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.5B-RaER, 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 antiangiogenic 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 \textit{in vivo} may be related to its ability to block microvascular 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 (Ser218/222) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-CD146 monoclonal antibody (clone P1H12) was obtained from Chemicon International, Inc. (Temecula, CA). Monoclonal antibody to phospho-ERK1/2 was obtained from Cell Signaling Technology (Beverly, MA). Nitrogen-100 (type 1 collagen) was from Cohesion Technologies (Palo Alto, CA).

**Cell Culture.** Primary adult human dermal microvascular endothelial cells were obtained from VEC Technologies, Inc. (Rensselaer, NY). Endothelial cells were maintained in MCDB-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 µg/ml heparin and cultured in a humidified incubator at 37°C in an atmosphere of 5% CO\(_2\) on collagen-coated (20 µg/ml) glass cover slips in the presence of complete medium and allowed to adhere overnight. Cells were serum-starved for 24 hours, treated with 20 µM b Raf:ER for 24 hours in serum-free MCDB-131, then treated for 1 hour with 1 µM 4-hydroxymisonoxifen followed by an additional 1 hour with 20 µM IC\(_{13}\) or IC\(_{22}\) 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 Ecoscent 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 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 \(
\begin{align*}
\beta_1, \beta_3, \\alpha_2
\end{align*}
\) integrin subunits. F-actin was visualized with Alexa Fluor 594-conjugated phalloidin (Molecular Probes, Eugene, OR). Cell layers were examined using an Olympus BXM-60 microscope equipped with a cooled CCD 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 on 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 μg/mL) glass cover slips in complete medium (24-well tissue culture dishes). After 4 hours, the medium was replaced with fresh medium containing either IIIC (20 μM) 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 DNase I 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 μg/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 μg/mL) tissue culture dishes in complete medium and maintained in a humidified incubator at 37°C and 5% CO2. After 4 hours, the medium was replaced with fresh medium containing either IIIC (20 μM) 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). Cells 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 (IIIC) Inhibits Microvascular Endothelial Cell Proliferation.** In order to investigate the effect of IIIC 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 IIIC or IIION. The IIION fragment used as control in this experiment is derived from the amino terminus of the tenth type III homology repeat of fibronectin (IIION) 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 IIIC peptide, the rate of endothelial cell growth was significantly decreased. Endothelial cell growth was essentially blocked at 20 μM IIIC. Incubation with the control fragment, IIION, had only a slight effect on endothelial cell growth. As shown in Fig 1B, the effects of IIIC were dose-dependent with half-maximal inhibition seen at approximately 10 μM IIIC. Similar results were also obtained with large vessel endothelial cells and bovine lung microvessel endothelial cells (data not shown). In contrast, IIIC 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 IIIC on endothelial cell growth was associated with an increase in apoptosis, microvessel cells were cultured in complete medium in the presence of 20 μM IIIC 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 IIIC or staurosporine treatment was then compared with that of control cells (C, Fig 2B). IIIC 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 DNase I 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 μM IIIC for 2 days or
induce apoptosis in adherent microvessel endothelial cells. Serum-starved microvessel endothelial cells were treated for its effect on the levels of cyclin D1, cyclin A, cdk4, and p21cip1, and p27kip1. Immunoblot analysis indicated that in the absence of III1C treatment, the addition of 10% serum to induce a mitogenic response. 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 48 hours in the presence (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.

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). Anastellin causes G1 modulation of F-actin. Under conditions shown to decrease expression of cell cycle proteins and prevent 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.

Figure 2. The effect of anastellin on apoptosis of adherent microvessel endothelial cells. Human dermal microvessel endothelial cells were plated on collagen-coated glass cover slips 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 (III1C) or absence (C) of 20 μM III1C, or 24 hours with 100 nmol/L staurosporine, were assayed for caspase-3 activity 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 (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, III13C. 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 (Fig. 5C), 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-Raf:ER 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-Raf:ER (30, 43). Control cells were infected with an adenoviral construct containing the gene for GFP. Infected cells were plated on collagen-coated tissue culture dishes (1 × 105 cells/mL) in complete 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 α5, β3, 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 IIIc 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, IIIc 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 IIIc-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
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). Anastellin has been 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

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**Figure 6.** \(\Delta B\)-Raf:ER rescues G1-S progression but not proliferation in anastellin-treated microvessel cells. Adherent microvessel endothelial cells were infected with either Ad.GFP or Ad.\(\Delta B\)-Raf:ER under serum-free conditions for 24 hours. Ad.\(\Delta B\)-Raf:ER–infected cells were incubated in the presence or absence of 1 \(\mu\)M 4-hydroxytamoxifen for 1 hour. Following a 1-hour treatment with 20 \(\mu\)M III\(_1\)C, cells were stimulated with 10% serum for an additional 24 hours. Cell lysates were prepared and immunoblotted with phosphospecific antibodies to ERK and MEK (A) or antibodies to cyclin D1, cyclin A and cdk4 (B). Similarly, Ad.\(\Delta B\)-Raf:ER–infected cells treated with 1 \(\mu\)M 4-hydroxytamoxifen were treated with 20 \(\mu\)M III\(_1\)C for 1 hour, stimulated with 10% serum for 16 hours, and then assayed for \[^{3}H\]-thymidine incorporation (C). Alternatively, Ad.\(\Delta B\)-Raf:ER–infected cells treated with 4-hydroxytamoxifen were cultured in complete media in the presence of 20 \(\mu\)M III\(_1\)C for 5 to 7 days and assayed for endothelial cell proliferation (D). Values were normalized to those obtained for control cells in the absence of III\(_1\)C.
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.1 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 angiogenic 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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References

31. Krumain L, Guo Q, Mattson MP. Calcium and reactive


40. Balbmann K, Cook SJ. Sustained MAP kinase activation is required for the expression of cyclin D1, p21Cip1 and a subset of AP-1 proteins in CCL39 cells. Oncogene 1999;18:3085–97.


42. Boehler RM, Scharf E, Assoian RK. Cytoskeletal integrity is required throughout the mitogen stimulation phase of the cell cycle and mediates the anchorage-dependent expression of cyclin D1. Mol Biol Cell 1996;7:101–11.


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