A could also induce morphologically identical sprouts, but only at concentrations that were two- to three-fold higher than those for unmodified Con A.

Induction of EC sprouts by Matrigel is associated with cessation of DNA synthesis [29]. We sought to determine whether or not DNA synthesis is similarly inhibited by Con A at concentrations that induce sprouts. We found that there was a dose-dependent decrease in [^{3}H]thymidine uptake as Con A concentrations were increased from 0–20 mg/ml (fig. 7). As in the case of sprout formation, this decrease in DNA synthesis rate could be abrogated by increasing the FBS concentration from 2 to 10%. This reduction in DNA synthesis is not due to a cytotoxic effect of Con A, as the numbers of viable REVC cells measured with Alamar Blue were unaffected by sprout-inducing concentrations of Con A after incubation for as long as 5 days (data not shown).

Identification of Con A Binding Cell Surface Glycoproteins

Con A-binding glycoproteins derived from REVC cells were isolated from detergent-generated cell lysates using affinity chromatography with Con A-Sepharose 4B. When the a-methylmannoside-eluted glycoproteins were resolved by SDS-PAGE under non-reducing conditions, they migrated as major bands with estimated molecular weights of 200, 31 and 25 kDa. Using Superose-12 HPLC, we were able to generate two pools of proteins termed HMW (>400 kDa) and LMW (<400 kDa). These were used to immunize 2 groups of mice for hybridoma production of monoclonal antibodies.

As a result of 2 fusions with spleens of mice immunized separately with either HMW or LMW, a panel of 19 monoclonal antibodies (16 IgG and 3 IgM) were generated that bound to the REVC-derived glycoproteins. Two of the IgM monoclonal antibodies (1G7 and 2E8), generated from the HMW fraction, reacted with purified rabbit plasma fibronectin but not with human or bovine fibronectin on Western blot and ELISA (data not shown). Both of these antibodies, if added prior to addition of Con A, could prevent the formation of sprouts (fig. 8). These antibodies, at the same concentration, also blocked the Con A-induced suppression of thymidine uptake in REVC cells (table 1). In contrast, neither PBS or mouse IgM monoclonal antibodies to irrelevant antigens nor purified mouse serum IgM had any effect on REVC sprout induction on DNA synthesis inhibition mediated by Con-A (data not shown). That several of the mAbs raised against REVC-derived Con A-binding proteins recognized fibronectin on immunoblots suggested that Con A binds to rabbit fibronectin. This was confirmed by finding that purified rabbit plasma fibronectin was retained on a Con A-Sepharose 4B column, and could be eluted with 400 m*M* a-methylmannoside (data not shown).

Table 1. Capacity of Anti-REVC Monoclonal Antibodies to Block Con A-Induced Inhibition of REVC Cell [^{3}H]Thymidine Uptake

Antibody Added	With Con A (cpm (sd))	Percent Change**	Without Con A (cpm (sd))	Percent Change*
PBS (control)	340 ± 12	0%	7869 ± 588	.0%
1G7 (IgM)	3940 ± 211	+1058%	7444 ± 710	-5.4%
2E8 (IgM)	7548 ± 343	+2120%	8710 ± 600	+10.6%
4G2 (IgG1)	310 ± 33	-9%	6700 ± 955	-14.8%
16C12 (IgG1)	204 ± 13	-40%	5325 ± 1992	-32.0%

* DNA synthesis measured by [^{3}H]thymidine uptake in 4-6 replicate samples and expressed as counts/min (standard deviation per 10^5 cells plated.

** Percent change in DNA synthesis in the presence of various monoclonal antibodies is expressed relative to that seen for REVC cells incubated with PBS (control).

fibronectin and other mannose-containing glycoproteins that are present in serum. Cross-linking of mannose-containing glycoproteins does not appear to be essential for this effect, in that succinyl-Con A, which is monovalent, could also induce sprouts. That an equivalent morphologic effect required two to three times as much succinyl-Con A as Con A suggests that the stoichiometric binding of this lectin to a critical number of mannose residues on rabbit fibronectin is required for induction of sprout formation in these rabbit EC. Fragments of fibronectin that include the cell attachment, RGD-containing domain would be expected to compete with intact, substratum-adsorbed fibronectin for integrin binding. Likewise, soluble ProNectin F would compete with substratum-adsorbed fibronectin molecules for binding to REVC integrins. In each case, we propose that loss of attachment of REVC cells to substrate-immobilized RGD sequences contributes to generation of a series of intracellular signals that ultimately lead to the sprout phenotype, with its attendant depression of DNA synthesis. These signals depend upon more than simple competition between soluble and substrate-bound RGD sequences, however. Monomeric RGD-containing peptides, such as FNfn7–10 and echistatin, did not induce sprouts, even at concentrations that led to cell retraction and release from the plastic substrate. Likewise, only those fractions of plasmin-digested rabbit fibronectin that contained both the cell binding and carboxy-terminal heparin binding domains induced sprouts. This apparent paradox can be explained by proposing that sprout formation requires not only displacement of REVC cells from substrate-bound fibronectin but also cross-linking of fibronectin-binding integrins by soluble dimeric or multimeric RGD-containing peptides.

Fibronectin is a dimer that is disulfide cross-linked at the carboxy terminus [11]. Our model would therefore predict that fragments of fibronectin which induce sprouts must contain not only the cell binding domain, but also the carboxy-terminal heparin-binding domain, where the disulfide cross-link is located. As figure 8 demonstrates, this was indeed observed. Support for this proposed mechanism also comes from previous

studies which have shown that EC sprout and tube formation is enhanced by antibodies to the a_2b_1 and a_vb_3 integrins [30] and by a reduction in the relative density of substrate-bound fibronectin molecules [31].

The role of the ECM in determining EC morphology has been extensively investigated by Ingber and his colleagues [8, 13, 31–33]. These authors have proposed that EC shape is determined by the balance between external forces, generated by ECM protein binding to integrins, and internal forces, generated by the cytoskeleton. Reducing the ECM-generated external forces by trypsinization or treatment with millimolar concentrations of RGD-containing peptides would disrupt this balance and lead to cell retraction and loss of sprouts. This was observed using both intact EC and saponin-permeabilized cell preparations that were induced to contract with ATP and calcium [33]. Furthermore, these authors noted that sprout formation was produced by plating EC on a fibronectin-coated substrate in the presence of bFGF. Although our model differs significantly from that proposed by these authors, this difference may be due to the effect of bFGF on EC. Thus, bFGF at the concentrations used by these authors has been documented to induce EC to secrete proteases and their activators into the medium [34–37]. These include plasminogen activator, which would be expected to activate plasmin. As we have demonstrated, activated plasmin cleaves fibronectin and induces EC to form sprouts. Further support for our model comes from earlier work by Maciag et al. [7]. These authors demonstrated that both trypsin and plasmin could accelerate the formation of sprouts and tubes in HUVECs in culture. Our work significantly extends these observations by demonstrating that the physiologically relevant protease plasmin is capable of cleaving endothelial-associated fibronectin, that the sprout-forming fractions of a plasmin digest of REVC-conditioned medium co-migrate on HPLC with these fibronectin fragments, and that fragments of fibronectin produced by trypsin are by themselves capable of both inducing sprouts and suppressing DNA synthesis.

an endogenous lectin derived from baby hamster kidney binds to fibronectin and laminin and alters cellular adhesion and morphology [41].

Our study would lend support to the notion that altering the balance between extracellular and intracellular tensile forces in REVC cells leads to a series of intracellular signals that ultimately produce EC sprouts. Myosin II, the major form of cytoplasmic myosin, provides intracytoplasmic tensile forces in conjunction with the actin-based cytoskeleton [42]. It thus represents an important component in the balance of forces between the ECM and the cytoplasm. Its activity is dependent on phosphorylation of its regulatory light chain [43], and the degree of light chain phosphorylation might therefore be expected to change in response to a disturbance of this balance. As our subsequent paper will demonstrate, this was the case for REVC cells that undergo sprout formation in response to Con A.

Acknowledgments

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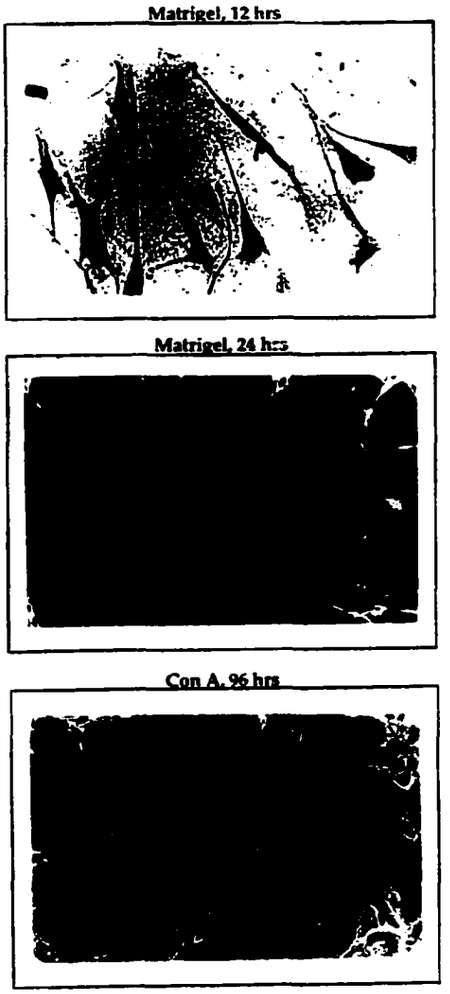

Fig. 1. Effect of Matrigel on REVC morphology. **Top** REVC cells plated on Matrigel in DMEM/F12 + 2% FBS demonstrated formation of EC sprouts. Formation of sprouts on Matrigel was rapid and complete by 12 h after plating. **Center** After 24 h, REVC cells formed tube-like structures. **Bottom** Addition of 8 mg/ml Con A to confluent cultures of REVC cells led to formation of tube-like structures after 4–5 days in culture. These structures were not observed with subconfluent cultures of REVC cells. Magnification: x 250.

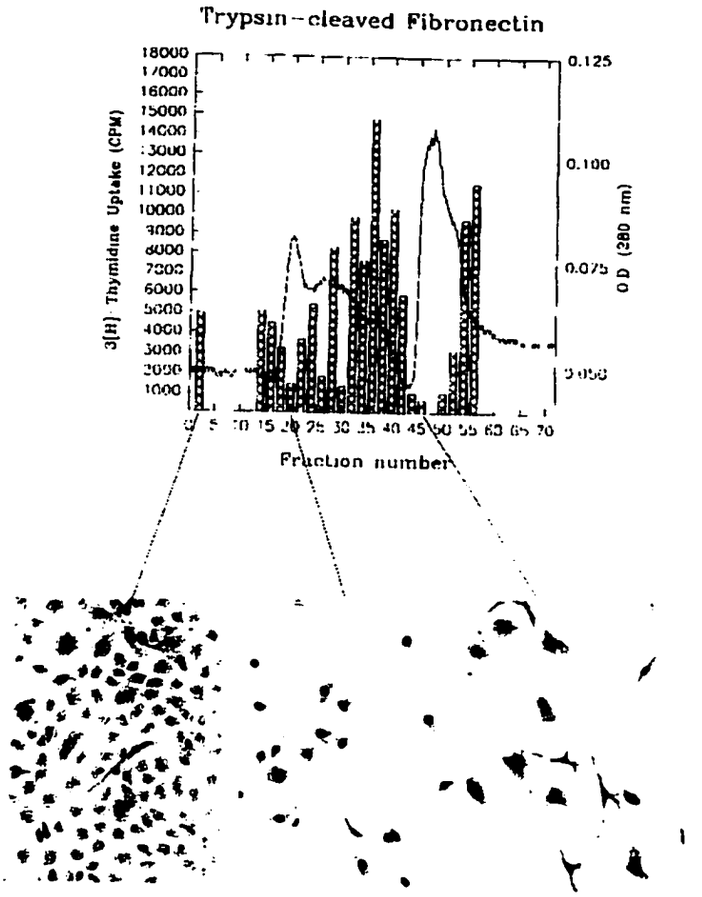

Fig. 2: The effect of trypsin-cleaved fibronectin fragments on REVC morphology and proliferation. Trypsin-cleaved fibronectin was separated on a Superose 12 HPLC sizing column and collected in 500 ml fractions. Absorbance at 280 nm is represented by the solid line. Fractions were assayed for their effects on REVC cell [^{3}H]thymidine uptake (cross-hatched bars) and on REVC cell morphology. Representative fractions are portrayed. Formation of sprouts was observed in fractions 42-48 and was accompanied by a decrease in tritiated thymidine uptake.

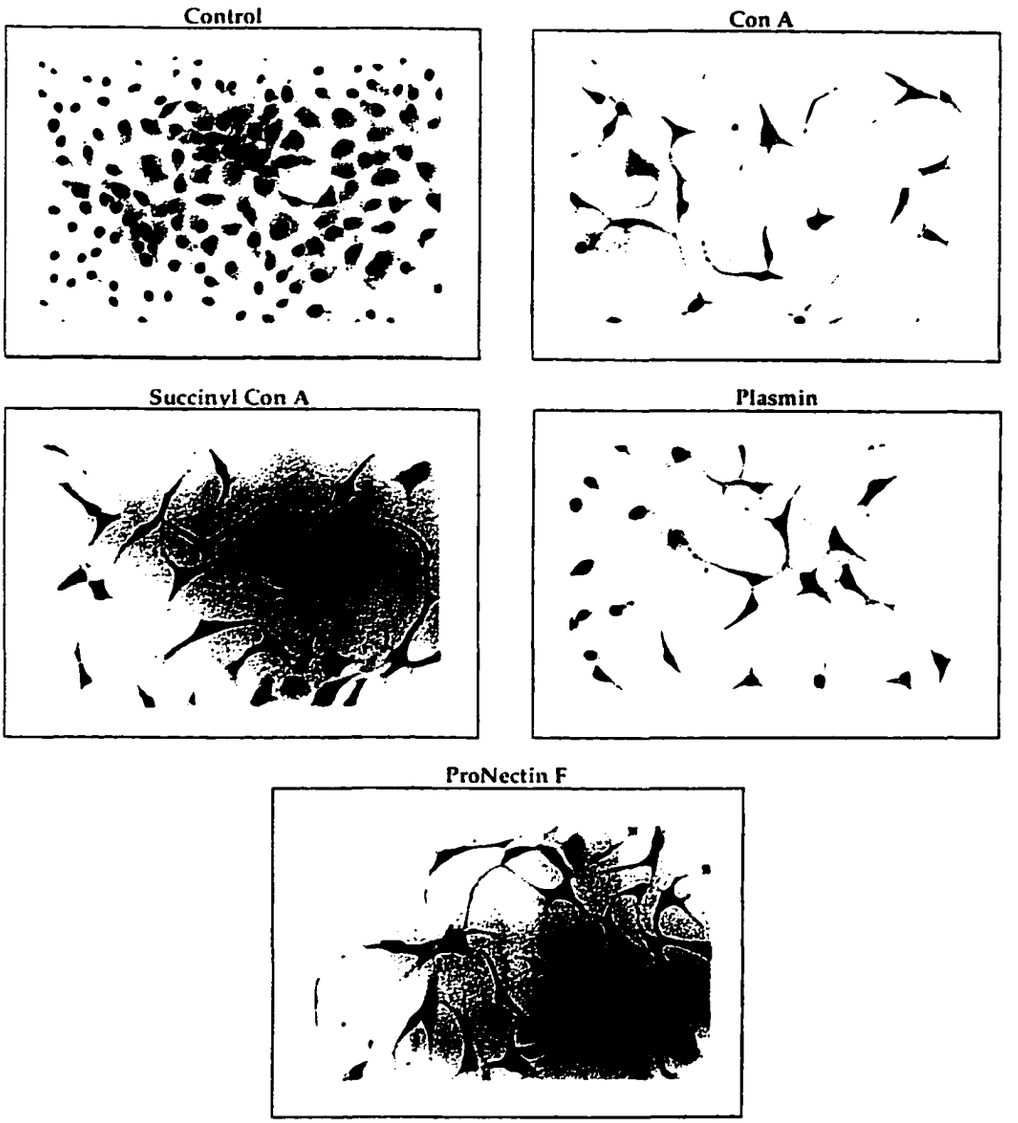

Fig. 3. Sprout induction in REVC cells by Con A, succinyl Con A, Plasmin, and ProNectin F. Control: REVC cells cultured to confluence on tissue culture plastic in DMEM/F12 + 2% FBS assumed a flattened cobblestone morphology typical of endothelial cells grown in culture. Sprouts could be induced in these cells by adding 8 μg/ml of Concanavalin A (Con A), 20 mg/ml succinyl Con A (succinyl Con A), 2 mg/ml plasmin (plasmin), or 2.5 mg/ml of soluble ProNectin F (ProNectin F). Magnification: x 100.

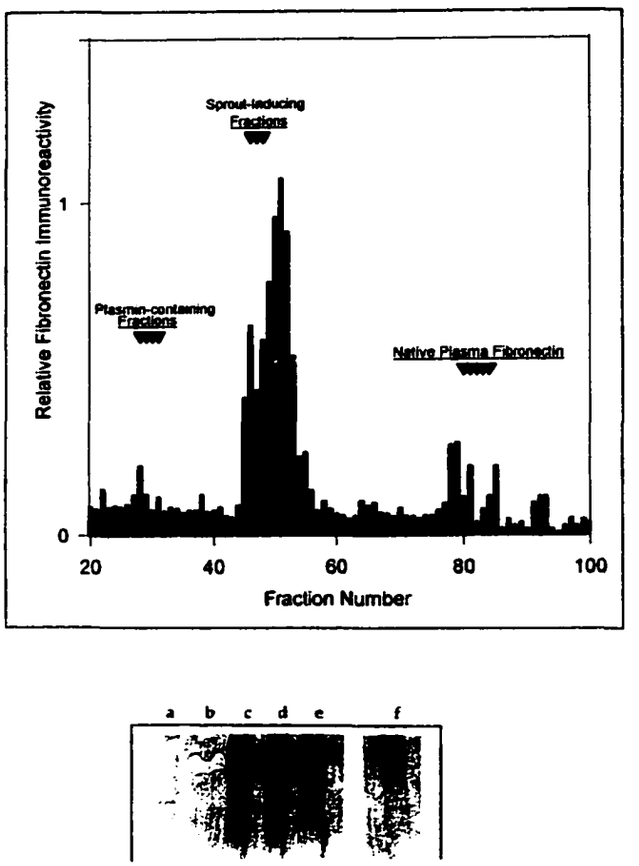

Fig. 4. Reversed-phase chromatograph of plasmin-digested REVC-conditioned medium. **Top** Plasmin-treated REVC-conditioned medium was fractionated by reversed-phase chromatography. Fractions were assayed by ELISA for fibronectin immunoreactivity using polyclonal goat anti-rabbit fibronectin antibody and were also assayed for sprout-forming activity. Fractions, which induced sprouts as indicated in the figure, coincided with the peak of fibronectin immunoreactivity. Elution positions of undigested fibronectin and plasmin, also indicated, were well-separated from fractions that induced sprouts. **Bottom** Immunoblot of sprout-forming fractions 47-49 (lanes c, d, and e), performed with the polyclonal anti-rabbit fibronectin antibody, revealed a series of bands in the range of 180-60 kD, which were not present in fraction 35 (lane a) or fraction 75 (lane b), neither of which induced sprouts. An immunoblot of native fibronectin (lane f) is included for comparison.

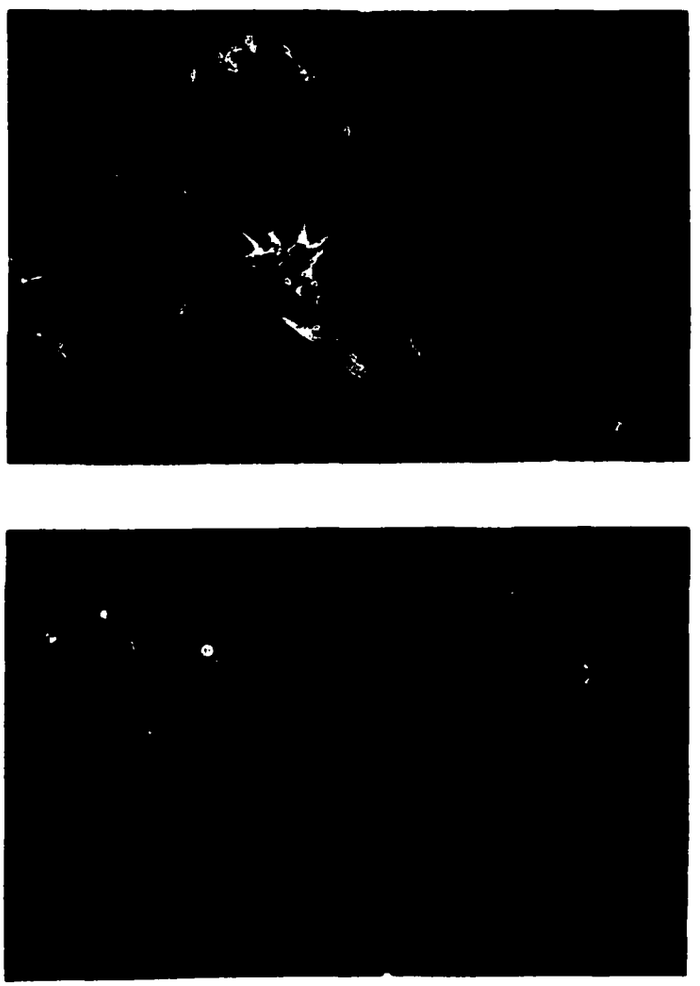

Fig. 5. Immunofluorescence microscopy of fibronectin staining on REVC cells ± plasmin. **Top** Fluorescence microscopy of REVC cells treated with anti-fibronectin mAb 4G7 and stained with FITC-labeled goat anti-mouse secondary antibody. Fibronectin staining showed a reticulated pattern which was largely restricted to the extracellular space and which outlined several of the cells in the field of view. **Bottom** Fluorescence microscopy of REVC cells treated with plasmin prior to fibronectin staining. Extracellular fibronectin was absent, with mild background staining of cells that demonstrated sprout formation. Magnification: x 250.

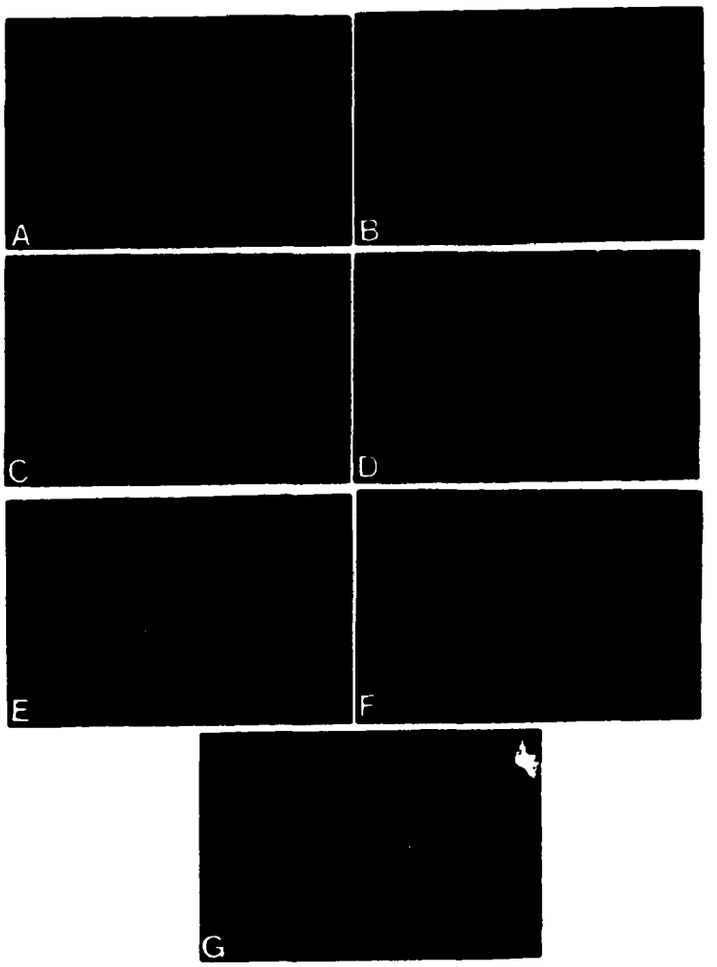

Fig. 6. Time course of actin rearrangement in REVC cells after addition of Con-A induced morphological change. There is a characteristic stress pattern formation in REVC cells (**A**), that rapidly dissolves after 5 min (**B**). There is the formation of lamellapodia after 3 h (**C**), with the formation of new cellular contacts (**D,E**) after 18 h. Elaborate sprout networks are obvious after 36–48 h (**F**). At low concentrations of Con-A (6 mcg/ml), the return to a cobblestone morphology (**G**) is observed after 72 h.

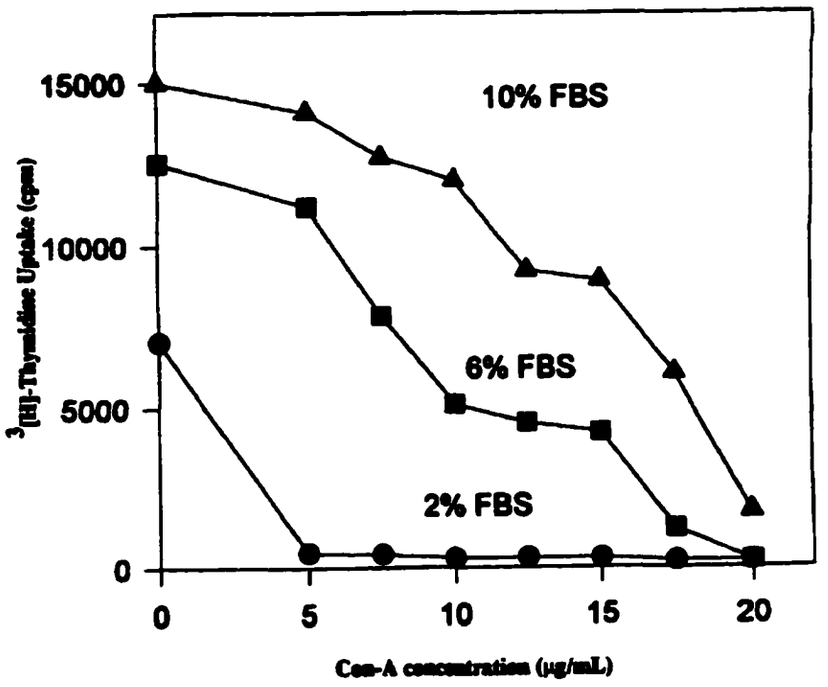

Fig. 7. Dose response curve demonstrating alterations in [^{3}H]thymidine uptake in REVC cells at varying Con A and FBS concentrations. Con A induced suppression of REVC cell [^{3}H]thymidine uptake could be blocked by increasing the concentration of FBS in the culture medium.

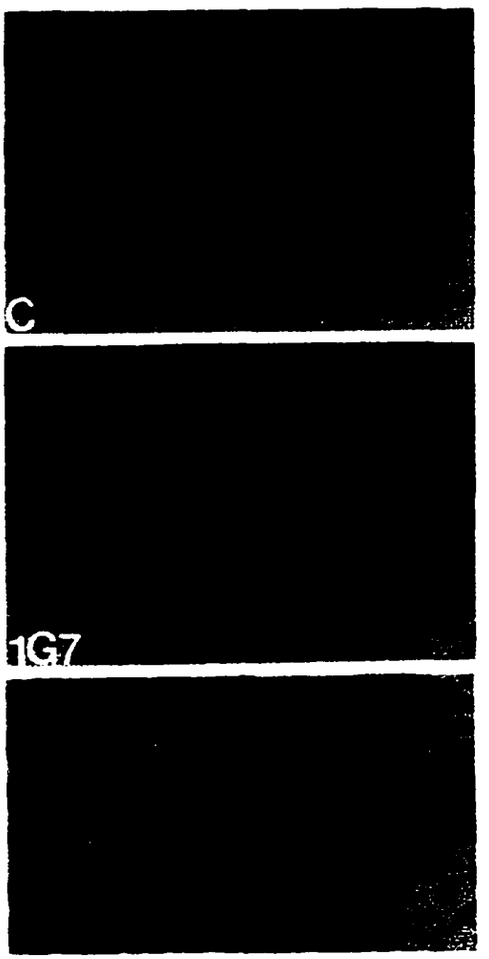

Fig. 8. Effect of anti-REVC IgM mAbs on the Con A effect. Addition of 45 mg/ml of IgM mAbs 1G7 (**middle**) or 2E8 (**bottom**) (reactive with rabbit plasma fibronectin) to cultures of REVC cells in DMEM/F12 + 2% FBS + 8 mg/ml of Con A blocked Con A-induced sprout formation, when compared to control cultures treated with Con A without antibody (**top**). Magnification: x 100.

EPIDERMAL GROWTH FACTOR STIMULATES VASCULAR ENDOTHELIAL GROWTH FACTOR PRODUCTION BY HUMAN MALIGNANT GLIOMA CELLS: A MODEL OF GLIOBLASTOMA MULTIFORME PATHOPHYSIOLOGY

by

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Abstract

Hypervascularity, focal necrosis, persistent cerebral edema, and rapid cellular proliferation are key histopathologic features of *glioblastoma multiforme* (GBM), the most common and malignant of human brain tumors. By immunoperoxidase and immunofluorescence, we definitively have demonstrated the presence of vascular endothelial growth factor (VEGF) and epidermal growth factor receptor (EGFr) in five out of five glioma cell lines (U-251MG, U-105MG, D-65MG, D-54MG and CH-235MG) and in eight human GBM tumor surgical specimens. In vitro experiments with glioma cell lines revealed a consistent and reliable relation between EGFr activation and VEGF production; namely, EGF (1-20 ng/ml) stimulation of glioma cells resulted in a 25–125% increase in secretion of bioactive VEGF. Conditioned media (CM) prepared from EGF-stimulated glioma cell lines produced significant increases in cystolic free intracellular concentrations of Ca^{2+} ($[Ca^{2+}]_i$) in human umbilical vein endothelial cells (HUVECs). Neither EGF alone or CM from glioma cultures prepared in the absence of EGF induced $[Ca^{2+}]_i$ increases in HUVECs. Preincubation of glioma CM with A4.6.1, a monoclonal antibody to VEGF, completely abolished VEGF-mediated $[Ca^{2+}]_i$ transients in HUVECs. Likewise, induction by glioma-derived CM of von Willebrand factor release from HUVECs was completely blocked by A4.6.1 pretreatment. These observations provide a key link in understanding the basic cellular pathophysiology of GBM tumor angiogenesis, increased vascular permeability, and cellular proliferation. Specifically, EGF activation of EGFr expressed on glioma cells leads to enhanced secretion of VEGF by glioma cells. VEGF released by glioma cells in situ most likely accounts for pathognomonic histopathologic and clinical features of GBM tumors in patients, including striking tumor angiogenesis, increased cerebral edema and hypercoagulability manifesting as focal tumor necrosis, deep vein thrombosis, or pulmonary embolism.

[15] have shown an increased proliferative index for EGFr-positive malignant glioma cells as compared with glioma cells that do not express this receptor. The implication is that activation of EGFr may play a role in the persistent proliferation of GBM. EGF is a low molecular weight (6 kDa) growth factor that is a normal constituent of human plasma [16] and is mitogenic for cells expressing EGFr. Several groups have also demonstrated that some gliomas show increased mRNA levels and express transforming growth factor-α (TGF-α), which can activate EGFr in vitro [17] in an equimolar fashion equivalent to EGF. Therefore, the ligand(s) responsible for activating EGFr, i.e. EGF and TGF-α, are either constitutively present in plasma and plasma ultrafiltrates or can be produced by glioma cells themselves.

Until now, there has been no demonstrated relation between EGFr expression in gliomas and associated vascularity of these tumors. In this study we demonstrate the simultaneous expression of VEGF and EGFr in five out of five brain tumor cell lines examined. In eight postoperative surgical specimens, both VEGF and EGFr were determined by immunohistochemical means to be variably expressed. More importantly, we demonstrate for the first time that activation of EGFr by EGF leads to increased secretion of bioactive VEGF by malignant glioma cells. This physiological relation provides a useful paradigm to explain the paradoxical existence of intense neovascularity and necrotic foci in GBM tumors.

Materials and Methods

Biochemical and Biological Reagents

Dulbecco's modified Eagle's medium mixed 1:1 with Ham's F-12 (DMEM/F12; Sigma Chemical Co., Inc., St. Louis, Mo., USA) and MCDB 105 (Sigma) supplemented to 2 m*M* with *L*-glutamine were used for culture of all glioma cells. Medium 199 (M199, GIBCO, Inc., Grand Island N.Y., USA) was used as the culture medium for human umbilical vein endothelial cells (HUVEC). Fetal bovine serum (FBS) and calf serum

(Hyclone, Inc., Logan, Utah, USA) were heat inactivated (56°C, 45 min). EGF (human, receptor grade; Collaborative Research, Inc., Bedford, Mass., USA), dexamethasone (Sigma), β-Phorbol (Sigma), and phorbol myristate acetate (PMA, Sigma) were all diluted at specified concentrations in Dulbecco's phosphate-buffered saline (PBS, pH 7.2). Transforming growth factor-β1 (TGF-β1) (R&D Systems, Inc., Minneapolis, Minn., USA) was prepared as 1 μg/ml stock solution by dilution in exipient (25% isopropyl alcohol + 0.1% trifluoracetic acid). Further dilution was carried out in PBS containing 3 mM CHAPS (Boehringer Mannheim, Inc., Mannheim, Germany). Recombinant VEGF (rhVEGF; 165 amino acid isoform) was purified at Genentech (South San Francisco, Calif.). Purified guinea pig VPF was used as a positive control for vWF assay and was obtained as a generous gift from Dr. Donald Senger (Beth Israel Hospital, Boston, Mass., USA). Fura-2/AM was purchased from Molecular Probes (Junction City, Ore., USA). Culture media and sera were determined to have < 100 pg/ml of endotoxin by the Limulus amoebocyte lysate assay.

Cell Lines

GBM cell lines U-251MG, U-105MG, D-65MG and D-54MG were obtained as a gift from Dr. Darell D. Bigner (Duke University, Durham, N.C.) and have been described in detail [1, 18, 19]. CH-235MG was cultured from a portion of a GBM tumor removed in 1979 from a 60-year-old female and has recently been characterized [20]. Karotypes for U-251MG, &105MG, D-65MG, and D54MG were similar to those previously reported [21, 22]. Moreover, restriction fragment length polymorphism mapping profiles produced by restricting cell line DNA with *Hae* III and probing Southern blots with YNH24 (locus D2S44, 0.96 polymorphic discrimination) and pH30 (locus D4S139, 0.99 polymorphic discrimination) were nonidentical, corroborating that these tumor cell lines originated from different patients. All of the glioma cell lines have been passaged >40

times and, for experiments, the cell lines were maintained in 150-cm^2 plastic tissue culture flasks (Falcon Plastics, Inc., Lincoln Park, N.J., USA) in complete culture media (DMEM/F12 + 2m*M* *L*-glutamine + 8% FBS) at 37°C and 7.5% CO_2. Antibiotics were not used routinely, and all cell lines were found by routine testing to be negative for mycoplasma by Hoechst 33258 stain [23]. Cells were grown to postconfluence with medium changes every third day and were harvested using brief incubation (10 min, room temperature [RT]) in 7 ml 0.05% Trypsin/0.53 mM EDTA solution (GIBCO). FBS (2 ml) was added to the flask to inactivate the trypsin, and adherent cells were released by trituration, pelleted by centrifugation (200 x *g*, 8 min, 22°C), and resuspended in 10 ml of DMEM/F12. Viable cell counts were determined by trypan blue exclusion.

Human Endothelial Cell Cultures

Primary cultures of human endothelial cells (HUVECs) were established from pooled umbilical cords and were propagated (passages 2-5) as previously described [24]. Medium 199 was supplemented with 15% FBS, 10 μM thymidine (Sigma), 100 μg/ml porcine heparin (Sigma), 50 μg/ml endothelial cell growth factor (Biomedical Technologies, Inc., Stoughton, Mass., USA), 100 μg/ml of streptomycin, and 100 μ/ml of penicillin (GIBCO). HUVECs were serially passaged twice per week by harvesting with trypsin/EDTA and seeding at a 1:4 ratio into gelatin (0.1%)-coated 75-cm^2 flasks. For these experiments, HUVECs between passage levels 2 and 6 were seeded into 35- or 100-mm gelatin-coated dishes, medium was exchanged every other day, and the cells were used after 6–8 d.

VEGF Secretion by U-105MG and U-251MG Cell Lines In Vitro

Glioma cells were plated at 2.5 x 10^5 cells/well in 24-well microtrays (Costar 3524, Costar, Cambridge, Mass., USA) in 400 μl of DMEM/F12 + 2m*M* *L*-glutamine + 10%

FBS, and cells were incubated for 3 days at 37°C in 7.5% CO_2. Monolayers were rinsed once with serum-free MCDB 105 medium, and 400 µl of fresh MCDB 105 + 2 m*M* *L*-glutamine containing 0, 2, 5, or 10% FBS was added to triplicate wells of each plate. At 24-h intervals, the medium and cell monolayers in one microplate were harvested as follows. Medium from each well was clarified by centrifugation (3,000 x *g*; 15 min, 4°C) in 1.5-ml polypropylene microcentrifuge tubes (Fisher Scientific Co., Pittsburgh, Penn., USA). Individual aliquots (350 ml) were stored frozen (-20°C) in separate microcentrifuge tubes until analyzed for VEGF by enzyme-linked immunosorbent assay (ELISA). Glioma cells were harvested from monolayer cultures with 1 or 2 ml of trypsin/EDTA solution, and viable cell numbers were determined by trypan blue exclusion.

Growth Factor and Pharmacologic Stimulation of Glioma Cell Lines

The effect of specific stimuli on VEGF secretion was assessed in glioma cell lines U-251MG, U-105MG, D-65MG and D-54MG. Each glioma cell line was plated at 3 X 10^5 cells/well in 6-well plates (No. 3407 Falcon, Inc.) in 2 ml of complete culture medium and incubated (37°C; 7.5% CO_2) undisturbed for 72 h. Monolayers were rinsed once with serum-free DMEM/F12 and 1 ml of serum-free DMEM/F12 containing either recombinant human EGF (20 ng/ml), TGF-β1 (1 ng/ml), dexamethasone (200 ng/ml), PMA (160 nM), β-phorbol (160 nM), or PBS was added to replicate wells. After a 5-day incubation (37°C, 7.5% CO_2), the conditioned media (CM) were collected, clarified by centrifugation, transferred to clean polypropylene microcentrifuge tubes, and held frozen (-20°C) until assayed for VEGF by ELISA.

Various EGF concentrations were used to induce VEGF in the U-105MG cell line as follows. U-105MG monolayers were established in 24-well plates by incubating (72 h, 37°C) 2.5 x 10^5 cells/well in 400 µl of DMEM/F12 containing 10% FBS. Monolayers

were rinsed once with serum-free medium and 400 µl of DMEM/F12 containing 3% FBS was added to each well. EGF (0–30 ng/ml) or PBS was added (10 µl) to triplicate wells to augment EGF concentrations by 0–30 ng/ml. After incubation (120 h, 37°C, 7.5% CO_2) media from individual wells were harvested separately. Clarified supernates were stored frozen (-0°C) until VEGF levels were quantified by ELISA.

Sandwich ELISA for VEGF

Ninety-six-well microtiter plates (Maxisorb; Nunc, Kamstrup, Denmark) were coated with monoclonal antibody (mAb) 4.6.1 [25] by incubating overnight at 4°C with 100 µl/well of antibody at 2.5 mg/mL in 50 mmol/liter of sodium carbonate buffer, pH 9.6 (coat buffer). After removal of the coating solution, the coated plates were blocked with 150 µl/well of 5% BSA in PBS for 1 h at RT and washed six times with 0.5% Tween 20 in PBS (wash buffer).

Standards were freshly prepared by dilution of rhVEGF (1 mg/ml established by quantitative amino acid analysis) with assay buffer (PBS containing 5% BSA, 0.05% Tween 20 and 0.01% Thimerosal) to 50, 25, 12.5, 6.25, 3.12, 1.56, 0.78 ng/ml. Diluted standards and samples were dispensed onto coated wells (100 µl/well), and plates were sealed and incubated at room temperature for 2 h with gentle agitation. Horseradish peroxidase (HRPO) labeled mAb 3.13.1 [25] was then added (100 µl/well). After further incubation (1 h, RT), the plates were washed. Freshly prepared OPD substrate solution (0.4 g of o-phenylenediamine dihydrochloride/l of PBS plus 0.4 ml of 30% hydrogen peroxide) was added (100 ml/well) and incubation (15 min, RT) carried out in the dark. The reaction was stopped by the addition of 100 µl of 2.25 mol/l sulfuric acid and absorbance at 490 nm determined on a V_{max} plate reader (Molecular Devices, Menlo Park, Calif., USA). A standard curve was generated by plotting absorbance vs. log of rhVEGF concentration, using a four-parameter nonlinear regression curve fitting program

(developed at Genentech). Sample concentrations were obtained by interpolation of their absorbance on the standard curve.

Bulk Production of Glioma Cell Line Conditioned Medium

U-251MG or U-105MG glioma cell lines were grown to confluence (72 h, 37°C) on 225-cm^2 flasks in DMEM/F12 containing 10% FBS. Cell monolayers were washed twice with serum-free DMEM/F12, and 35 ml of serum-free medium containing 20 ng/ml of EGF or PBS were added to each flask and incubated undisturbed for 72 h (U-105MG) or 96 h (U-251MG). This CM was collected, clarified by centrifugation (800 x *g*, 10 min, RT) and concentrated with extensive dialysis against PBS (Centriprep-10; Amicon Division, Beverly, Mass., USA). Final concentration was achieved with Centricon-10 microconcentrators (Amicon). Total volume reduction for U-251MG CM was 70-fold, and for U-105MG CM was 84.5-fold. A 150-µl aliquot of each concentrate was stored at 4°C before calcium flux assay, and the remainder was stored at -20°C prior to quantification of VEGF by ELISA.

Measurement of Ca^{2+}-Sensitive Fura-2 Fluorescence

Fura-2, a Ca^{2+}-sensitive fluorescent dye [26], was used to monitor changes in cytosolic free intracellular calcium concentration (Ca^{2+}_i) in HUVEC suspensions as described by Brock and Capasso [24]. For these experiments, HUVEC were grown on gelatin (0.1%)-coated 100 mm (diam) dishes. To detach cells, six to eight dishes (~1.5-3 x 10^7 cells) were briefly exposed to Hank's balanced salt solution without calcium or magnesium and with 2 mM EDTA and 0.1% BSA. Cells were pelleted by centrifugation (200 x *g*, 3 min) and resuspended in serum-free M199 (5 x 10^6 cells/ml). The cell suspension was incubated with fura-2/AM (0.1 µM, 20 min, 37°C) then diluted tenfold with M199 and incubated for an additional 10 min. Cells were pelleted by low-speed

coated plates were blocked with 200 µl /well of 5% BSA in PBS (1 h, RT), and washed four times with wash buffer.

Standards were freshly prepared by diluting human vWF with assay buffer (PBS containing 5% BSA, 0.05% Tween 20) to 6.25, 3.12, 1.56, 0.78 and 0.39 mU/ml. The diluted standards and samples were dispensed onto the coated wells (100 µl/well). Plates were sealed and incubated at room temperature for 2 h with gentle agitation. Plates were washed as above and HRPO-labeled goat Fab anti-vWF was then added (100 µl/well). After further incubation, (1 h, RT), the plates were washed, freshly prepared OPD substrate solution was added (100 µl/well), and incubation was carried out in the dark (15 min, RT). Enzymatic reaction was stopped by addition of 2.25 M sulfuric acid (100 µl/well) and absorbance (490 nm) determined using a microtiter plate reader (model 450; BIO-RAD, Richmond, Calif., USA). A standard curve was generated by plotting absorbance vs. vWF concentration; sample concentrations were obtained by interpolation of their absorbance relative to standards.

Immunohistochemistry/Immunofluorescence

Immunohistochemical staining for VEGF or EGFr was performed on glioma cell lines U-251MG, U-105MG, CH-235MG, and D-65MG, or on eight different GBM tumors obtained at surgery. Freshly resected GBM tumors were debrided, blocked, and frozen in O.C.T. mounting medium (Miles Laboratories, Inc., Naperville, Ill., USA). Frozen sections (10 µm thick) of GBM tumors that were mounted on poly-*L*-lysine coated slides or saline-rinsed confluent glioma cell line monolayers grown on glass slides (8-place Lab-Tek [Naperville, Ill.] culture chambers) were immersed in ice-cold 100% methanol for 30 s and allowed to dry. Slides were incubated in 3% BSA/PBS (30 min, RT) to block nonspecific binding, and excess blocking solution was replaced by a primary antibody solution in 3% BSA/PBS containing either 10 µg/ml of A.4.6.1 (anti-

VEGF), 10 μg/ml of Mab 425, (anti-EGFr, [27]), 1:2000 dilution of 29.1.1 (anti-EGFr ascites; Sigma), or a phycoerythrin-conjugated control polyclonal mouse IgG fraction (Becton Dickinson Co., Mountain View, Calif., USA). Glioma tissue sections or glioma cell line monolayers were incubated overnight in a humid atmosphere at RT and then extensively rinsed with PBS. For fluorescence staining, the slides were incubated (1 h, RT) with rabbit FITC-conjugated anti-mouse IgG (Southern Biotechnology, Inc., Birmingham, Ala., USA) at 10μg/ml in 3% BSA/PBS, then rinsed three times with PBS. For immunoperoxidase staining, the manufacturer's instructions were followed as described for the Vectastain Elite ABC IgG kit (Vector Laboratories, Inc., Burlingame, Calif., USA). All slides were coverslipped with 10% polyvinyl alcohol.

Alternatively, we have used the antigen retrieval system (BioGenex, San Ramon, Calif., USA) to demonstrate immunoperoxidase staining in formalin-fixed paraffin-embedded specimens of GBM tissues obtained through the UAB Tissue Procurement Facility (University of Alabama, Birmingham, Ala., USA). Histopathological diagnoses were provided by UAB neuropathologists or outside consultants. Paraffin tissue sections, cut at 10 μm, were mounted on TEPSA (3-aminopropyltriethoxysilane; Aldrich Chemical, Milwaukee, Wis., USA)-coated slides and baked 1h, 58°C. Mounted sections were soaked for 3 min each in two changes of xylene for deparaffinization, then dehydrated in graded changes of ethanol (70, 95, 100, and 100%) and rehydrated in PBS. Endogenous peroxidase was blocked by soaking mounted sections in 1.5% H_2O_2 in methanol. Each slide was then washed in deionized water and processed according to manufacturer's instructions for the antigen retrieval system. Nonspecific binding was blocked by incubating sections in 5% BSA/PSA for 40–60 min. mAbs A.4.6.1 (α-VEGF) or OKT4 (α-CD4; hybridoma obtained from American Type Culture Collection, Rockville, Md., USA) were diluted in 5% BSA/PBS, and each section was incubated with one of the mAbs (100 μl, 1 h, 37°C). Tissue sections were washed in two changes of 1% Triton-X100/PSA for 10 min each, followed by two rinses with PBS. Vectastain Elite

ABC kit was used as described above the complete the immunoperoxidase staining. Tissue sections were counterstained with aqueous hematoxylin and coverslips mounted with Permount (Fisher Scientific, Pittsburg, Penn., USA).

Results

Glioma Cell-derived VEGF

VEGF was detected at various concentrations in the CM of U-251MG, U-105MG, D-65MG and D-54MG glioma cell lines as detected by a VEGF-specific ELISA. VEGF accumulation in U-251MG conditioned medium (fig. 1A) correlated positively with increasing FBS concentrations (0–10%). Assays performed with diluted FBS indicated that culture medium not exposed to cells did not have detectable immunoreactive VEGF. The mean concentration of VEGF in medium from glioma cells (initial density: 2.5 x 10^5/well) grown in serum-free conditions averaged 2.3 ng/ml on day 3. In contrast, the CM from cells grown in 10% FBS contained an average VEGF concentration of 10.2 ng/ml by day 3. This represented a 340% increase in the amount of VEGF produced by these cells. To determine whether this increase reflected enhanced VEGF production or improved cell survival and proliferation, we concurrently counted viable cells trypsinized from the same wells from which the supernates were harvested. Growth curves (fig. 1B) of U-251MG cells in medium containing varying FBS concentrations revealed that, by day 3, cell number was greater in 10% FBS (1.23 x 10^6 cells/well) as compared with that of serum-free medium (9.8 x 10^5 cells/well). However, this only represented a 22% increase in cell number. These data indicated that modest increases in glioma cell numbers did not fully account for increased VEGF production; rather, a significant portion of the threefold increase in VEGF appeared to be due to increased secretion. Similar comparisons for the U-105MG cell line (not shown) yielded qualitatively similar results.

Stimulation of VEGF by EGF

Induction of VEGF secretion by various stimuli was examined using four glioma cell lines maintained in serum-free media. Both PMA (160 nM; fig. 2) and EGF (20 ng/ml; fig. 2) stimulated VEGF secretion in all four cell lines when compared with that induced by either beta phorbol (160 nM) or PBS. Increased VEGF secretion by U-105MG was induced by EGF at physiological concentrations of 1–20 ng/ml (fig. 3). VEGF secretion was not consistently suppressed or enhanced after administration of dexamethasone or TGF-β to glioma cells (table I).

Table 1. Effect of dexamethasone or TFG-β on secretion of VEGF by glioma cell lines

		Percent change in VEGF secretion[a]	
Glioma cell line	Unstimulated secretion (ng/ml)	Dexamethasone (2 mM)	TFG-β1 (2 ng/ml)
U-251MG	3.84	+116[b]	-20[b]
U-105MG	6.22	-20	-40
D-65MG	1.02	ND[c]	+77
D-54MG	3.97	+56	+15

[a] VEGF secretion measured by ELISA in 72–96 h CM.
[b] Values are percents.
[c] ND, not detectable.

Functional Activities of Glioma-derived VEGF

Although ELISA can provide a very accurate estimate of immunoreactive VEGF, biological activity of VEGF could not be determined using this assay. Therefore, we assayed changes of $[Ca^{2+}]_i$ within, and vWF secretion by, HUVECs as a measure of VEGF activity produced by cultured human glioma cells.

Changes in $[Ca^{2+}]_i$. As illustrated in figure 4, CM obtained from U-105MG glioma cells stimulated for 72 h with EGF induced an increase in $[Ca^{2+}]_i$ in HUVEC suspensions. Furthermore, this effect was not obtained unless the glioma cells were stimulated with

EGF; EGF itself did not alter HUVEC $[Ca^{2+}]_i$. Qualitatively similar fluorescence tracings were obtained using supernates prepared in an identical fashion from U-251MG cell line cultures.

To confirm that VEGF in glioma CM was responsible for the $[Ca^{2+}]_i$ increase, we used an anti-VEGF monoclonal antibody to neutralize VEGF [25]. As demonstrated in figure 5, mAb A.4.6.1 completely inhibited the ability of EGF-induced glioma CM to increase $[Ca^{2+}]_i$ in HUVECs. Addition of an antigen-irrelevant monoclonal antibody (α-S100 protein; IgG_{2a}) was without effect, yielding a transient similar to that of medium only. Thrombin (1 U/ml) was added at 240 s to demonstrate that the lack of VEGF-induced $[Ca^{2+}]_i$ change was not due to a nonspecific effect of the antibody. These experiments confirmed that alterations in $Ca^{2+}{}_i$ were specifically due to VEGF in glioma CM.

vWF Secretion. As previously described [10], another biological activity of VEGF is its ability to stimulate release of vWF from HUVECs. Enhanced release of vWF was induced by EGF-stimulated glioma cell CM (fig. 6). Again, as demonstrated above, vWF release was blocked by anti-VEGF mAb A.4.6.1 but not by an antigen irrelevant antibody specific for S-100 protein. As previously described [10], both thrombin and purified VEGF were effective stimulants for vWF release in HUVECs.

Immunohistochemical Staining

The presence of VEGF in frozen sections of two human GBM surgical specimens was demonstrated using both fluorescein (fig. 7, left) and peroxidase-labeled immunohistochemical staining methods. Both methods produced similar results; namely, a diffuse cytoplasmic and diffuse pericellular staining pattern. Normal brain grey matter from several patients undergoing routine temporal lobectomy for intractable seizures was consistently negative. Attempts to use the A.4.6.1 mAb for staining formalin-fixed

paraffin-embedded tissue sections were initially unsuccessful. However, when six archival GBM tissue sections were processed using an antigen retrieval system (BioGenex), we could reliably and reproducibly identify VEGF in all six of these human brain tumor sections (fig. 7, right). Again, normal cerebral cortex did not stain with this antibody. To date, we have demonstrated VEGF by various immunohistochemical methods in all eight human GBM tumor specifmens examined (Table 2).

Table 2. Immunohistochemical demonstration of VEGF and EGF4 in malignant gliomas

Tumor tissue designation	Diagnosis	Immunofluorescence results		Immunoperioxidase reults	
		VEGF	EVGr	VEGF	EGFr
Frozen sections					
90-112141	GBM	++	++	+	++
92-001461	GBM	+	+	+	ND[a]
Archival paraffin sections					
89-006119	GBM	ND	ND	+++	ND
90-003221	GBM	ND	ND	ND	++
91-06A011	GBM	ND	ND	++	ND
91-10A149B	GBM	ND	ND	+	ND
92-06A102	GBM	ND	ND	+++	ND
92-07A002	GBM	ND	ND	++++	0
92-08A182	GBM	ND	ND	+++	ND
Total Tumors Positive/Total Tested		2/2	2/2	8/8	2/3

Frozen sections tested by both indirect immunofluorescence and indirect immunoperioxidase; paraffin sections tested after antigen retrieval by indirect immunoperoxidase only; scoring based on intensity with 0, negative; +, light positive staining; ++, moderate staining; +++, strong positive staining, ++++, intense positive staining.

[a] ND = not done

EGFr were also detected in two of three human GBMs examined. mAb 29.1.1 is able to recognize EGF4 in human formalin-fixed paraffin-embedded GBM tissue sections without using the antigen retrieval system; furthermore, its application in this instance was not helpful in improving the strength or pattern of reactivity that was observed through the cytoplasm of the tumor cells, as well as localized to their surfaces (fig. 8). All four glioma cell lines examined (U-251MG, D-65MG, D-54MG, and CH-235MG)

stained positively for VEGF (fig. 9, A and B) and EGFr (fig. 10, A and B). Anti-VEGF staining of glioma cell lines revealed diffuse cytoplasmic distribution with focally intense perinuclear staining, and a delicate cell surface localization. Anti-EGFr stained coarsely in clumps on cell surfaces of glioma cell lines with diffuse cytoplasmic staining that was less dense than that of intracytoplasmic VEGF staining. A polyclonal mouse IgG_1 fraction used as the first antibody did not react with glioma cell lines.

Of the five glioma cell lines examined, we have detected production of VEGF by ELISA, immunohistochemistry, or calcium mobilization assays in all five (Table 3).

Table 3. Summary of detection methods used to demonstrate production of VEGF by five glioma cell lines.

Cell line designation	ELISA	Immunohistochemistry	Calcium release
U-251MG	+	+	+
U-105MG	+	+	+
D-65MG	+	+	NE[a]
D-54MG	+	NE	NE
CH-235MG	NE	+	NE

[a]NE, not examined

Discussion

Angiogenesis plays a critical role in tumor progression by providing oxygen and nutrients to rapidly dividing tumor cells and allowing for rapid elimination of CO_2 and waste metabolites. Folkman [28] initially hypothesized that tumors liberate specific substances that stimulate and maintain a continued blood supply to the tumor bed. Since then, numerous angiogenic substances have been identified, including fibroblast growth factor (FGF) [29], TGF-α [30], TGF-β [31], angiogenin [32], platelet-derived growth factor (PDGF) [33], platelet derived-endothelial cell growth factor (PD-ECGF) [34] and

for secretion and are immunochemically localized to glioma cell nuclei and cytoplasm. As a further point of distinction, Connolly [9] notes that VEGF is a more potent angiogenic growth factor than HBGF on a molar basis. Additionally, although HBGF and TGF-β act on numerous cell types, it appears that VEGF mitogenic activity is restricted to endothelial cells. Furthermore, HBGF and TGF-β induced angiogenesis is accompanied by fibrosis, a histopathological feature conspicuously absent in GBM tumors. Other putative angiogenic substances include angiogenin, which has not been demonstrated to have any direct activities on endothelial cells, and TNF-α and TGF-β, both of which can actually suppress endothelial cell proliferation. Lastly, although VEGF, histamine, and thrombin are all potent vasoactive substances that directly increase $[Ca^{2+}]_i$ in HUVECs, we have never observed a similar $[Ca^{2+}]_i$ increase of HUVECs by HBGF, TGF-β, EGF, or TNF-a (unpublished observations).

In addition to our conclusive demonstration that VEGF is present in, and secreted from, glioma cells, we report for the first time that constitutive production of VEGF by four human glioma cell lines was enhanced by EGF. This increased VEGF secretion was documented by ELISA, by changes in $[Ca^{2+}]_i$ in HUVECs, and by increased secretion of vWF from HUVECs. We are currently investigating whether the amount and/or persistence of mRNA encoding for various isoforms of VEGF [38] is also regulated by EGF.

Our finding that EGF stimulates VEGF elaboration has important implications in understanding the biology and basic physiology of malignant glial tumors. EGF is a normal constituent of human plasma and may enter tumor stroma via tumor bed capillaries that have been induced to be "leaky" by VEGF. EGF infiltrating the tumor stroma would be available to bind to glioma cells to increase cellular proliferation and VEGF production. This would represent a positive feedback mechanism and provides a highly plausible explanation for uncontrolled tumor growth, angiogenesis, and other pathologic sequelae present in brain tumors and perhaps other malignant cancers (fig. 11).

Our paradigm predicts that plasma EGF would be maintained within the intravascular space and would not enter interstitial spaces unless vascular permeability is induced by bioactive compounds (e.g., VEGF) or vessel trauma. Once in the extravascular space, EGF would become biologically important only to cells expressing EGFr. It is relevant that malignant gliomas have been shown by several groups to express or overexpress EGFr constitutively [13–15].

Some GBM tumors, as well as other malignancies, secrete TGF-α, which may further augment tumor growth, angiogenesis, edema, and other vascular sequelae. Thus, TGF-α may enhance the positive feedback loop depicted in figure 11.

In an attempt to explore this working hypothesis further, we have preliminary data that VEGF is present in the MCF-7 human breast carcinoma cell line that has functional EGF receptors that may increase VEGF production (Goldman and Gillespie, unpublished data). Interestingly, expression of functional EGFr in these cells is under hormonal regulation and may represent a ubiquitous mechanism whereby vascularity and cellular proliferation are coregulated in hormone sensitive tissues, such as breast, endometrium, and ovary. Intensive efforts in our laboratory are currently aimed at identifying intra- and intercellular mechanism(s) regulating VEGF-mediated neovascularity in hormone sensitive tissues.

Peritumoral cerebral edema that is characteristically associated with malignant brain tumors presents an immediate and serious life-threatening complication of this disease process. Dexamethasone, a potent glucocorticoid, is clinically useful in ameliorating symptoms in brain tumor patients of increased intracranial pressure. In our experiments, dexamethasone did not reliably diminish the constitutive secretion of VEGF by glioma cell lines. Osnishi et al. [39] have shown that the C_6 rat glioma cell line expresses a protein factor, possibly VEGF, that stimulated vascular permeability in vivo. The activity of this factor was inhibited by pretreating animals with dexamethasone. They speculate that dexamethasone may act directly on endothelial cells to prevent vascular permeability

or indirectly to inhibit glioma cell production of VEGF. Furthermore, Criscuolo et al. [6] have shown that dexamethasone completely blocked human glioma-induced alterations in $[Ca^{2+}]_i$ in HUVECs. Together with our results, these data would suggest that dexamethasone does not reduce cerebral edema by limiting VEGF production but rather serves to antagonize the ability of VEGF to induce vascular permeability related changes in brain capillary endothelial cells.

VEGF-mediated activation of endothelial cells provides a mechanistically simple explanation for many of the pathognomic histopathological and clinical findings in patients with brain tumors. For example, we have shown that bioactive glioma-derived VEGF produced a marked increase in vWF secretion from endothelial cells and was specifically inhibited by anti-VEGF antibody. In addition to the procoagulant effect of increased endothelial cell vWF secretion, VEGF has been demonstrated to stimulate thromboplastin activity in human endothelial cells [11]. Concerted augmentation of platelet aggregation/adherence activity by vWF, and enhanced thromboplastin activity (extrinsic cascade) in response to VEGF may lead to increased levels of fibrin split products [38] and contribute to the increased incidence of thromboembolic phenomena (deep vein thrombosis and pulmonary embolism) seen in brain tumor patients.

As another example, it is a paradox that, despite abundant proliferation of capillaries in GBM, these tumors have variable degrees of necrosis. Although it has been speculated that this necrosis is a result of uncontrolled tumor growth disproportional to blood supply, this explanation does not account for the intense vascularity of these tumors. We contend that the focal necrosis seen in human GBM tumors is due in large part to multiple foci of thrombus resulting from hypercoagulability in the abnormal tumor vessels within the tumor bed. This concept is supported by the work of Clauss et al. [11], who demonstrated that low level TNF-α infusion induces occlusive thrombosis in a VEGF-secreting tumor. Their studies have also shown that VEGF synergizes with TNF-α in stimulating thromboplastin activity in vitro. Furthermore, although TNF-α has

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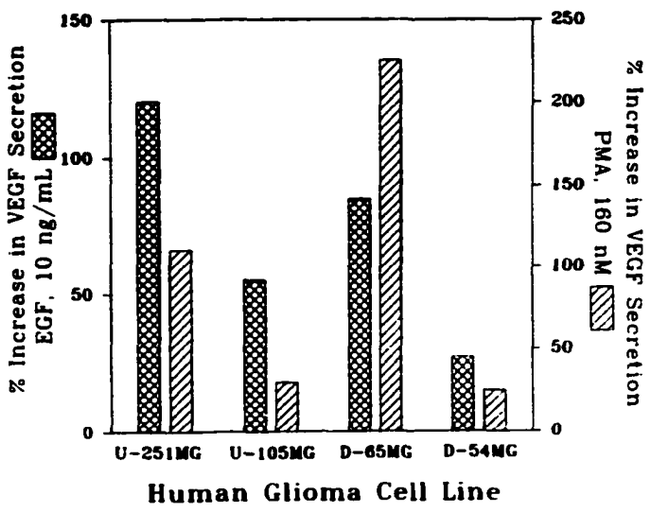

Fig. 2. Ability of stimuli to induce VEGF secretion by human glioma cell lines. PMA at 160 nM and EGF at 10 ng/ml enhanced VEGF secretion by each of the four glioma cell lines examined. VEGF in culture supernates was quantified by ELISA, and percentage increases were calculated in comparison to stimulation by β-phorbol (for PMA) or PBS (for EGF).

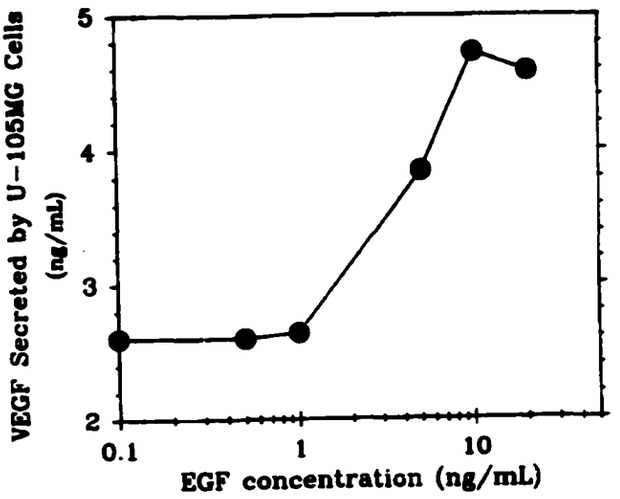

Fig. 3. EGF induced VEGF secretion by U-105MG glioma cells in a dose-dependent fashion with maximal stimulation at 10 ng/ml. VEGF in 96-h glioma cell CM was quantified by ELISA.

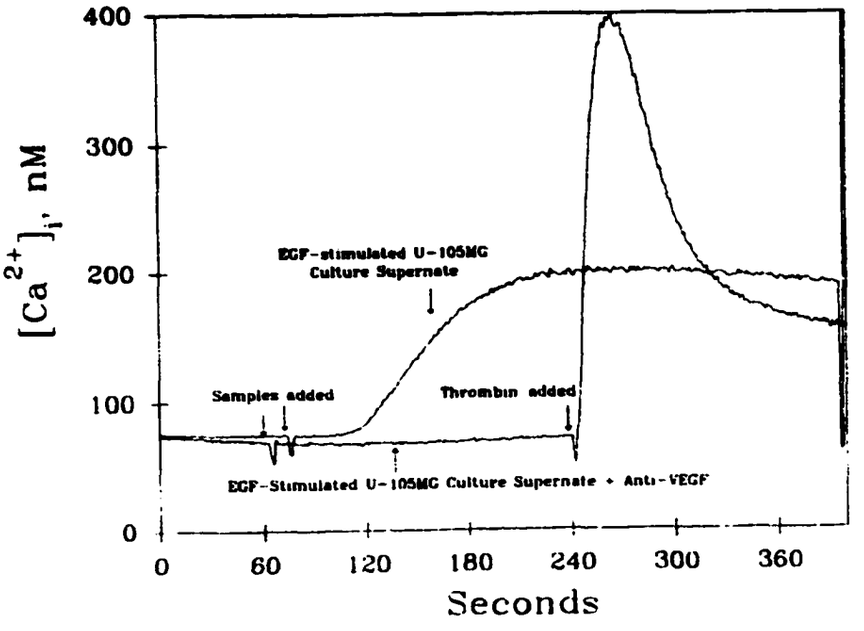

Fig. 5. The $[Ca^{2+}]_i$ mobilization transient in HUVECs induced by EGF stimulated U-105 MG glioma cell CM was abolished by preincubation of CM with 1 μg/ml of anti-VEGF antibody A4.6.1. In contrast, A4.6.1. did not affect the thrombin-(1 U/ml) induced $[Ca^{2+}]_i$ transient.

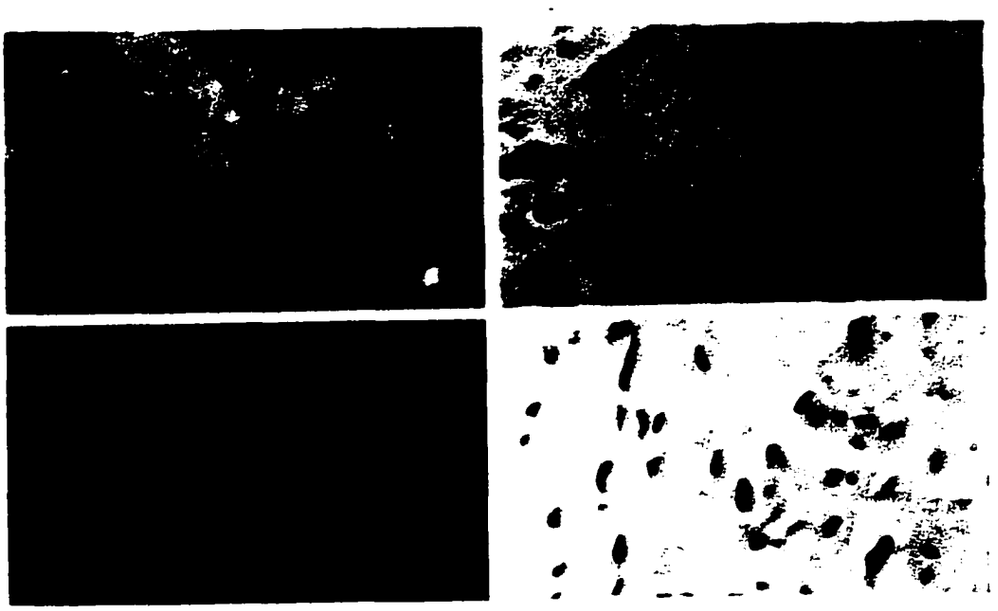

Fig. 7. Methanol-fixed frozen sections of a GBM tumor showed a diffuse cytoplasmic staining pattern with anti-VEGF antibody A4.6.1 and FITC-labeled goat anti-mouse IgG (upper left). Polyclonal mouse IGG1 did not bind as assessed with FITC-rabbit anti-mouse IgG (lower left). Binding of A4.6.1 in paraffin sections of GBM tissue (upper right) was observed by immunoperoxidase staining after antigen retrieval processing; anti-CD4 monoclonal mouse (IgG1) antibody was not reactive (lower right). Paraffin sections were counterstained with aqueous hematoxylin

Fig. 8. EGF receptors in formalin-fixed, paraffin-embedded sections from a GBM tumor section. Monoclonal antibody 29.1.1 reacted positively with cell surface EGFr as detected by indirect immunoperoxidase staining techniques.

Fig. 9. Immunoperoxidase detection of VEGF in methanol-fixed human glioma cell lines. Staining was more pronounced in the U-251MG cell line (left panel) than in the CH-235MG glioma cell line (right panel). Note the distinctive nuclear outline generated by the diffuse cytoplasmic staining pattern.

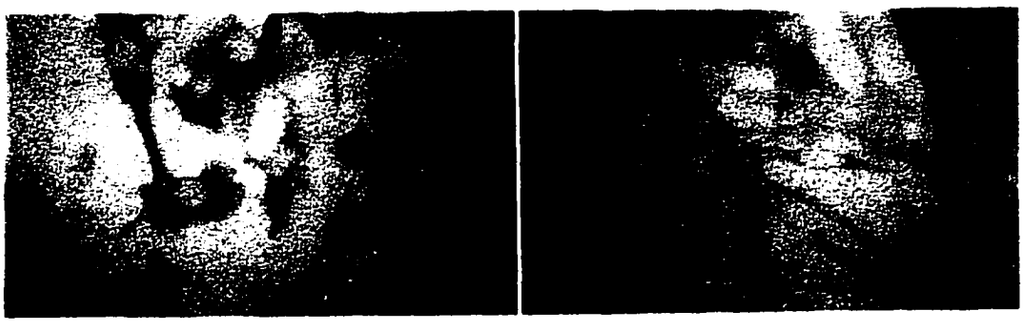

Fig. 10. Immunoperoxidase detection of EGFr using monoclonal antibody mAb425 methanol-fixed human glioma cell lines. EGFr were present in all glioma cell lines examined; staining patterns for the U-251MG (left panel) and CH-235MG (right panel) glioma cell lines are shown here.

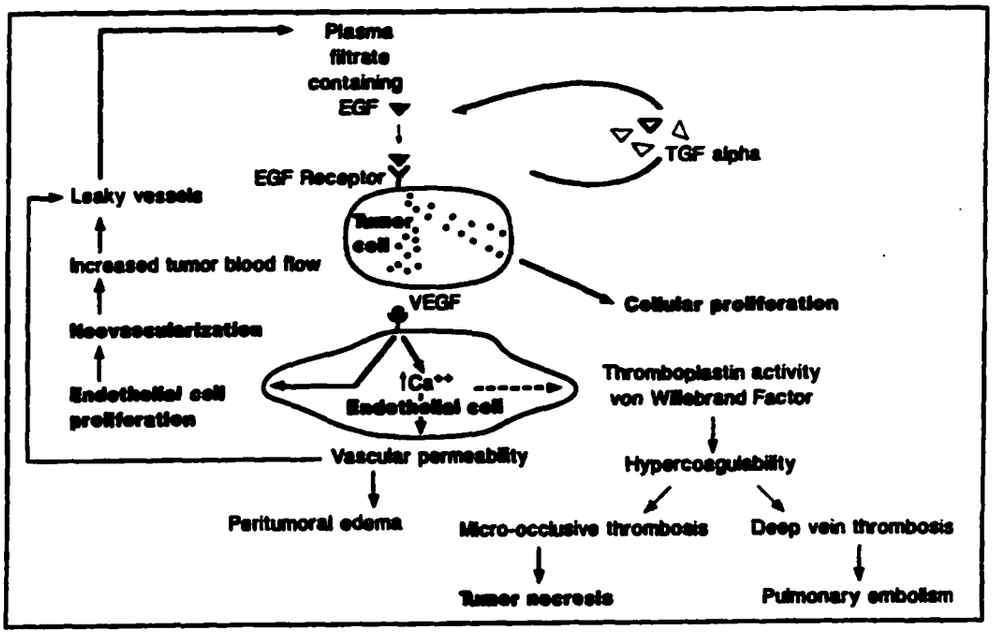

Fig. 11. A schematic representation of proposed intercellular mechanisms in the development of vascular pathophysiology characteristic of GBM tumors.

SUMMARY

In summary, angiogenesis, or formation of capillaries in vivo, is a process initiated by "angiogenic" factors that stimulate neovascularization. Direct angiogenic factors also stimulate proliferation of endothelial cells and enhance release of proteolytic enzymes from endothelial cells. While FGF is a prototypic direct angiogenic growth factor, its physiologic role as an angiogenic factor in vivo remains controversial. VEGF is a secreted and selective growth factor that is probably more relevant physiologically for many tumors including GBM.

Following the "proliferative phase" of angiogenesis mediated by VEGF, endothelial cells enter a "transitional phase." For operational purposes, the transitional phase is characterized by diminished cellular proliferation and sprout formation, as this dissertation will demonstrate, which is a result of the action of fibronectin fragments on endothelial cells. The final stage of angiogenesis is the "differentiation phase," which is characterized by the formation of capillary tubes composed of endothelial cells. Current research indicates that laminin [6, 25] is an important mediator of the differentiation phase or tube formation, and that fibronectin fragments, likely produced as a result of proteases secreted in the proliferative phase, are capable of stimulating the production of laminin. This dissertation set out to establish a meaningful and organized relation among all phases of angiogenesis.

The first published paper established by multiple criteria that REVC cells are endothelial in origin and that they exhibit most phenotypic characteristics commonly observed in classical EC. For example, REVC cells were capable of forming branching tube-like structures when plated on Matrigel, thus demonstrating their usefulness for

investigating tube formation. Additionally, REVC cells were proven capable of secreting fibronectin. Using this cell line as a stable, reproducible assay system, these cells were used to explore possible links between mitogen-induced endothelial cell proliferation, protease secretion and extracellular matrix-induce sprout formation.

Unlike primary endothelial cells, REVC cells provide a homogeneous population of endothelial cells that can be used to perform a large number of experiments that previously could not be performed due to the short life span of primary endothelial cell cultures. In the second manuscript, we have taken advantage of the homogeneous nature of REVC cells to dissect the morphological changes coincident for the formation of endothelial sprouts.

By screening single compounds, we determined that Concanavalin-A, a lectin that binds to unique carbohydrate determinants, bound to REVC cells and induced the formation of endothelial sprouts. In the following paper, we demonstrated that Concanavalin-A bound to fibronectin on the surface of REVC cells.

We hypothesized that alterations in fibronectin conformation were responsible for the induction of sprouts. To alter the conformation of fibronectin in a more physiologically relevant manner, we used proteases to cleave fibronectin and then screened for fibronectin fragments that had the ability to induce sprouts. Our findings, that protease-generated fibronectin fragments induce endothelial sprouts, provide a simple and relevant explanation of how endothelial cells receive a signal to change their shape.

The second paper demonstrated protease-digested fibronectin and Con-A induced sprout formation by REVC endothelial cells. Since Con-A bound predominantly to fibronectin on the REVC cell surface, it is likely that Con-A induced REVC shape changes by altering the conformation of native fibronectin.

Our finding that proteases cleave fibronectin into active fragments that induce sprout formation and suppress endothelial cell proliferation is important for

understanding the process of angiogenesis. This finding serves as an explanation of how endothelial cells shift from a proliferative phase to a differentiation phase. The second paper established a relation between the mitogen-associated release of proteases in the proliferative phase of angiogenesis with sprout formation and diminished cell proliferation that is characteristic of the differentiation phase of angiogenesis. With this relation established, a comprehensive view of the angiogenesis process can be proposed that may help to explain the role of other growth factors and extracellular matrix components in angiogenesis.

In the third paper, we investigated a direct angiogenic factor, VEGF, in glioblastoma multiforme. This particular growth factor not only induces endothelial cell proliferation, but also induces the release of proteases such as collagenase and plasminogen activator from endothelial cells. The presence of VEGF and its associated proteases in glioblastoma may contribute to the formation of fibronectin fragments in the tumor matrix and facilitate the differentiation of endothelial cell into capillary sprouts. The third paper therefore demonstrates the presence of biologically active VEGF in glioblastoma multiforme and examines it in relation to tumor cell proliferation.

The results of the final paper demonstrated three pertinent findings: 1) glioma cells secrete a form of VEGF that is biologically active for EC as measured by intracytoplasmic calcium flux and von Willebrand Factor release. 2) EGF (for which most gliomas express the appropriate receptor) was able to increase the amount of secreted VEGF markedly. This suggests that glioma cell growth and elaboration of an angiogenic factor may be co-regulated in malignant gliomas. Initial studies suggest that other growth factors capable of stimulating glioma cell proliferation, such as PDGF and FGF are also capable of stimulating VEGF production. 3) VEGF protein could be demonstrated conclusively and, in some cases, in abundance in malignant glioma tissue specimens.

In the model I propose, endothelial cells enter a "proliferative phase" of angiogenesis mediated by VEGF, or alternatively FGF. In response to VEGF, endothelial cells release collagenase and plasminogen activator. These enzymes act locally on the extracellular matrix. One component of the extracellular matrix is fibronectin. Endothelial cells produce an abundance of fibronectin in vitro and in vivo. Circulating blood also contains high levels of fibronectin as well, which is in direct contact with endothelial cells in vivo. As demonstrated in this dissertation, endothelial cells enter a transitional phase characterized by diminished cellular proliferation and sprout formation that is a result of the direct action of fibronectin fragments on endothelial cells. Austria and Couchman [49] have demonstrated that fibronectin fragments have the capacity to induce the secretion of laminin. This finding is important because laminin is thought to be a mediator of the differentiation phase or tube formation [10, 27, 39, 51].

Using this model describing the angiogenic process, it should be possible to determine the mechanism by which other angiogenic factors act to produce capillaries. For instance, this model allows one to predict that capillaries should form if proteases were activated in the absence of EC mitogens.

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