Anastellin, a Fragment of the First Type III Repeat of Fibronectin, Inhibits Extracellular Signal-Regulated Kinase and Causes G₁ Arrest in Human Microvessel Endothelial Cells

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Abstract

The formation of a microvascular endothelium plays a critical role in the growth and metastasis of established tumors. The ability of a fragment from the first type III repeat of fibronectin (III₁C), anastellin, to suppress tumor growth and metastasis in vivo has been reported to be related to its antiangiogenic properties, however, the mechanism of action of anastellin remains unknown. Utilizing cultures of human dermal microvascular endothelial cells, we provide evidence that anastellin inhibits signaling pathways which regulate the extracellular signal-regulated (ERK) mitogen-activated protein kinase pathway and subsequent expression of cell cycle regulatory proteins. Addition of anastellin to primary microvascular endothelial cells resulted in a complete inhibition of serum-dependent proliferation. Growth inhibition correlated with a decrease in serum-dependent expression of cyclin D1, cyclin A and the cyclin-dependent kinase, cdk4, key regulators of cell cycle progression through G₁ phase. Consistent with a block in G₁-S transition, anastellin inhibited serum-dependent incorporation of [³H]-thymidine into S-phase nuclei. Addition of anastellin to serum-starved microvessel cells resulted in a time-dependent and dose-dependent decrease in basal levels of phosphorylated MEK/ERK and blocked serum-dependent activation of ERK. Adenoviral infection with Ad.ΔB-RaEER, an inducible estrogen receptor-B-Raf fusion protein, restored levels of active ERK in anastellin-treated cells, rescued levels of cyclin D1, cyclin A, and cdk4, and rescued [³H]-thymidine incorporation. These data suggest that the antiangiogenic properties of anastellin observed in mouse models of human cancer may be due to its ability to block endothelial cell proliferation by modulating ERK signaling pathways and down-regulating cell cycle regulatory gene expression required for G₁-S phase progression. (Cancer Res 2005; 65(1): 148-56)

Introduction

Fibronectin is a large molecular weight mosaic protein which is organized into multiple repeats of type I, type II and type III domains representing regions of amino acid homology [reviewed by Pankov and Yamada (1)]. Fibronectin is found as a soluble protomer in the blood plasma, but is actively polymerized into large disulfide stabilized polymers found in the extracellular matrix of most tissues [reviewed by Wierzbicka-Patynowski and Schwarzbauer (2)]. The fibronectin matrix undergoes continual remodeling in response to a variety of biologically active molecules including cytokines and lipids (3, 4). Remodeling events include changes in the level of fibronectin matrix (3), changes in the local synthesis of particular fibronectin isoforms (5), as well as changes in the overall organization of the matrix (6). Changes in the amount, type, and organization of the fibronectin matrix affects cellular differentiation, apoptosis, and cell cycle progression (7–12). Fibronectin matrix, therefore, provides a dynamic biological scaffold from which cells receive signals critical for cell growth and survival.

Interactions of cells with matrix occur through a family of cell adhesion receptors termed integrin receptors. Integrins are transmembrane receptors which consist of an α and β subunit. The “classical” fibronectin receptor, α₅β₁, mediates adhesion of cells to fibronectin, participates in the assembly of the fibronectin matrix, and transmits structural and mechanical information back into the cell by interacting with intracellular signaling pathways which regulate cytoskeletal organization and growth factor signaling. Specific pathways regulated by α₅β₁ include the extracellular signal-regulated (ERK) mitogen-activated protein kinase pathways (13, 14) and small molecular weight Ras subfamily members such as Rac and Rho (15). Remodeling of the fibronectin matrix, which occurs in association with tumor angiogenesis, includes changes in the expression of fibronectin isoforms as well as changes in the levels of fibronectin present in the tumor stroma (16, 17). Antagonists of the integrin receptor for fibronectin, α₅β₁, have been shown to disrupt tumor angiogenesis in mouse models of human cancer (18, 19). In addition, a genetic knockout of the α₅ subunit of the integrin results in a lethal phenotype which is associated with impaired vascular development (20).

Recently, several studies have indicated that fragments of extracellular matrix molecules may function as potent angiogenic inhibitors. These inhibitors include fragments of collagen (21–23), laminin (24), as well as fibronectin (25, 26) and have been effective in preventing the growth of several types of human tumors in mice. Anastellin is a fragment of fibronectin derived from the carboxyl-terminal two-thirds of the first type III homology repeat (III₁C) and has been shown to suppress tumor growth and metastasis in mouse models of human cancer (25, 26). The effects of anastellin on tumor growth have been proposed to result from an inhibition of tumor angiogenesis; however, there has been little characterization done on how anastellin might regulate endothelial cell biology. In this study, we tested the effects of anastellin on the growth of cultured human microvascular endothelial cells and have found that it completely blocked serum-dependent growth. Treatment of cells with anastellin resulted in a rapid decrease in the basal levels of phosphorylated ERK and prevented serum-mediated ERK activation. Inactivation of ERK was associated with a drop in the...
levels of cyclin D1, cyclin A, and cdk4, and an inhibition of \(^{3}H\)-thymidine incorporation into S-phase nuclei. Expression of constitutively active B-Raf rescued levels of phosphorylated ERK, expression of cyclin D1, cyclin A, and cdk4, and rescued \(^{3}H\)-thymidine incorporation in the presence of anastellin. These results suggest that the antiangiogenic properties of anastellin observed in vivo may be related to its ability to block microvessel endothelial cell growth by modulating ERK-dependent expression of cell cycle regulatory proteins.

### Materials and Methods

**Reagents.** Unless indicated otherwise, reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Mouse monoclonal antibody to cyclin D1 was obtained from BD Biosciences (San Jose, CA). Rabbit polyclonal antibodies to cyclin A, cdk4, phospho-FAK (Tyr927), MEK1, ERK2, and phospho-MEK1/2 (Ser217/222) were obtained from Santa Cruz Biotechnolog.

**Cell Culture.** Primary human adult dermal microvascular endothelial cells were obtained from VEC Technologies, Inc. (Rensselaer, NY). Endothelial cells were maintained in MCD-131, 20% defined fetal bovine serum (HyClone Laboratories, Logan, UT), 2 mmol GlutaMAX (Invitrogen, Carlsbad, CA) and EGM-2MV SingleQuots growth factor cocktail (Cambrex Corp., East Rutherford, NJ; complete medium) supplemented with 10 mg/mL heparin and cultured in a humidified incubator at 37°C in an atmosphere of 5% CO\textsubscript{2} on collagen-coated (20 μg/mL) tissue culture dishes. In most experiments, human dermal microvascular endothelial cells were plated on collagen-coated dishes in complete medium at a density of 1 × 10\textsuperscript{4} cells/well (12-well tissue culture dish), cultured overnight, and serum-starved for 30 hours.

**Generation and Purification of Recombinant Fibronectin Fragments.** Recombinant I\textsubscript{IIIC} (anastellin), I\textsubscript{IIIA}D, and I\textsubscript{IIIA}H fibronectin fragments were generated by PCR amplification of the human fibronectin cDNA clone pH1120 (27). Primers used to generate recombinant fragments I\textsubscript{IIIC}, I\textsubscript{IIIA}D, and I\textsubscript{IIIA}H were as described by Morla et al. (28) and Klein et al. (29). PCR products were cloned into bacterial expression vectors pQE-70 (I\textsubscript{IIIC} and I\textsubscript{IIIA}D) and pQE-30 (I\textsubscript{IIIA}H; Qiagen, Inc., Valencia, CA) in frame with a bacterial 6×His tag, and the sequences were confirmed by automated dyeoxy sequencing. Recombinant His-tagged fibronectin fragments were expressed in M15 bacterial cells (Qiagen) and purified to homogeneity by standard Ni-NTA, size-exclusion, and cation-exchange chromatography as previously described (29).

**Generation of Recombinant Adenoviruses.** The DNA for Δβ-RafER (30) was a generous gift from Martin McMahon (University of California–San Francisco). Δβ-RafER DNA was subcloned into pAdTrack-cytomegalovirus and the recombinant adenoviruses (AdΔβ-RafER) were produced using the AdEasy system as previously described (31, 32). Viruses were titered and used at a multiplicity of infection of 5 (33). The green fluorescent protein (GFP) adenovirus (AdGFP) was obtained from Q-Biogene (Carlsbad, CA).

**Endothelial Cell Proliferation Assay.** Microvascular endothelial cells were plated on collagen-coated (20 μg/mL) 96-well tissue culture dishes (500 cells/well) in complete medium and allowed to attach for 3 to 4 hours prior to addition of recombinant fibronectin fragments. On each of six consecutive days following initial plating and treatment, cells were fixed for 15 minutes in 3% paraformaldehyde (37°C), washed thrice with phosphate-buffered saline (PBS), and stored at 4°C until assayed. Endothelial cell proliferation was determined indirectly by ELISA using a mouse antiendothelial cell (anti-CD146) monoclonal antibody. Briefly, cell layers, blocked in PBS containing 3% BSA, were incubated with 0.2% BSA, PBS containing 0.75 μg/mL monoclonal anti-CD146 antibody for 1 hour at room temperature, washed four times with PBS, followed by incubation with 0.2% BSA, PBS containing horseradish peroxidase–conjugated goat anti-mouse IgG (1000-fold dilution; BioRad, Hercules, CA) for 1 hour at room temperature. Cell layers were washed five times with PBS and incubated with substrate [0.1 mL of 0.1 mol/L citrate (pH 5); 0.5 mg/mL o-phenylenediamine, and 1 μL hydrogen peroxide] for 2 to 6 minutes. The reaction was terminated with 50 μL of 2N H\textsubscript{2}SO\textsubscript{4}, and the absorbance determined (A\textsubscript{490nm}).

**Immunoblot and Expression Analysis.** In most experiments, cleared cell lysates were prepared prior to protein separation by SDS-PAGE. Briefly, cell layers were washed with ice-cold PBS containing 1 mmol Na\textsubscript{3}VO\textsubscript{4} before solubilization in lysis buffer [20 mmol Tris-Cl (pH 7.4), 1% Triton X-100, 0.5% Nonidet P-40, 0.1 mol/L NaCl, 40 mmol NaF, 30 mmol Na\textsubscript{2}PO\textsubscript{4}, 2 mmol EGTA, 1 mmol Na\textsubscript{3}VO\textsubscript{4}, and 0.5 mmol phenylmethylsulfonyl fluoride containing one tablet of Complete Mini (Roche Biochemical, Indianapolis, IN) protease inhibitor cocktail per 10 mL]. Cell lysates were then run by centrifugation at 20,000 × g for 20 minutes at 4°C and the insoluble pellets discarded. Cleared lysates were stored at -80°C until use. The protein concentration of cell lysates was determined with a bicinchoninic acid protein assay reagent kit (Pierce, Rockford, IL) using BSA as standard. In some experiments, whole cell lysates were prepared. Typically, cell layers were initially washed with ice-cold PBS containing 1 mmol Na\textsubscript{3}VO\textsubscript{4} immediately prior to solubilization in Laemmli sample buffer. Following PAGE, proteins were transferred to nitrocellulose membranes (Schleicher & Schuell Bioscience, Keene, NH). Membranes were blocked with TBST (Tris-Cl (pH 7.4), 150 mmol NaCl, 0.1% Tween 20) containing 5% (w/v) BSA and processed for Western analysis using an enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ). In some instances, blots were reprobed after stripping for 30 minutes in 62.5 mmol Tris-Cl (pH 6.7) and 2% SDS containing 10 mmol β-mercaptoethanol at 60°C. Digital image analysis of immunoblots was done using Scion Image software (shared NIH software; Scion Corp., Frederick, MD).

**Measurement of DNA Synthesis.** Human dermal microvascular endothelial cells were plated on collagen-coated (20 μg/mL) 24-well dishes at 1.25 × 10\textsuperscript{4} cells/well in the presence of complete medium and allowed to adhere overnight. Cells were serum-starved for 30 hours, treated with 20 μM I\textsubscript{IIIC} or I\textsubscript{IIIA}H for 1 hour then stimulated with 10% fetal bovine serum for an additional 16 hours. In some experiments, cells were infected with AdGFP or AdΔβ-RafER for 24 hours in serum-free MCD-131, then treated for 1 hour with 1 μM 4-hydroxytamoxifen followed by an additional 1 hour with 20 μM I\textsubscript{IIIC} or I\textsubscript{IIIA}H prior to serum stimulation. S-phase nuclei were labeled by incubating cells with 1 μCi of \(^{3}H\)-thymidine for 6 hours. Cells were treated with 10% TCA and recovered in 1N NaOH. Samples were neutralized and transferred to Ecosint A (National Diagnostics, Atlanta, GA) scintillation fluid. Incorporation of \(^{3}H\)-thymidine was determined by liquid scintillation.

**Fluorescence Microscopy.** Human dermal microvascular endothelial cells were plated on collagen-coated (20 μg/mL) glass cover slips in the presence of complete medium and allowed to adhere overnight. Cells were then cultured in the presence of 10% serum ± 20 μM I\textsubscript{IIIC} for up to 24 hours. Cells were fixed for 20 minutes in 3% paraformaldehyde, permeabilized in 0.5% Triton X-100 for 10 minutes, blocked in 1% BSA, and immunostained with monoclonal antibodies against β\textsubscript{3}, α\textsubscript{5}, and α\textsubscript{2} integrin subunits. F-actin was visualized with Alexa Fluor 594-conjugated phalloidin (Molecular Probes, Eugene, OR). Cell layers were examined using an Olympus BUX-60 microscope equipped with a cooled CCD sensor-camera (Cooke, Auburn Hills, MI) and images acquired using Slidebook software (Intelligent Imaging Innovation, Denver, CO).

**TUNEL Assay.** Human microvascular endothelial cells were analyzed for apoptosis according to the manufacturer's protocol using the DeadEnd Fluorometric TUNEL System assay kit (Promega, Madison, WI). Experimental conditions were based upon those used in the proliferation assay.
with minor modifications. Briefly, \(2 \times 10^4\) human dermal microvessel endothelial cells were plated on collagen-coated (20 \(\mu\)g/mL) glass cover slips in complete medium (24-well tissue culture dishes). After 4 hours, the medium was replaced with fresh medium containing either III\(_{1C}\) (20 \(\mu\)M) or PBS (buffer control). Cells were cultured for an additional three days prior to staining. As a positive control, staurosporine (100 nmol/L) was added to nontreated cells during the last 24 hours to induce apoptosis. Some cells were treated with DNaseI to fragment chromosomal DNA and served as positive assay control. After fluorescein-12-dUTP nick end labeling was completed, the cells were counterstained with 1 \(\mu\)g/mL propidium iodide and the cover slips were mounted on glass slides with ProLong antifade mounting medium (Molecular Probes). Images were viewed and captured using an Olympus BMX-60 microscope equipped with a cooled CCD sensi-camera (Cooke).

**Determination of Caspase-3 Activity.** Human dermal microvessel endothelial cells were plated on collagen-coated (20 \(\mu\)g/mL) tissue culture dishes in complete medium and maintained in a humidified incubator at 37°C and 5% CO\(_2\). After 4 hours, the medium was replaced with fresh medium containing either III\(_{1C}\) (20 \(\mu\)M) or PBS (buffer control). Cells were cultured for an additional 2 days. Staurosporine (100 nmol/L) was added to nontreated cells during the final 24 hours to induce apoptosis (positive control). Cell layers were washed with PBS containing 0.5 mmol EDTA and released with 0.05% trypsin (HyClone), PBS, 0.5 mmol EDTA. Cells were then pelleted, washed thrice in PBS, and assayed for caspase-3 activity using the CaspACE Assay System kit (Promega) according to the manufacturer’s protocol.

**Results**

**Anastellin (III\(_{1C}\)) Inhibits Microvascular Endothelial Cell Proliferation.** In order to investigate the effect of III\(_{1C}\) on endothelial cell proliferation, primary human dermal microvessel endothelial cells were plated on collagen-coated 96-well tissue culture dishes and grown for 6 days in the presence of recombinant fibronectin modules III\(_{1C}\) or III\(_{10N}\). The III\(_{10N}\) fragment used as control in this experiment is derived from the amino terminus of the tenth type III homology repeat of fibronectin (III\(_{10}\)) and is truncated just prior to the integrin-binding site (29). On each of six consecutive days, cells were washed and fixed, and endothelial cell growth was determined indirectly by ELISA. As shown in Fig 1A, endothelial cell number doubled every 25 to 30 hours over the 6-day period. In the presence of the III\(_{1C}\) peptide, the rate of endothelial cell growth was significantly decreased. Endothelial cell growth was essentially blocked at 20 \(\mu\)M III\(_{1C}\). Incubation with the control fragment, III\(_{10N}\), had only a slight effect on endothelial cell growth. As shown in Fig 1B, the effects of III\(_{1C}\) were dose-dependent with half-maximal inhibition seen at approximately 10 \(\mu\)M III\(_{1C}\). Similar results were also obtained with large vessel endothelial cells and bovine lung microvessel endothelial cells (data not shown). In contrast, III\(_{1C}\) had no effect on the growth of human fibrosarcoma (HT-1080) cells (data not shown).

**Anastellin Does not Induce Apoptosis of Adherent Microvessel Endothelial Cells.** To determine whether the effect of III\(_{1C}\) on endothelial cell growth was associated with an increase in apoptosis, microvessel cells were cultured in complete medium in the presence of 20 \(\mu\)M III\(_{1C}\) for 3 days and apoptotic cells were visualized using the terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) technique. Control cells received an equivalent amount of PBS. As a positive control, some cells were treated with 100 nmol/L staurosporine, an inhibitor of cellular protein kinases which has been shown to induce apoptosis in a variety of different cell types (34, 35). Cells were then fixed and labeled with fluorescein-12-dUTP, TUNEL-positive cells, as indicated in Fig 2A, were counted and expressed as the percentage of the total number of cells viewed in a given field. The proportion of fluorescein-12–labeled cells following III\(_{1C}\) or staurosporine treatment was then compared with that of control cells (C, Fig 2B). III\(_{1C}\) was found to have no significant effect on the proportion of TUNEL-positive cells (\(P > 0.01; n = 3\)). In contrast, cells treated with staurosporine exhibited a severalfold increase in the proportion of cells undergoing apoptosis. No significant difference was observed between nontreated and PBS-treated control cells (data not shown). In addition, cells treated with DNaseI to induce chromosomal DNA fragmentation exhibited 100% TUNEL-positive staining (data not shown).

As an alternative to the TUNEL assay, caspase-3 activity was also used as an indicator for apoptosis. Cell lysates were prepared from cells treated with either 20 \(\mu\)M III\(_{1C}\) for 2 days or...
Anastellin Down-regulates Cyclin D1, Cyclin A, and cdk4 Levels and Blocks G1-S Phase Progression. Proliferation of endothelial cells is tightly controlled by the expression and activation of a variety of cell cycle regulatory proteins including cyclins, cyclin-dependent kinases (cdk), and inhibitors of cyclin-cdk complexes (i.e., p21cip1 and p27kip1). In particular, there is growing evidence that activation of cdk4 and induction of cyclin D1 protein synthesis plays a critical role in G1-S phase progression. These events are, in turn, controlled by a complex series of signal transduction events (reviewed in ref. 36). To determine whether the growth inhibition observed in III1C-treated microvessel endothelial cells was accompanied by changes in the level of cell cycle regulatory proteins, III1C was tested for its effect on the levels of cyclin D1, cyclin A, cdk4, p21cip1, and p27kip1. Serum-starved microvessel endothelial cells were treated for 1 hour with 20 μM III1C, then stimulated with 10% serum to increase the expression of these proteins, but typically reduced their levels significantly below basal levels. As control, cells were incubated with III13 module for 1 to 24 hours and immunostained for changes in cell shape or integrin-based adhesion, microvessel cells were treated for 1 hour with 20 μM III1C, then stimulated with 10% serum to induce a mitogenic response. Immunoblot analysis indicated that in the absence of III1C treatment, the addition of serum induced an increase in the level of cyclin D1, cyclin A, and cdk4 protein as much as 2-fold above that of control cells (Fig. 3A). Treating cells with III1C not only blocked the ability of serum to increase the expression of these proteins, but typically reduced their levels significantly below basal levels. As control, cells were incubated with the III13 module of fibronectin. This module was chosen because of its ability to bind to the extracellular matrix as has been shown for III1C (29). The III13 fragment had no effect on the expression of any of the cell cycle proteins tested. Cell lysates were also examined for changes in p21cip1 and p27kip1 expression (Fig. 3B). As shown in Fig. 3B, there was little or no change in the expression of either of these cdk inhibitors. Similar results were observed in more detailed time course experiments (data not shown). In addition, III1C blocked serum-dependent incorporation of [3H]-thymidine into S-phase nuclei (Fig. 3C) consistent with a block in S-phase entry. These results suggest that the ability of III1C to block endothelial cell proliferation may be linked to changes in cell cycle regulatory protein expression.

Anastellin does not affect α5β1 integrin clustering or actin stress fiber formation. Previous studies have shown that tension-dependent changes in cell shape and integrin-mediated adhesion play a critical role in regulating cell cycle progression in endothelial cells (37, 38). In particular, disruption of the actin cytoskeleton or loss of integrin-based adhesion signaling through the fibronectin receptor (α5β1 integrin) has been shown to block cyclin D1 biosynthesis and prevent progression through the G1 phase (37, 38). To investigate the possibility that down-regulation of cyclin D1, cyclin A, and cdk4 expression by III1C could be mediated through changes in cell shape or integrin-based adhesion, microvessel cells were incubated with III1C for 1 to 24 hours and immunostained for α5, β1, and α2 integrin subunits. Changes in cell shape were determined by phalloidin staining of F-actin. Under conditions shown to decrease expression of cell cycle proteins and prevent incorporation of [3H]-thymidine (Fig. 3), as well as block endothelial cell proliferation (Fig. 1), III1C had no effect on cell adhesion, actin cytoskeletal organization or cell shape (Fig. 4A). Clustering of α5 and β1 integrin subunits into focal complexes and focal adhesions was unaffected by either short-term (1 hour) or long-term (24 hours) exposure to III1C (Fig. 4A). Although only little clustering of α2 could be found associated with actin stress fibers, there was no apparent change in the level or distribution as a result of III1C treatment.

Previous studies have shown that FAK is phosphorylated at Y397 in response to α5β1 ligation to fibronectin and accumulates at focal

100 nmol/L staurosporine for 24 hours and assayed for the induction of caspase-3 activity (Fig. 2C). Compared to control cells (C), III1C had no significant effect on caspase-3 activity (P > 0.01; n = 3). Staurosporine treatment (100 nmol/L), however, increased caspase-3 activity severalfold above that observed in control cells (Fig. 2C). These results agree with those obtained by TUNEL staining and indicate that the III1C peptide does not induce apoptosis in adherent microvessel endothelial cells.

Figure 2. The effect of anastellin on apoptosis of adherent microvessel endothelial cells. Human dermal microvessel endothelial cells were plated on collagen-coated glass coverslips and cultured for 3 days in the presence or absence of 20 μM III1C (III1C). PBS (C) was used as vehicle control. As positive control, 100 nmol/L staurosporine was added during the last 24 hours to induce apoptosis. Cell layers were washed, fixed, and stained. TUNEL-positive cells (A), were counted and normalized to the total number of cells (counterstained with propidium iodide; not shown) in a given field and averaged over three or more independent fields. The relative proportion of TUNEL-positive cells obtained was then expressed as fold increase over that obtained for control cells (B). Alternatively, lysates generated from cells treated 48 hours in the presence of III1C or absence (C) of 20 μM III1C, or 24 hours with 100 nmol/L staurosporine, were assayed for caspase-3 activity, a proteolytic indicator for apoptosis. Caspase-3 activity was then compared with that obtained for control cells (C). Results are the mean ± SE of three determinations. *P values < 0.01 (Student’s t test) when compared with control cells.
complexes and focal adhesions (38). Consistent with III1C having no effect on the localization and clustering of integrins in adherent cells (Fig. 4A), serum-starved microvessel cells treated with III1C for up to 90 minutes exhibited no change in the level of Y397 phosphorylation (Fig 4B). These results suggest that the ability of III1C to down-regulate levels of cyclin D1, cyclin A, and cdk4 are unlikely to be a result of a loss of cell adhesion or change in cell shape.

**ERK Is Inactivated in Anastellin-Treated Adherent Microvascular Endothelial Cells.** Induction of cyclin D1 expression during G1-S progression has been shown to depend on sustained activation of the ERK signal transduction pathway in several cell types (39–42). Down-regulation of cyclin D1 expression, as well as cyclin A and cdk4, in III1C-treated microvessel endothelial cells (Fig. 3) suggests that signal transduction pathways leading to ERK activation may be affected by III1C treatment. Consistent with this idea, serum-starved adherent microvessel cells treated with III1C exhibited a time-dependent decrease in basal levels of phosphorylated ERK and its upstream kinase, MEK (Fig. 5A and B). Inactivation of basal ERK and MEK occurred within 10 minutes and was nearly complete within 20 minutes following treatment with 20 μM III1C. In contrast, no effect was observed on the activation state of ERK in microvessel cells treated with the control fragment, III10C. Serum-starved cells treated with increasing concentrations of III1C exhibited a dose-dependent decrease in both phosphorylated MEK and ERK with half-maximal activity observed at 7.5 μM III1C, similar to the IC50 observed for endothelial cell growth arrest (Fig. 1). To determine whether III1C can modulate the effect of serum on ERK activation, serum-starved microvessel cells were treated with 20 μM III1C for up to 90 minutes were lysed and immunoblotted with phosphospecific antibodies to Y397FAK (B). Immunoblots were stripped and reprobed with antibodies to FAK for loading control.

**ΔB-RafER Rescues G1-S Phase Progression but not Proliferation in Anastellin-Treated Microvessel Cells.** Several studies have indicated that sustained activation of MEK/ERK is required for cyclin D1 induction during mid-G1 phase (40, 42). In order to determine whether the ability of III1C to down-regulate levels of cyclin D1, cyclin A, and cdk4 is a consequence of an inhibition of the MEK/ERK signal transduction pathway (Fig. 5), microvessel endothelial cells were infected with an adenoviral construct expressing an inducible estrogen receptor-B-Raf fusion protein, ΔB-RafER (30, 43). Control cells were infected with an adenoviral construct containing the gene for GFP. Infected cells

![Figure 3](image-url)  
**Figure 3.** Anastellin down-regulates cyclin D1, cyclin A, and cdk4 expression and blocks G1-S phase progression. Human dermal microvessel endothelial cells were plated on collagen-coated tissue culture dishes (1 × 10⁵ cells/mL) in complete medium for 24 hours then serum-starved for an additional 24 hours in MCDB-131, 0.5% BSA (A and B). Microvessel cells treated for 1 hour with either 20 μM III1C or 20 μM III13 as control were stimulated with 10% fetal bovine serum for 24 hours to initiate a mitogenic response. Nontreated serum-starved microvessel cells served as negative control for induction of cell cycle protein expression. Cell lysates were generated and immunoblotted (15 μg/lane) for cyclin D1, cyclin A, and cdk4 (A). Lysates were also blotted for changes in the level of cyclin-dependent kinase inhibitors, p21cip1 and p27kip1 (B). Similarly, serum-starved cells were treated with 20 μM III1C or III13 for 1 hour prior to stimulation with 10% serum (16 hours). Cells were pulsed with 1 μCi of [³H]-thymidine for 6 hours. Incorporation of [³H]-thymidine into S-phase nuclei was determined by liquid scintillation (C).

![Figure 4](image-url)  
**Figure 4.** The effect of anastellin on cell shape and integrin clustering. Microvessel endothelial cells were cultured in serum-containing medium for 24 hours followed by either a 1- or 24-hour treatment with 20 μM III1C in serum-free medium. Cells were immunostained with monoclonal antibodies to β1, α5, or α2 integrin subunits. F-actin was visualized with Alexa Fluor 594-conjugated phalloidin (A). Arrows, areas where integrin subunits cluster into focal adhesions. Serum-starved microvessel cells treated with 20 μM III1C for up to 90 minutes were lysed and immunoblotted with phosphospecific antibodies to Y397FAK (B). Immunoblots were stripped and reprobed with antibodies to FAK for loading control.
expressing ΔB-RafER were treated with 1 μM 4-hydroxytamoxifen for 1 hour to induce activation of ΔB-RafER then treated for 1 hour with 20 μM III1C to block serum-dependent activation of ERK. Infected cells were then stimulated with serum (10%) and cultured for an additional 24 hours. Cell lysates were generated and immunoblotted with phosphospecific antibodies to ERK and MEK to verify ΔB-RafER-induced activation of MEK/ERK (Fig. 6A). Anastellin had no effect on the ability of ΔB-RafER to activate ERK or MEK. To evaluate the effects of ΔB-RafER-induced ERK on cell cycle proteins, lysates were also immunoblotted for cyclin D1, cyclin A, and cdk4. Consistent with the results shown in Fig. 3, III1C treatment blocked serum-dependent expression of cyclin D1, cyclin A, and cdk4 in GFP-infected control cells; however, activation of ΔB-RafER with 4-hydroxytamoxifen rescued expression of cyclin D1, cyclin A, and cdk4 (Fig. 6B). Similarly, expression of ΔB-RafER rescued [3H]-thymidine in III1C-treated cells (Fig. 6C). Interestingly, ΔB-RafER expression did not rescue cell proliferation (Fig. 6D). These data indicate that re-expression of ERK activity is sufficient to rescue levels of cell cycle proteins and S-phase entry of anastellin-treated microvessel cells but not proliferation.

**Discussion**

Anastellin, a fragment derived from the carboxyl terminus of the first type III homology repeat of fibronectin, blocks tumor growth, and metastasis in mouse models of several human cancers; however, the molecular mechanisms underlying this activity are not well understood. In this study, we found that addition of anastellin to cultured microvascular endothelial cells completely blocked serum-dependent growth providing direct evidence that anastellin's effects on tumor growth result from inhibition of angiogenesis. Inhibition of cell growth was not associated with an induction of apoptosis, suggesting that the effects of anastellin on growth were due to effects on cell cycle progression rather than on activation of apoptotic pathways. We found that anastellin quickly

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**Figure 5.** Anastellin inactivates MEK/ERK and blocks serum-mediated ERK activation in adherent microvessel endothelial cells. Human dermal microvessel endothelial cells were cultured in 24-well dishes (5 × 10^5 cells) for 24 hours in complete medium, serum-starved an additional 24 hours in MCDB-131, 0.5% BSA, and then treated with either 20 μM III1C or 20 μM III10N for up to 90 minutes (A and B) or with increasing concentrations of III1C for 60 minutes (C). D, serum-starved cells were treated for 1 hour with 20 μM III1C prior to stimulation with serum for up to 90 minutes. Whole-cell lysates were prepared as described and immunoblotted with phosphospecific antibodies to ERK (A, C, and D) and MEK (B and C). Immunoblots were then stripped and reprobed with antibodies to MEK1 and ERK2 for loading control. Expression levels were determined and expressed as the percentage of those obtained in the absence of treatment.
and completely reduced basal levels of phosphorylated MEK and ERK in serum-starved microvessel cells with half-maximal inhibitory effects seen between 10 and 20 minutes. Inactivation of ERK was dose-dependent and the doses of anastellin required for MEK/ERK inactivation closely correlated with the doses required for inhibition of cell proliferation. The decrease in endothelial cell proliferation was associated with an inhibition of \[^{3}H\]-thymidine incorporation as well as decreases in the level of cyclin D1, cyclin A, and cdk4 protein. As these proteins are key regulators of cell cycle progression from G1 into S phase [reviewed by Lloyd (44), and Cooper and Shayman (45)], it seems that the effects of anastellin on cellular growth are due to an inhibition of cell cycle progression. These findings are consistent with earlier reports demonstrating inhibitory effects of anastellin on the activity of cyclin E/cdk2 complexes in human umbilical vein endothelial cells (9) and on the growth of human vascular smooth muscle cells (46).

The anastellin-induced decrease in \[^{3}H\]-thymidine incorporation and cyclin/cdk levels directly correlated with an inhibition of serum-dependent activation of ERK. Earlier reports have suggested that ERK-dependent induction of cyclin D1 is rate-limiting for progression through G1 and entry into S phase and is required for cell cycle progression in adherent cells (47). Levels of cyclin D1 are up-regulated in response to serum activation and appropriate cytoskeletal organization (48, 49). Recent studies suggest that cyclin D1 protein is regulated at both the transcriptional and translational levels. Transcriptional regulation of cyclin D1 requires sustained levels of active ERK (42), whereas translational regulation of cyclin D1 is independent of ERK and is dependent on Rac signaling pathways (38). Taken together, these data suggest that the effect of anastellin on cyclin D1 levels is due to a block in ERK-dependent transcription of cyclin D1. Upstream regulators of ERK-dependent cyclin D1 expression include Rho/Rho kinase and FAK (41, 50, 51). Anastrat was shown to affect both Rho as well as FAK signaling pathways (9); however, the relationship of these pathways to the expression of cyclin D1 in microvessel cells is not yet known. Although overexpression of \(\Delta B\)-Raf:ER rescued ERK activation, cyclin D1 protein levels, and DNA synthesis in anastellin-treated cells, it did not rescue cell division. The inability of \(\Delta B\)-Raf:ER to rescue cell growth indicates that anastellin may have inhibitory effects on ERK-independent pathways which regulate cell cycle events beyond S phase.

Previous studies using anastellin have shown that it binds to fibronectin and can either promote or inhibit the deposition of fibronectin into extracellular matrix (9, 28). Under the conditions of our experiments, we have found that anastellin had little effect on the levels of fibronectin present in the extracellular matrix. However, we have recently reported that anastellin binds directly to matrix fibronectin and alters its conformation (29). Changes in the conformation of the preestablished matrix may be one possible mechanism whereby anastellin can affect cell growth. Previous studies have indicated that changes in the organization of the fibronectin matrix can affect cell cycle progression (11). Alternatively, anastellin may bind directly to
cell surface receptors and modulate intracellular signaling pathways. Earlier studies have shown that anastellin can bind to the α5β1 fibronectin receptor and support adhesion (52). However, microvesSEL cells were able to adhere and spread normally on fibronectin in the presence of anastellin, suggesting that anastellin was not interfering with integrin binding to fibronectin. Effects of anastellin on the level of phosphorylated ERK were not accompanied by changes in integrin clustering, actin stress fibers or FAK phosphorylation, indicating that the anastellin-induced decrease in basal levels of active ERK did not result from changes in cell adhesion.

Very recent reports have shown that the matrix-derived inhibitors of angiogenesis, endostatin and tumstatin, work through distinct integrin receptors to block endothelial cell migration and promote apoptosis, respectively (53, 54). One possible interpretation is that a generic mechanism of action of these peptides may be to disrupt matrix-derived signals which regulate proliferation, migration, and survival (53, 55). Indeed, we have recently shown that in dermal fibroblasts, anastellin can induce conformational changes in matrix fibronectin which are accompanied by the activation of p38 (29). The α5β1 fibronectin integrin receptor has been shown to signal p38 (56), PI3 kinase, and ERK (57), raising the possibility that anastellin-induced conformational changes in matrix fibronectin may act to modulate integrin-mediated signaling events without causing any disruption in integrin-dependent adhesion. Taken together, the results reported here suggest that the antiangiogenic properties of anastellin observed in mouse models of human tumors may be due, in part, to a cell cycle block at G1-S, subsequent to the inhibition of ERK-dependent cell cycle gene expression. Further studies should provide a better understanding of the molecular basis linking the regulation of ERK signaling pathways to the antiproliferative activity of anastellin. Mapping these pathways will aid in the identification of target molecules that can modulate tumor angiogenesis.

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Anastellin, a Fragment of the First Type III Repeat of Fibronectin, Inhibits Extracellular Signal-Regulated Kinase and Causes G₁ Arrest in Human Microvessel Endothelial Cells

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