Clinical Neurosciences in the Decade of the Brain: Hypotheses in Neuro-Oncology

VEG/PF Acts Upon the Actin Cytoskeleton and Is Inhibited by Dexamethasone: Relevance to Tumor Angiogenesis and Vasogenic Edema

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Hypothesis: We have proposed that VEG/PF acts by transforming the cytoskeletal architecture of microvascular endothelial cells.

Background: Evidence supporting a pivotal role for vascular endothelial growth/permeability factor (VEG/PF) in tumor angiogenesis and edemagenesis is compelling. VEG/PF exhibits specific endothelial cell mitogenicity and is expressed by brain tumors exhibiting increased vascularity and microvascular extravasation. The mechanistic cascade that follows VEG/PF-tyrosine kinase receptor binding remains uncertain, however. Actin is a cytoskeletal protein that regulates cellular motility, shape and vesicular transport. Regulation of actin stress fibers, cell-surface focal adhesions and plasmalemmal "ruffles" is mediated by tyrosine kinase activation of GTP-binding proteins that are in turn linked to intracellular calcium flux. As VEG/PF is known to induce cytosolic calcium ion transients in endothelial cells, actin microfilaments would appear to be logical candidates for study of a cytocontractile response mediated by calcium signal transduction.

Methods: VEG/PF-induced endothelial actin cytoskeletal changes were studied using rhodamine phalloidin staining and fluorescence photomicrography.

Results: When exposed to VEG/PF, cultured endothelial cells from human umbilical veins and rat brain microvessels exhibited a reversible, dose-related reorganization of actin stress fibers, cell contraction and rounding, and widening of the intercellular spaces. VEG/PF perturbation also induced plasmalemmal "ruffling." All VEG/PF-induced cytoskeletal changes were inhibited by preincubating endothelial cells with dexamethasone or anti-VEG/PF IgG antibody.

Conclusion: The findings support a role for VEG/PF-induced cytoskeletal alterations in the pathophysiology of brain tumor angiogenesis and edemagenesis. These observations are likely to be directly linked to VEG/PF-induced endothelial cytosolic calcium flux. Insight into the mechanism of dexamethasone's clinical efficacy is also provided.

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[b]*Abbreviations:* VEG/PF, vascular endothelial growth/permeability factor; GTP, gaunosine triphosphate; mRNA, messenger ribonucleic acid; IgG, immunoglobulin G; G-actin, globular or monomeric actin, F-actin, fibrous or polymerized actin; HUVEC, human umbilical vein endothelial cell; RBMVEC, rat brain microvessel endothelial cell; HBSS, Hanks balanced salt solution; BSA, bovine serum albumin; DNase, deoxyribonuclease; GFAP, glial fibrillary acidic protein; SMA, smooth muscle actin; GGT, gamma-glutamyl transpeptidase; ras, rac, rho and rab, GTP-binding proteins; PLC, phospholipase-C; IP3, inositol 1,4,5-triphosphate; DAG, diacylglycerol; LSCEM, laser scanning confocal epifluorescence microscopy.

INTRODUCTION

A number of common mediators of inflammation and microvascular extravasation have previously been implicated in tumor edemagenesis [1-6]. Studies demonstrating vascular endothelial growth/permeability factor (VEG/PF)[b] mRNA expression, and VEG/PF protein expression and secretion, support VEG/PF's role in the genesis of malignant effusions such as ascites, pleural effusions, tissue edema, and the edema accompanying many systemic and central nervous system neoplasms [7-30]. VEG/PF is expressed by benign and malignant tumors alike, and its presence correlates closely with the presence and extent of microvascular extravasation in the form of peritumoral edema or tumor cyst formation [6, 14, 28, 29]. VEG/PF acts specifically on vascular endothelial cells and has recently been implicated in the pathogenesis of aneurysms and vascular malformations arising in the central nervous system [15, 19, 20, 24, 31-35, 55]. Its pluralistic actions are essential for tumor growth, as it facilitates both the rapid proliferation of blood vessels (angiogenesis), and a means of developing the extracellular fibrin matrix (microvascular extravasation or edemagenesis) requisite for the ingrowth of new tumor elements.

Actin is one of the three major constituents of the cytoskeleton. The actin cytoskeleton of endothelial cells plays essential roles in cellular function. Whereas its participation in cellular motility, chemotaxis, and cell division appears most relevant to angiogenesis, its role in cellular secretion, endocytosis and permeability more likely relate to edemagenesis. Actin fibers play an essential role in cell dynamics and the maintenance of cell shape. Any change in cellular shape can therefore be expected to be accompanied, if not dictated, by a structural and functional change in actin protein conformation. Regulation of actin's repertoire of cellular functions is only partially understood. It involves interactions with a variety of specific actin binding or regulatory proteins which modulate intracellular calcium flux, protein phosphorylation, and ultimately, the balance between the monomeric (G-actin) and polymerized (F-actin) forms. The studies described herein were designed to ascertain and characterize the effects of VEG/PF perturbation on the endothelial actin cytoskeleton, and to explore this cytocontractile mechanism as a plausible target for dexamethasone's clinical efficacy.

MATERIALS AND METHODS

Working hypothesis, rationale and objectives

We hypothesized that VEG/PF acts by changing endothelial cellular shape by an actin cytoskeletal contractile mechanism [14-16]. Actin fibers are crucial components of the cytoskeleton in living cells. By altering their three-dimensional structural conformation, actin molecules can regulate motility, shape, and vesicular transport in vascular endothelial cells [4, 23, 36-40]. VEG/PF is capable of inducing cytosolic calcium transients in endothelial cells [16]. Actin, in turn, interacts with a variety of calcium-activated proteins. The actin state of polymerization is readily studied by rhodamine phalloidin fluorescence staining [39, 41-43]. Highly polymerized filamentous actin (F-actin) forms a structural network of stress fibers throughout the endothelial plasmalemma, whereas actin depolymerization into the monomeric or globular (G-actin) form results in a bright diffuse "ground glass" staining pattern without apparent structural integrity.

Our experimental objectives were: (1) to examine and define histamine-induced effects on endothelial actin stress fibers, in order to serve as positive controls, (2) to establish, define, and interpret VEG/PF-induced effects on endothelial F-actin stress fibers, and (3) to determine whether dexamethasone exhibited any inhibitory action on VEG/PF-induced actin changes.

Endothelial cell isolation, culture and characterization

Human umbilical vein endothelial cells (HUVEC) were obtained commercially as primary or low passage (#1-3) cryopreserved inoculums (Clonetics Corporation, San Diego, CA) that were reconstituted and cultured in T75 flasks coated with two percent gelatin. Culture medium consisted of E-199 basal medium, supplemented with 20 percent fetal bovine serum (Gibco, Grand Island, NY), 2 mM glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 75 µg/ml endothelial cell growth supplement (Collaborative Biomedical, Bedford, MA) and 150 µg/ml heparin (Sigma, St. Louis, MO). When the cells were confluent as judged by phase contrast microscopy, they were propagated by 1:3 in new flasks. Sterile 35 mm diameter plastic tissue culture wells were coated with fibronectin by incubating the wells with a 0.1 mg/ml fibronectin solution in Hank's balanced salt solution (HBSS) for 30 min. The solution was removed and the wells washed once with HBSS. At passage two or three the cells were plated onto the wells at a density of 1.2 x 10^5 cells per well. One or two days were allowed for the cells to grow into a confluent monolayer.

Rat brain microvascular endothelial cells (RBMVEC) were isolated and characterized in our laboratory employing a previously described method [44]. Four 3-month-old female white Lewis rats were anesthetized with Nembutal and thereafter decapitated. The rat brains were extracted using sterile surgical instruments and immediately immersed in an oxygenated sterile buffer solution consisting of HBSS with 10 mM HEPES buffer, 100 U/ml Penicillin, 100 µg/ml Streptomycin and 0.5 percent bovine serum albumin (BSA). Rat brains were isolated in a sterile environment and minced into 2 to 3 mm pieces in a separate beaker containing buffer solution. The resultant mixture was transfered into a sterile tube and centrifuged 5 min at 1500 rpm. The buffer was poured off and the cortex tissue placed in an oxygenated enzyme solution consisting of HBSS containing 1 mg/100 ml collagenase/dispase (Boerhinger Mannheim, Indianapolis, IN), 10 mM HEPES buffer, 100 U/ml Penicillin, 100 µg/ml Streptomycin, 0.147 µg/ml TLCK (Sigma, St. Louis, MO) and 20 U/ml DNase (Boerhinger Mannheim, Indianapolis, IN) at 37°C for 1 hr. The resultant solution was aspirated up and down a Pasteur pipet to produce a suspension that was centrifuged again at 1500 rpm for 5 min. The supernatant was poured off and the pellet mixed with a 25 percent BSA solution after which this mixture was centrifuged again for 15 min at 2900 rpm. The latter step resulted in the separation of myelin from a capillary pellet at the bottom of the tube. The myelin-rich supernatant was poured off and the capillary pellet resuspended in buffer solution that was centrifuged for five min at 1500 rpm. The buffer was poured off and the pellet was incubated with the enzyme solution at 37°C for 3 hr.

Two sterile ultracentrifuge tubes were then coated with protein by filling them with buffered BSA solution and gently shaking them for 20 min. Both were filled with a 50 percent isotonic percoll (Sigma, St. Louis, MO) mixture that was centrifuged for 1 hr at 16500 rpm. The cell-containing enzyme digest was centrifuged again for 5 min at 1500 rpm and thereafter the enzyme mix was poured off the resulting pellet. The pellet was resuspended in 2 ml of buffer, of which each half was gently dribbled on to the percoll gradients. These gradients were centrifuged again for 15 min at 2900 rpm. A Pasteur pipet was used to extract the capillary pellet bands from the tubes. These were mixed with buffer solution again and centrifuged for 5 min at 1500 rpm. The buffer solution was poured of and the cells mixed with culture medium consisting of HAMS nutrient mixture (Gibco, Grand island, NY) with 20 percent lymphocyte culture serum (Hyclone Labs, Logan, UT), 100 U/ml Penicillin, 100 µg/ml Streptomycin and 2 mM glutamine. The cells were plated on fibronectin-coated plastic tissue culture dishes and left to grow in an incubator with frequent changes of the culture medium for 1 to 2 weeks.

The identity of the endothelial cell cultures was confirmed by the typical "cobble-stone" (HUVEC) or "spindle" (RBMVEC) morphology, the F-actin distribution pattern of individual cells, and specific staining for both Factor VIII and di-acetylated-LDL. Contaminating cells (astroglia, smooth muscle, pericytes) were identified by specific staining for glial fibrillary acidic protein (GFAP), HAM-56, alpha-smooth muscle actin (alpha-SMA), and gamma-glutamyl transpeptidase (GGT). Cultures were randomly selected at regular intervals and screened for cellular homogeneity. Experiments were routinely performed on cultures exhibiting over 95 percent purity for vascular endothelial cells.

Rhodamine phalloidin fluorescence staining

Rhodamine-phalloidin, a fluorescent derivative of the phallotoxin from Amantia phalloides, binds with high affinity to F-actin fibers. Ten microliters of rhodamine phalloidin (Molecular Probes, Eugene, OR) was evaporated in a small test tube and redissolved in 200 ul of PBS. Slides with fixed endothelial cells were covered with the rhodamine phalloidin solution for 20 minutes in a dark environment. Then, a 1:1 volume glycerol/PBS antifade reagent was placed on the cells, and a coverslip mounted on top. The cells were examined by fluorescence microscopy using an Olympus BH-2 fluorescence microscopy system matched to an Olympus PM-10AD photomicrographic system (Olympus Corporation, Lake Success, NY).

Experimental design and data analysis

VEG/PF165, the most biologically potent and abundant VEG/PF isoform (Genentech, San Francisco, CA), has been used for all experiments described herein. Several different concentrations of VEG/PF (10^{-9} to 10^{-7} M) or histamine (10^{-8} to 10^{-6} M) were added to the endothelial cell containing wells and incubated for varying intervals (1, 2, 5, and 10 min), after which they were fixed in a 4 percent paraformaldehyde (PFH) solution in full medium and left in the incubator for 15 min. After removing the PFH, the fixed cells were washed extensively with HBSS. Negative control groups were exposed to a volume of HBSS equal to that of the experimental treatment groups. Treatment with dexamethasone was achieved by preincubating the cells in media containing 10 µM dexamethasone for 2 hr prior to a VEG/PF perturbation. Histamine-induced cytoskeletal changes have previously been defined and therefore served as a positive control. Preincubation of the cells with a previosly defined rabbit polyclonal anti-VEG/PF IgG antibody (Monsanto, St. Louis, MO) in a dilution of 1:800 completely inhibited the VEG/PF-actin response and served as an additional control.

Microscopic examination of all slides was conducted by the two investigators who simultaneously viewed each slide and made comparisons between negative controls (HBSS), positive controls (histamine), and three experimental groups consisting of (1) VEG/PF treated, (2) dexamethasone-VEG/PF treated, and (3) anti-VEG/PF IgG-VEG/PF treated cells. All slides were examined under 200x, 400x and 1000x magnification. Results were tabulated by randomly choosing three to six fields from each fluorescent slide prep for photomicrography. Kodak Elite ISO 400 high-definition film (Eastman Kodak, Rochester, NY) was push-processed (2-2.5 x Iso) in order to optimize image exposure and resolution. The latter served to create a permanent image archive that would facilitate ongoing data interpretation without having to bleach the original fluorescent slide preparations. Representative transparency images were converted to 5 x 7 inch Cibachrome prints. Experiments were run simultaneously in quadruplicate on the same day. Each experiment and its relevant control was repeated at least 10 times. The database therefore consists of several hundred original glass fluorescent slide preps (more than 50 slides for each of four experimental groups) and well over two thousand photomicrographic slides

and prints. All VEG/PF-induced actin cytoskeletal changes were interpreted and described in accordance with discussions in the existing pertinent literature [37-40, 45-48].

RESULTS

Control and histamine studies

A series of subconfluent and confluent endothelial cell (HUVEC and RBMVEC) monolayer cultures were established as controls for the study of the normal actin cytoskeletal architecture. Unperturbed specimens exhibited a high degree of actin micro-filament organization. Extensive arrays of actin filament bundles were often oriented par-allel to one another and to the long axis of spindle-shaped cells. Cells displaying a poly-hedral architecture had a more radial alignment of actin fibers around a central or para-central nucleus (Figures 1A and 4A). Confluent monolayers exhibited minimal or no gaps between juxtapposed endothelial cells. A dense peripheral band of actin filaments encir-cled the perimeter of cells in confluent cultures and was sometimes more prominent than the coexisting stress fibers (Figures 1B and 1C). Histamine stimulation was used to estab-lish positive controls for comparison with VEG/PF studies. Histamine-perturbed cells showed evidence of cytoplasmic retraction in association with a reorganization of actin fil-aments characterized by a near complete dissolution or depolymerization. Diffusely fluo-rescent material in the perinuclear region imparted a characteristic "ground glass" appear-ance to the cytoplasm and served to accentuate the relatively dark nuclear profile. No evi-dence of plasmalemmal ruffling was detected, and the histamine-induced changes were not inhibited by preincubation with dexamethasone (Figure 2).

VEG/PF studies

After addition of nanomolar concentrations of VEG/PF, previously confluent endothelial cell monolayers responded with an extensive reorganization of actin stress fibers and cell retraction, with shrinkage and "rounding up" of their cell profile, and a marked widening of the interendothelial spaces. This response was rapid, with maximal actin changes occurring within 1 min of VEG/PF perturbation and complete reversal of the actin changes within 5 min). Diffusely fluorescent "ground glass" perinuclear staining and cellular clumping occurred typically (Figures 3A-C). In several instances, the endothelial cell plasmalemma exhibited a thickened, intensely staining peripheral band with a characteristic "ruffled" appearance (Figure 3D). Despite extensive cellular shrink-age and distortion, cell-to-cell contacts in the form of actin plasmalemmal microspikes were sometimes maintained, except in the most severely contracted cells (Figures 3B-D). There were no major qualitative or quantitative differences between the HUVEC and RBMVEC groups in their response to VEG/PF perturbation, except for the more spindle-like appearance of the RBMVEC both before and after perturbation (Figures 1C and 3C). In cells that showed a partial response, the actin dissolution was most obvious around the cell nucleus, thereby suggesting that depolymerization of actin fibers is initiated in this area. Dose-response experiments revealed progressively higher VEG/PF concentrations (10^{-9} to 10^{-6} M) to increase the extent of actin reorganization (pictorial data not shown). Overall, the endothelial actin changes induced by VEG/PF exhibited a structural similar-ity to those evoked by histamine treatment.

Preincubation of endothelial cells with a 10 micromolar concentration of dexametha-sone for at least 2 hr rendered them unresponsive to VEG/PF perturbation, an affect not reproduced in the histamine perturbed group (Figures 1A, 1C, 4A, 4B and 4C). The 2 hr temporal contingency suggests dexamethasone is acting either through receptor-mediated de novo synthesis of a VEG/PF inhibitory protein, or by down-regulation of VEG/PF receptor expression. A rapid and non-specific steroid-induced stabilization of the endothe-lial cell membrane would appear less plausible as dexamethasone's inhibitory action was
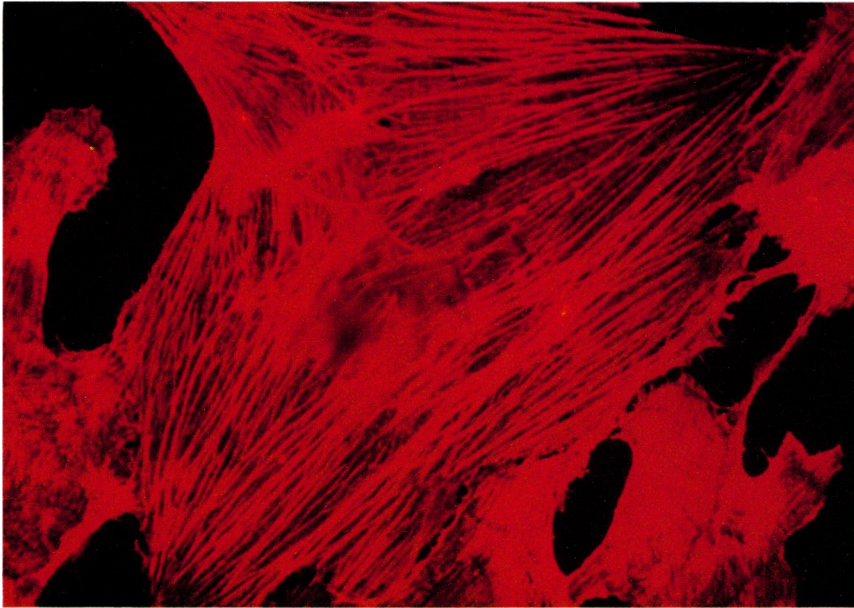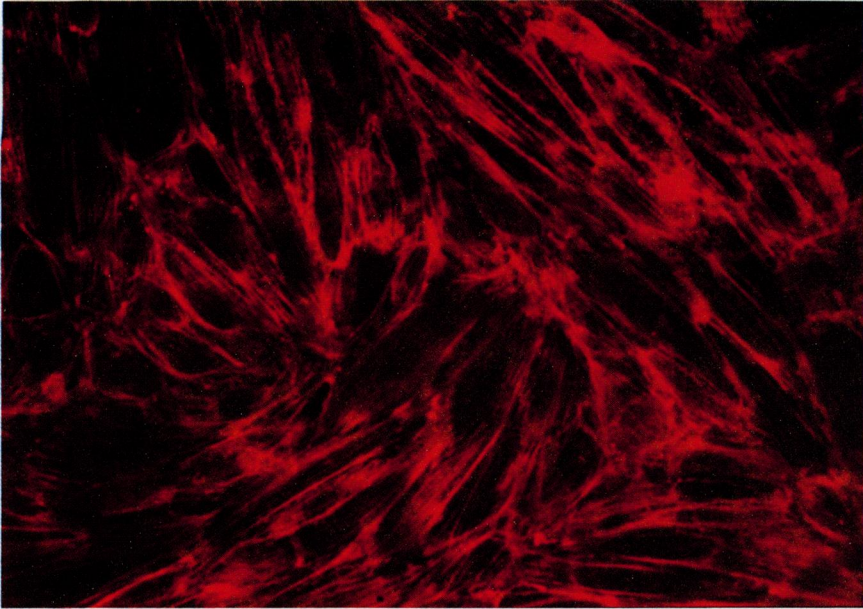
Figure 1A. Control HUV endothelial cell. Fluorescence photomicrograph (1000x) of a single human umbilical vein endothelial cell from a subconfluent monolayer stained with rhodamine phalloidin. This image illustrates the intricate actin microfilament (F-actin) network responsible for endothelial cytoskeletal integrity.
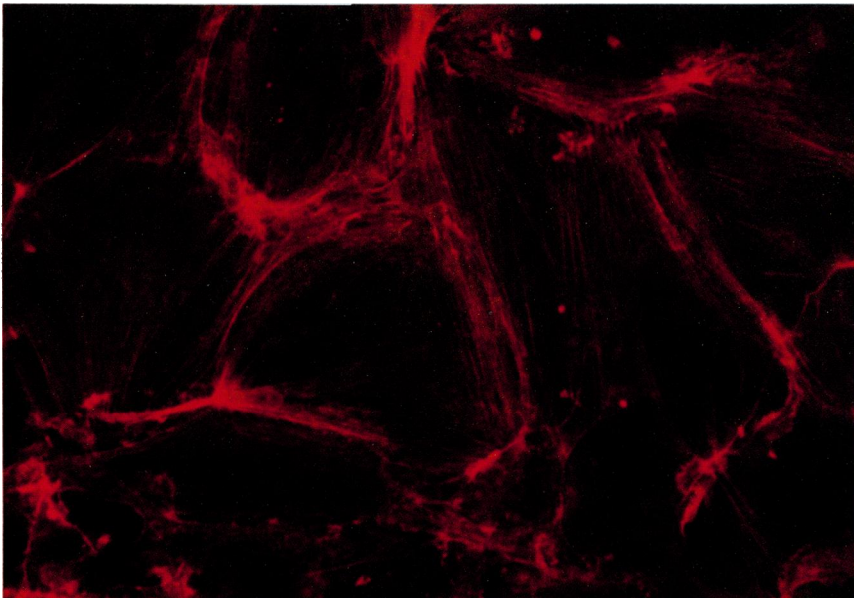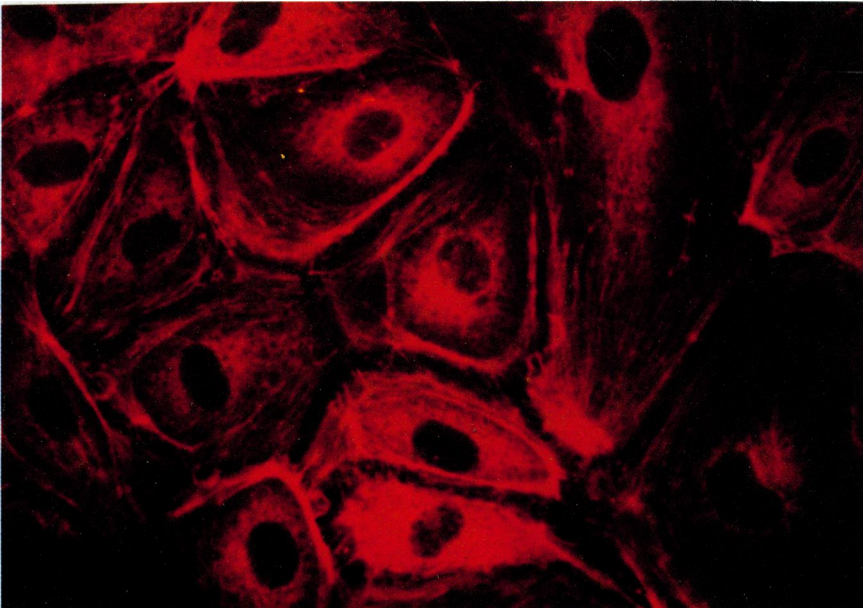

Figure 1B. Control HUV endothelial cells. Fluorescence photomicrograph (400x) of a confluent human umbilical vein endothelial cell (HUVEC) monolayer stained with rhodamine phalloidin. Note that in addition to an extensive organization of cytoplasmic actin stress fibers, there exists a well-defined peripheral F-actin band outlining the individual endothelial cell margins.

**Figure 1C. Control RBM endothelial cells. Fluorescence photomicrograph (200x) of a conflu-
ent rat brain microvessel endothelial cell (RBMVEC) monolayer stained with rhodamine
phalloidin.** Note the extensive organization of actin stress fibers and the well-defined endothelial
cell margins.

Figure 2. Histamine-treated HUV endothelial cells. Fluorescence photomicrograph (400x) of
HUV endothelial cells after exposure to histamine (10^{-7} M) for 1 min. This resulted in a general dis-
solution of actin stress fibers that was most apparent in the cytoplasm surrounding the endothelial
cell nucleus. The diffuse dissolution of actin microfilaments into monomeric G-actin is responsible
for the characteristic "ground glass" appearance imparted to the cytoplasm. The cellular margins are
contracted and more rounded resulting in significant enlargement of the interendothelial spaces.
This is known to result from histamine-induced calcium transients which in turn induce a reversible
depolymerization of F-actin to the monomeric G-actin form. Intercellular cross-linking actin fila-
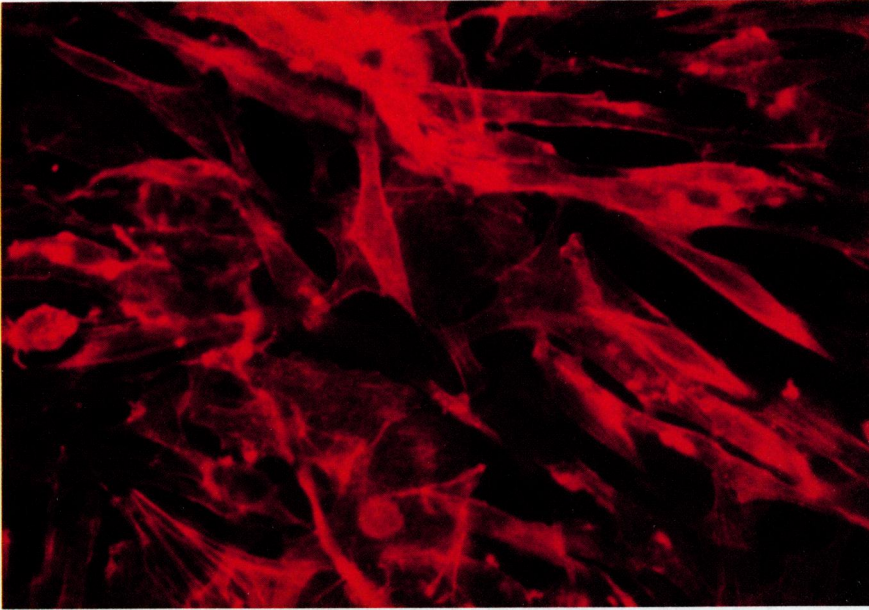ments or microspikes are clearly visible.
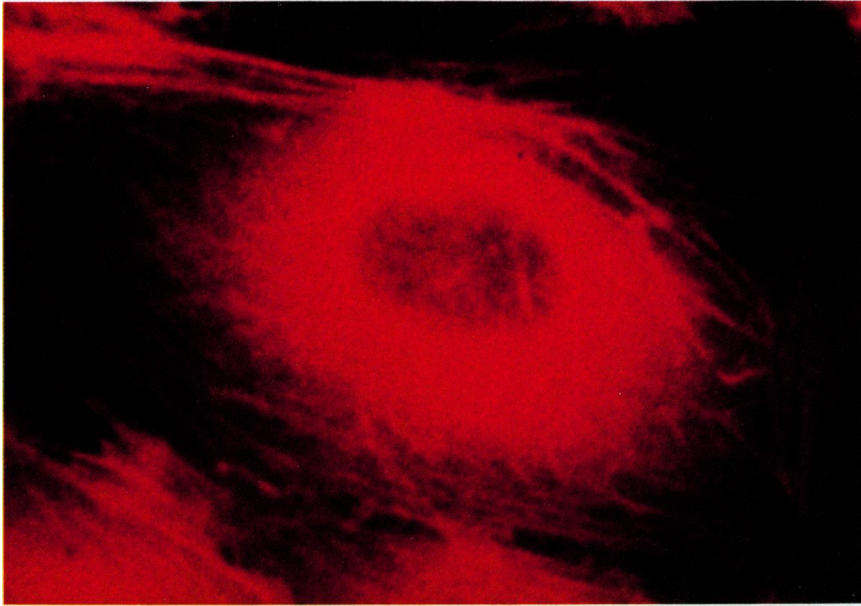
Figure 3A. VEG/PF-treated HUV endothelial cell. Fluorescence photomicrograph (1000x) of VEG/PF-treated HUV endothelial cells 1 min after exposure (8 x 10^{-9} M). Diffuse depolymerization of actin fibers has resulted in a characteristic "ground glass" appearance to the cytoplasm with accentuation of the nuclear outline, cell contraction, and rounding.
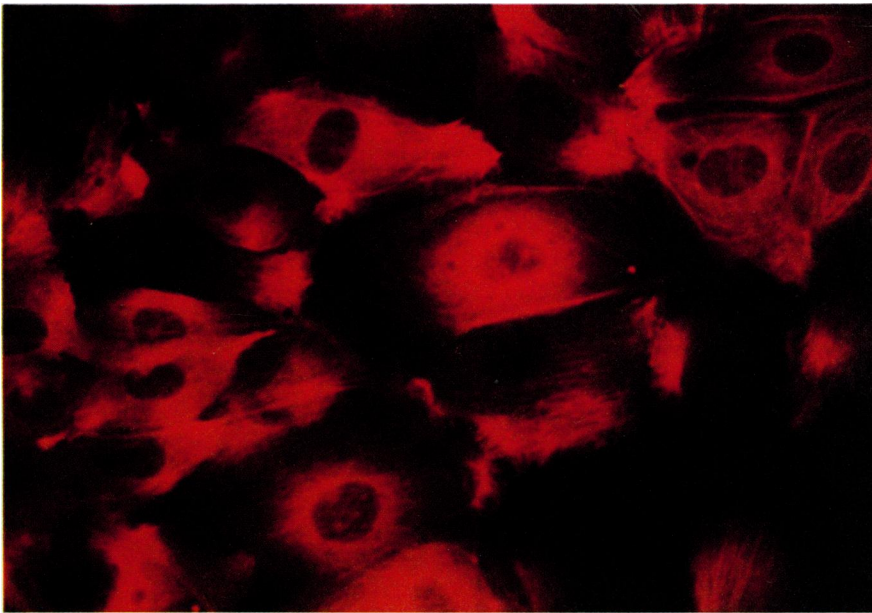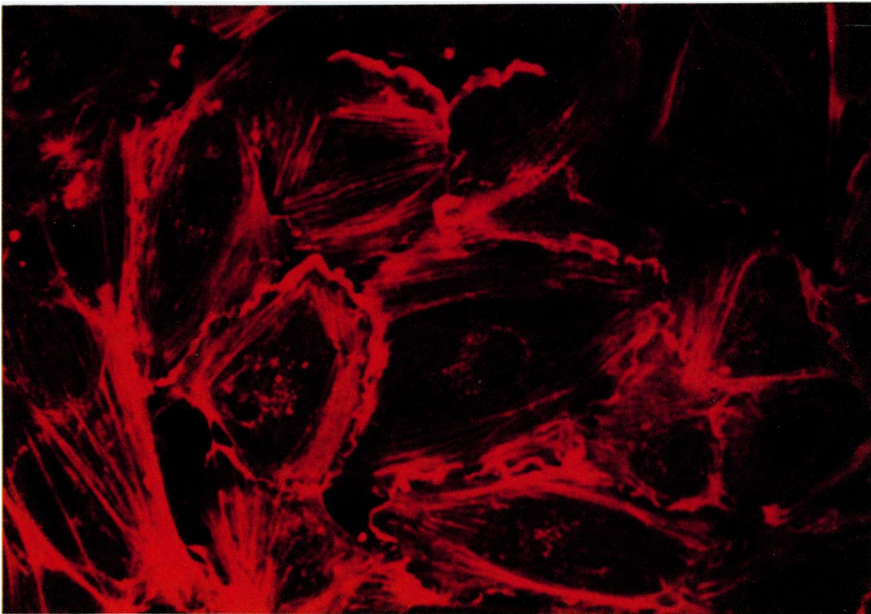

Figure 3B. VEG/PF-treated HUV endothelial cells. Fluorescence photomicrograph (400x) of VEG/PF-treated HUV endothelial cells 1 min after exposure (8 x 10-9 M). Groups of cells have reacted with depolymerization of actin fibers and cell contraction. Some cells appear to be held together by cross linking actin filaments or microspikes. Widening of interendothelial spaces is clearly evident.
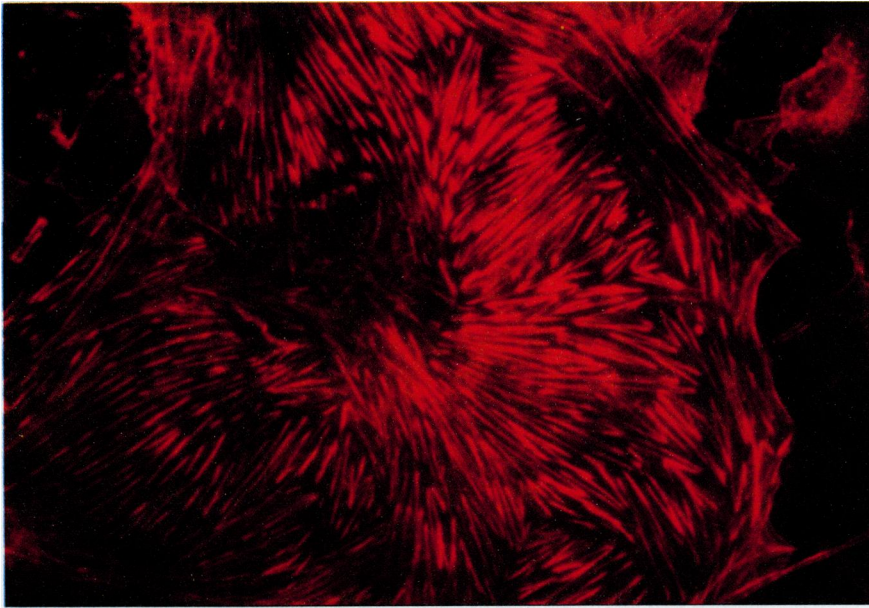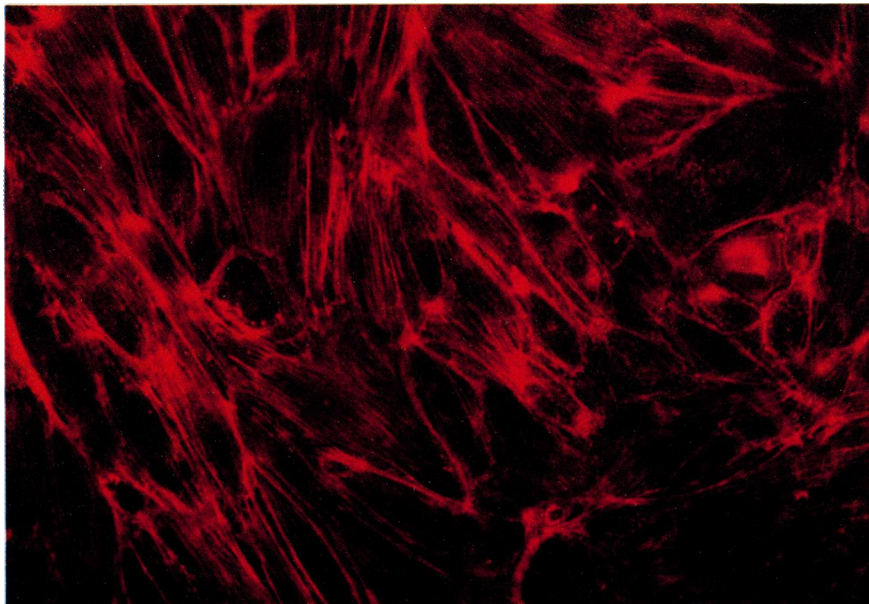
Figure 3C. VEG/PF-treated RBM endothelial cells. Fluorescence photomicrograph (400x) of VEG/PF-treated RBM endothelial cells 1 min after exposure (8 x 10^{-9} M). Groups of cells have reacted with varying degrees of depolymerizatiqn of actin fibers and cell contraction. Interendothelial gaps are very evident.
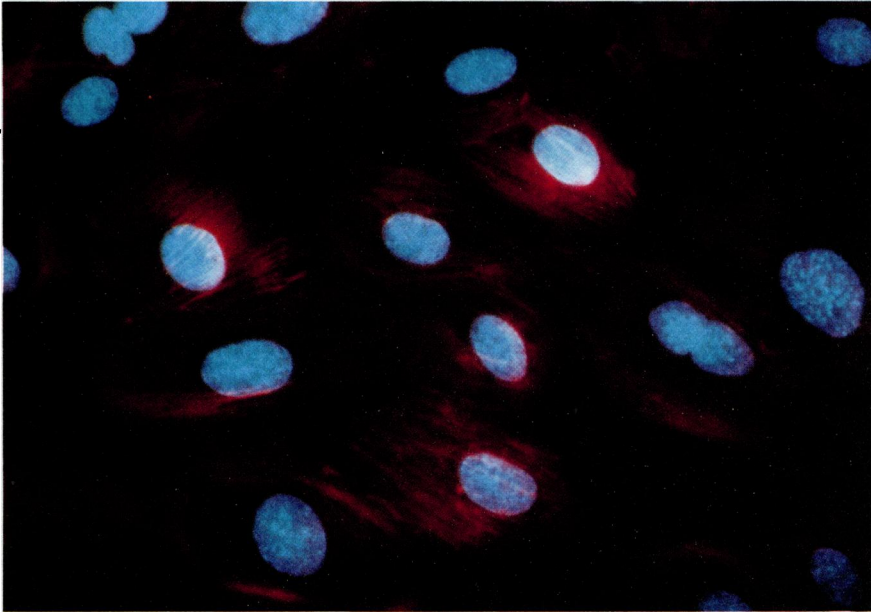

Figure 3D: VEG/PF-treated HUV endothelial cells. Fluorescence photomicrograph (400x) of VEG/PF-treated HUV endothelial cells 1 min after exposure (8 x 10^{-9} M). The entire field of cells has responded with depolymerization of actin. In addition, most of the endothelial cell plasmalemma are intensely stained at their periphery and exhibit a characteristic "ruffled" appearance.

Figure 4A. Dexamethasone-VEG/PF-treated RBM endothelial cell. Fluorescence photomicrograph (1000x) of a single rat brain microvessel endothelial cell from a subconfluent monolayer stained with rhodamine phalloidin. Preincubation with a 10 micromolar concentration of dexamethasone in media for 2 hr rendered the cells unresponsive to VEG/PF treatment. This image illustrates complete preservation of the intricate actin microfilament network. A similar lack of responsiveness to VEG/PF was demonstrated in cells coincubated with polyclonal anti-VEG/PF IgG antibody prior to VEG/PF exposure.

Figure 4B. Dexamethasone-VEG/PF-treated RBM endothelial cells. Fluorescence photomicrograph (400x) of VEG/PF-treated RBM endothelial cells 1 min after exposure (8 x 10^{-9} M). Preincubation with a 10 micromolar concentration of dexamethasone in media for 2 hours rendered the cells unresponsive to VEG/PF treatment. There is preservation of both the confluent monolayer and the individual cellular microfilament network.

Figure 4C. Dexamethasone-VEG/PF-treated HUV endothelial cells. Fluorescence photomicrograph (400x) of VEG/PF-treated HUV endothelial cells 1 min after exposure (8 x 10^{-9} M). Preincubation with a 10 micromolar concentration of dexamethasone in media for 2 hr rendered the cells unresponsive to VEG/PF treatment. There is preservation of both the confluent monolayer and the individual cellular microfilament network.

not conferred by incubation periods less than 1 hr. Preincubation of the cells with a polyclonal anti-VEG/PF IgG antibody completely inhibited the VEG/PF-actin response (Figure 5). Similar responses to VEG/PF perturbation and dexamethasone treatment occurred in both human umbilical vein and rat brain microvessel endothelial cells. We believe the results are noteworthy both for the extent and reproducibility of the actin changes.

DISCUSSION

Morphology of brain tumor microvessels

Implicit in the current discussion, as in prior discussions by the author, is the premise that VEG/PF-induced edemagenesis and angiogenesis have already been defined as not taking place in microvessels with homotypical blood-brain barrier features. The pathogenesis of peritumoral vasogenic edema is a only partially understood. Extravasated plasma fluid, electrolytes and proteins originate exclusively from the brain tumor microvasculature which differs both morphologically and physiologically from normal blood-brain barrier microvessels [47-53]. VEG/PF and other permeability mediators appear only to affect capillaries that do not exhibit homotypical blood-brain barrier features [14]. It has been hypothesized that their action on the normal brain microvasculature is mechanistically inhibited by the physical expression of pentalaminar tight-junctions between brain endothelial cells [14, 15]. Although phenotypic differences in brain tumor microvessels likely contribute to the increased permeability seen in the setting of peritumoral vasogenic edema, the mechanisms by which these vascular alterations either occur, or are sustained have hitherto been elusive. Phenotypic expression of tight junctions and other barrier features is contextual, and requires the establishment of physical contacts between brain
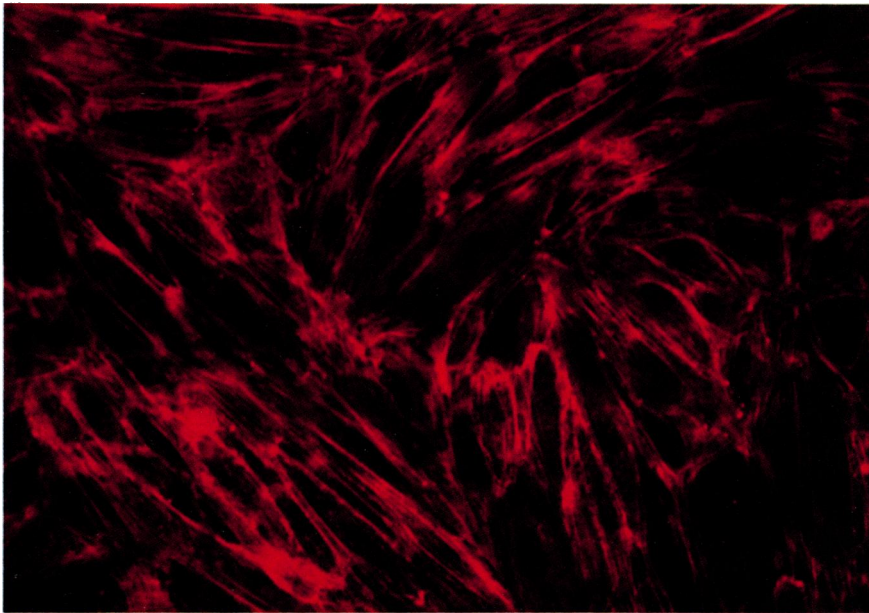
Figure 5. Anti-VEG/PF IgG-treated RBM endothelial cells. Fluorescence photomicrograph (400x) of VEG/PF-treated RBM endothelial cells 1 min after exposure (8 x 10^{-9} M). Preincubation with polyclonal anti-VEG/PF IgG antibody rendered the cells unresponsive to VEG/PF treatment. There is preservation of both the confluent monolayer and the individual cellular microfilament network.

endothelial cells and normal astroglial cells. Divergence from the typical barrier endothelial phenotype, as seen in brain tumors, appears to correlate with the extent of malignant astrocytic degeneration. Microvessels in low-grade gliomas exhibit more typical, albeit altered blood-brain barrier features, while those associated with highly malignant anaplastic astrocytomas and glioblastoma multiforme bear no semblance to normal blood-brain barrier microvessels and maintain many functional and phenotypic similarities to systemic microvessels [14, 15]. It is based upon our existing knowledge of these functional and phenotypic differences that we feel justified studying the action of VEG/PF action in other "unspecialized" endothelial cells such as HUVECs, or RBMECs that have not been co-cultured with normal astroglial cells. Hence, despite their different sites of origin, cultured HUVEC and RBMVEC likely share much in common both with each other and with brain tumor microvessels. The tissue culture environ essentially allows them to revert to a more generic phenotype lacking specialized "barrier" features. It follows, that experiments using endothelial cells with preserved homotypical blood-brain barrier features, would not acurately depict the actual neoangiogenic milieu that has been previously well-described in central nervous system neoplasms.

Regulation of the endothelial cell actin cytoskeleton

Actin is a 375 amino acid protein which polymerizes into alpha-helical microfilaments. Six actin isoforms exist with beta-actin occurring most commonly in endothelial cells, pericytes and fibroblasts, and alpha-actin occurring in vascular smooth muscle cells, cardiac and skeletal muscle. Actin is known to exist in a variety of structures and forms in many different cell types. In vascular endothelial cells, actin is compiled into several morphologically and functionally distinctive structures including: (1) short bundles in microvilli and stereocilia, (2) cortical actin stress fibers, (3) focal cell-matrix adhesion

sites, (4) contractile rings, (5) cellular leading edge, (6) cell surface invaginations, (7) filopodia, (8) microspikes protruding from the cell surface, (9) cellular membrane ruffles and lamellipodia, and (10) networks of highly organized filaments traversing the cortical cytoplasm [4, 36-43]. Cellular actin in its filamentous form (F-actin) is a dynamic protein that is continually shortening or depolymerizing at one end, while simultaneously growing (repolymerizing) at the other by a process called "treadmilling." The equilibrium between the F- and G-actin pools is a complex process involving several regulatory proteins.

Actin stress fibers are important in maintaining cellular shape and structural integrity. In tissue culture, they have been shown to run horizontally, in parallel with the cell membrane attached to the culture disk suface. In situ, actin stress fibers run parallel to the direction of blood flow, thus protecting the cell from flow-related shearing forces. Dissolution of actin stress fiber networks in response to environmental stimuli causes cells to contract in volume and "round up" in shape. Specific actin cytoskeletal changes have now been defined for perturbations involving histamine exposure, oxidant injury, oxyhemoglobin exposure, and ATP depletion [45-48, 54-58]. In addition, recent studies have shown that assembly of actin stress fibers, cortical networks and focal adhesions occurs rapidly in the presence of a ras-related GTP-binding protein designated rho [38]. Rac, a related GTP-binding protein, has been shown to increase actin accumulation in membrane "ruffles." cell surface microspikes, and increased micropinocytotic activity [39]. The G-protein Rab is believed to serve as a regulatory factor in the endocytotic pathway [55]. Severing of actin filaments into short fragments is accomplished in the presence of specialized actin binding proteins such as gelsolin and villin. Both proteins are activated in response to transient increases in endothelial cytosolic calcium ions [23, 36, 40, 42, 54-58]. It would therefore appear plausible for VEG/PF-induced actin changes to be mediated by the same calcium-linked G-protein cascade.

## Calcium signalling and the endothelial cytoskeleton

Calcium ions act as intracellular messengers that control cellular functions in many living systems [4, 16, 35-37, 39, 56, 58]. The calcium signalling motif postulated for electrically inexcitable vascular endothelial cells is initiated by agonist binding to a specific surface membrane receptor (VEG/PF-tyrosine kinase). A G-protein intermediate (rho, rac or rab) then activates phospholipase C (PLC) resulting in the release of the soluble messengers, inositol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). IP3 activates a specific receptor and releases calcium from the endoplasmic reticulum into the cytoplasm. DAG increases actin nucleation and influences cytoskeletal assembly [56]. Transient elevations in cytosolic Ca^{++} therefore result in alterations in cytocontractile proteins and consequent cellular deformation [12-15, 23, 32, 36, 57].

We previously hypothesized that a VEG/PF-induced increase in intracellular calcium concentration could lead to endothelial cytoskeletal reorganization, resulting in cellular contraction and distortion, opening of interendothelial clefts, and subsequent extravasation of plasma fluid and proteins into the tumor interstitium (edemagenesis) [13-17,28, 29]. This direct calcium-cytocontractile relationship was recently observed using real-time laser scanning confocal epifluorescence microscopy (LSCEM) employing a calcium probe (fluo-3), and optical disc image aquisition and storage. VEG/PF-induced cytosolic calcium release was readily visualized, and correlated with a dynamic change in endothelial cell shape including cellular shrinkage and "rounding up" reminiscent of that observed in the static rhodamine-phalloidin images (Figures 6A-C). Similar mechanisms may be responsible for the synchronous membrane alterations requisite for endothelial cytokinesis and microvascular growth (angiogenesis).
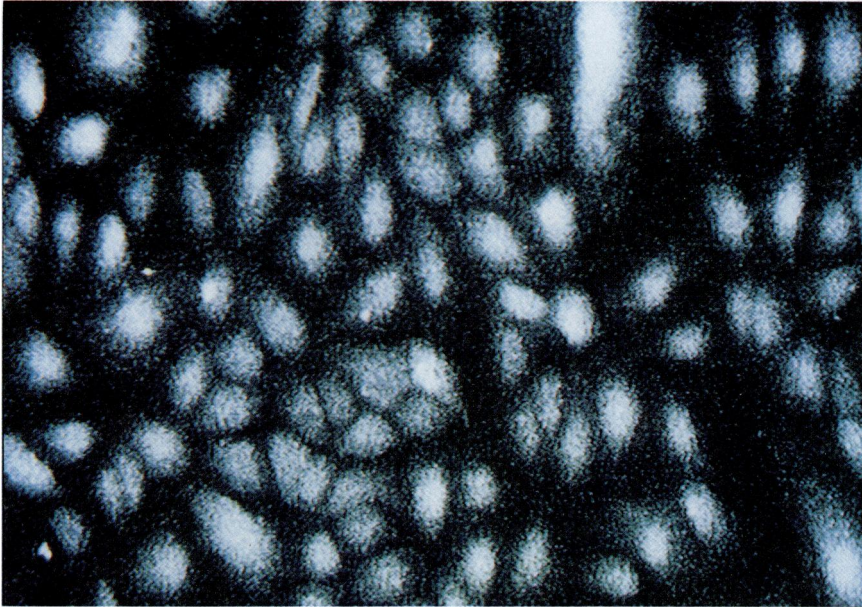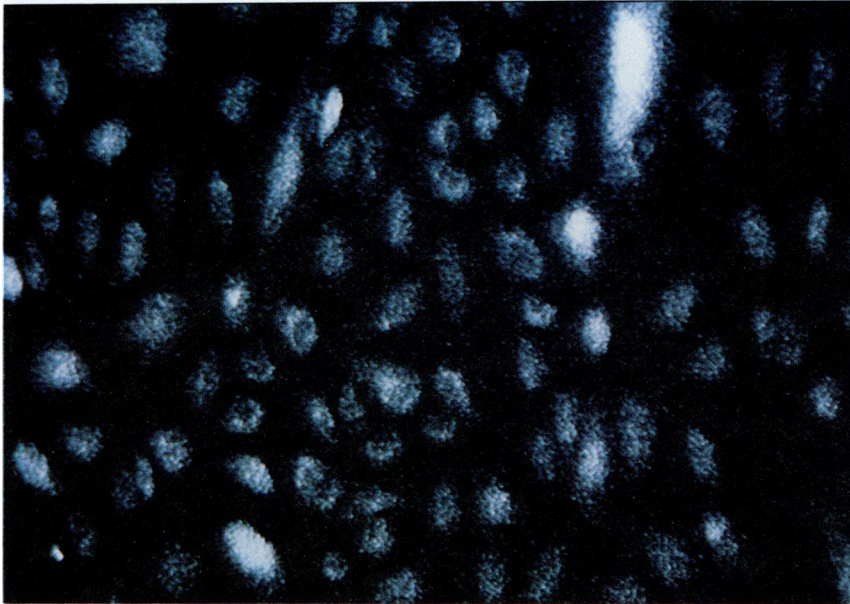
Figures 6A. VEG/PF-induced calcium flux in endothelial monolayers. Fluorescence photomicrograph (200x) of the direct VEG/PF-induced calcium-cytocontractile response was recently observed using real-time laser scanning confocal epifluorescence microscopy (LSCEM). Employing a calcium probe (fluo-3), and optical disc image aquisition and storage, VEG/PF-induced cytosolic calcium release was readily visualized and immediate in onset. These images were captured sequentially over 30 sec at 10 sec intervals. This image captured at 10 sec. Calcium flux correlated with a dynamic change in endothelial cell shape including cellular shrinkage and "rounding up" reminiscent of that observed in the static rhodamine-phalloidin images.
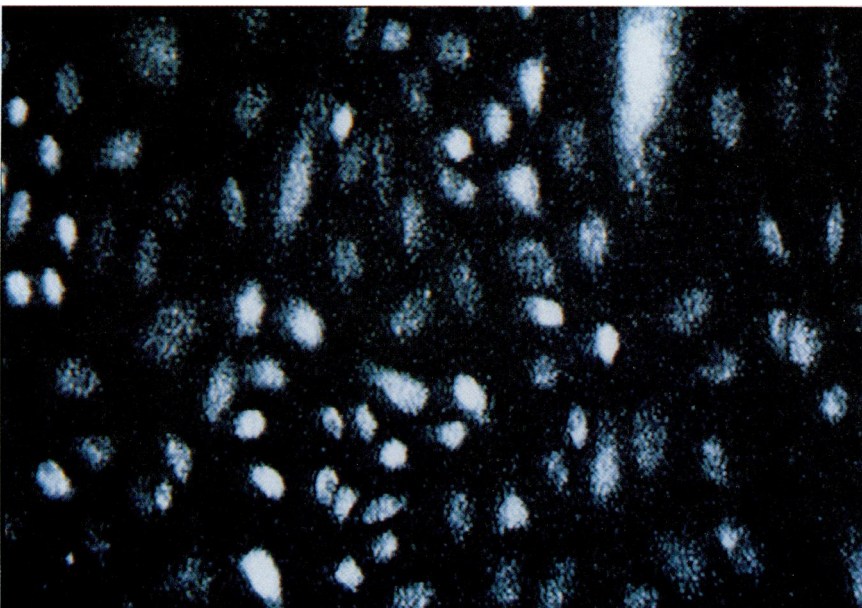
Figures 6B. VEG/PF-induced calcium flux in endothelial monolayers. Fluorescence photomicrograph (200x) of the direct VEG/PF-induced calcium-cytocontractile response was recently observed using real-time laser scanning confocal epifluorescence microscopy (LSCEM). Employing a calcium probe (fluo-3), and optical disc image aquisition and storage, VEG/PF-induced cytosolic calcium release was readily visualized and immediate in onset. These images were captured sequentially over 30 sec at 10 sec intervals. This image captured at 20 sec. Calcium flux correlated with a dynamic change in endothelial cell shape including cellular shrinkage and "rounding up" reminiscent of that observed in the static rhodamine-phalloidin images.

Figures 6C. VEG/PF-induced calcium flux in endothelial monolayers. Fluorescence photomicrograph (200x) of the direct VEG/PF-induced calćium-cytocontractile response was recently observed using real-time laser scanning confocal epifluorescence microscopy (LSCEM). Employing a calcium probe (fluo-3), and optical disc image aquisition and storage, VEG/PF-induced cytosolic calcium release was readily visualized and immediate in onset. These images were captured sequentially over 30 sec at 10 sec intervals. This image captured at 30 sec. Calcium flux correlated with a dynamic change in endothelial cell shape including cellular shrinkage and "rounding up" reminiscent of that observed in the static rhodamine-phalloidin images.

The efficacy of dexamethasone in the treatment of peritumoral vasogenic edema is widely recognized [59-65]. Clinical improvement is associated with a resolution of edema on CT images, and tumor enhancement related to increased microvascular permeability diminishes considerably after dexamethasone treatment. Despite its wide usage, the mechanism dexamethasone's beneficial effects has remained uncertain. Given the causal role proposed for VEG/PF in the pathogenesis of vasogenic edema, it is reasonable to consider whether dexamethasone's efficacy relates to an influence on VEG/PF expression by tumor cells, or VEG/PF receptor expression and other related VEG/PF-induced endothelial cell events. Dexamethasone inhibition of·VEG/PF expression in cultured human malignant glioma cells has previously been reported [11]. Indeed, preincubation of endothelial cells with dexamethasone for two hours completely abolishes VEG/PF-induced cytosolic calcium transients [16]. The latter observation led us to hypothesize that dexamethasone is able to close the blood-tumor barrier by preventing VEG/PF-induced cytoskeletal contraction [14, 15]. The data collected thus far appear to support that hypothesis.

SUMMARY

These observations newly describe a rapid change in endothelial actin cytoskeletal conformation in response to VEG/PG perturbation. The resultant induction of endothelial cell contraction and widening of the interendothelial spaces is reminiscent of changes induced by other potent mediators of microvascular permeability. VEG/PF-induced changes could be prevented by preincubation of endothelial cells with a polyclonal anti-VEG/PF IgG antibody,

and by pretreatment with dexamethasone. These findings support a specific permeability-inducing effect of VEG/PF on endothelial cell barriers. Rapidly proliferating endothelial cells responded more dramatically to VEG/PF perturbation than did slowly growing cells. Tumor endothelial cells, perhaps in response to VEG/PF stimulation, would be expected to show an enhanced proliferation rate. It is compelling to consider a relationship between active endothelial cell proliferation and VEG/PF receptor up-regulation. We have briefly reviewed the literature regarding the most relevant signal transducing pathways linking VEG/PF binding to its tyrosine kinase receptor, the induction of cytosolic calcium ion transients, and ultimately, to our hypothesized action for VEG/PF upon the endothelial actin cytoskeleton.

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