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FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

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GRADE - DEGREE

Department of Biochemistry, Microbiology and Immunology

FACULTÉ, ÉCOLE, DÉPARTEMENT - FACULTY, SCHOOL, DEPARTMENT

TITRE DE LA THÈSE - TITLE OF THE THESIS

Mechanism for the Inhibition of Angiogenesis by Endostatin

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DEAN OF THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

Mechanism for the Inhibition of Angiogenesis by Endostatin

Brodie T. Weagant

Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfillment of the requirements for the degree of Masters of Science

University of Ottawa
Ottawa, Ontario, Canada
2004

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ISBN: 0-494-01639-6
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ISBN: 0-494-01639-6

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Abstract

Angiogenesis, the formation of new blood vessels from pre-existing ones, is important in wound healing, pregnancy, tumour growth and metastases. This process involves the proliferation and migration of endothelial cells as new capillaries are formed. Endothelial cells rely on signals from surrounding cells and the extra-cellular matrix (ECM) for their growth, survival and migration. These signals are mediated in part by integrins, which are transmembrane heterodimeric proteins that link the cell to the ECM. Additionally the ECM may contribute components that are inhibitory to angiogenesis. This project focused on one of the anti-angiogenic molecules endostatin, which is a naturally occurring inhibitor of angiogenesis that works exclusively on endothelial cells. As endostatin is a cleavage product of the ECM protein collagen XVIII, there was a strong possibility that it may interfere with integrin signaling and function resulting in the inhibition of angiogenesis. It is also possible that the mechanism by which endostatin inhibits angiogenesis may be affected by the presence of different ECM proteins and thus different cellular signals via integrins. This study therefore sought to elucidate the mechanism of inhibition of angiogenesis by endostatin, specifically its effects on the integrin signal transduction pathway, and the subsequent effects of tumor versus normal ECM proteins on this mechanism.

In this study we sought to demonstrate the effect of endostatin on endothelial cell adhesion, proliferation, survival and migration in the presence of various ECM proteins. We found that endostatin was able to decrease VEGF-induced adhesion of endothelial cells to a variety of matrices including laminin, collagen IV, fibronectin, collagen I, tenascin-c and vitronectin. Endostatin was also able to inhibit the proliferation of

endothelial cells however, this function was more dependent on the ECM proteins upon which the endothelial cells were cultured with the strongest inhibitory effects noted following growth on plastic and the weakest inhibitory effects observed following growth on collagen I or tenascin C which are both tumor associated ECM proteins. We also showed that endostatin did not induce apoptosis of VEGF-treated endothelial cells and in parallel showed that endostatin did not reduce the VEGF-induced activation of the survival protein Akt. Migration of endothelial cells on gelatin, which is derived from the tumour matrix collagen I, in response to VEGF was decreased by addition of endostatin as determined by chemotaxis assays. These data suggested that one of the primary mechanisms of inhibition of angiogenesis by endostatin may be through its ability to inhibit endothelial cell adhesion and migration, both of which are processes that involve signaling via the integrin pathway. To test this possibility, the intracellular tyrosine kinase focal adhesion kinase (FAK) was examined. As the levels of total FAK in the cell did not change as a result of treatment with endostatin we examined the phosphorylation status of FAK using phospho-specific antibodies as it has been demonstrated that phosphorylation of certain sites are associated with FAK activity and signaling. We observed that FAK had decreased phosphorylation at its VEGF responsive site, Y861, after the addition of endostatin when endothelial cells were cultured on tumour-associated matrices. In parallel we did not observe any significant changes in FAK phosphorylation at Y397 which is the autophosphorylated site of the kinase and is associated with its activation. Thus the effects of endostatin on the adhesion and migration of endothelial cells may possibly act in part by decreasing the phosphorylation of FAK at Y861 resulting in an impairment of the ability of FAK to associate with other

scaffolding proteins in the cell. As mentioned, these effects were influenced by the ECM proteins upon which the endothelial cells were cultured, thus the effectiveness of endostatin as an anti-angiogenic agent may be impaired by the presence of certain tumor associated ECM proteins. These results highlight the importance of understanding the mechanisms by which anti-angiogenic agents function in order to help elucidate the means by which they will be most effective in a therapeutic regimen.

Acknowledgements

I wish to express my appreciation to my supervisor, Dr. Christina Addison, for her guidance, knowledge and endless patience, especially during the writing of this thesis.

I would also like to thank Dr. Michael McBurney for the opportunity to join the team at the Ottawa Regional Cancer Centre.

To Christie for her technical expertise and direction in the lab.

To Alenko and Chris for helping me to keep a constant level of insanity while working.

To everyone on the third floor who together know something about everything.

And especially to Lois, Clive, Trevor and Carrie and my family for their continual support and motivation.

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Introduction

Angiogenesis is the outgrowth of new vasculature from pre-existing blood vessels. This process is necessary for wound healing, tumour growth and metastases and as well plays a role in a number of other diseases. The balance between pro- and anti-angiogenic molecules is critical in regulating this process and any upset in the system can trigger an angiogenic switch and the growth of new vessels. Progression of a tumour is critically dependent on angiogenesis. A tumour lacking a capillary network cannot grow beyond $1mm^3$ without a blood supply of its own to bring oxygen and nutrients and remove waste by-products. This stage of tumour will be in equilibrium between proliferation and cell death and must pass the rate-limiting angiogenic switch for further cancer progression. Therefore, the tumour must release pro-angiogenic molecules such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), or angiopoeitins that induce capillary growth and invasion. Oncogene activation or tumour-suppressor mutations lead to pro-angiogenic gene expression (Thompson et al, 1989). Also, the hypoxic and ischaemic areas in and around a tumour can signal the release of growth factors (Shweiki et al, 1992). The extracellular matrix (ECM) surrounding the vessels must be degraded as well to allow for migration of endothelial cells into the tumour. However, the vasculature of a tumour differs from normal physiological vessels in several aspects. In normal angiogenesis the vessels mature rapidly and form neatly closed circulating networks with tightly associated pericytes while tumour angiogenesis remains active allowing for continued growth of new vasculature that leads to less

organized structures that are dilated, leaky or have blind ends with loosely connected pericytes (recently reviewed by Bergers and Benjamin, 2003).

The physiological process of normal angiogenesis involves several overlapping stages that lead to the seamless production of a functioning vasculature. The course of events in angiogenesis is the formation of new vessels, remodeling and branching, and stabilization. The formation of new vessel sprouts is usually triggered by hypoxia and is often initiated by VEGF activity (Shweiki et al, 1992). Pericytes that are in contact with capillary endothelial cells detach and assist in the guidance of new vessels through degradation of the endothelial basement membrane and surrounding ECM (Nehls et al, 1992). Remodeling and branching of the vasculature occurs to ensure that all cells in a tissue receive the oxygen and nutrients they require. The ECM guides the migration of vessels towards the hypoxic tissue by chemotaxis along increasing concentration gradients of pro-angiogenic factors, such as VEGF and bFGF. These pro-angiogenic factors can be sequestered in the matrix and are liberated following cleavage of matrix molecules by proteases that can also be produced by the tumour. Anti-angiogenic proteins may also be released through cleavage of matrix molecules and in fact many endogenous inhibitors of angiogenesis are matrix fragments such as endostatin, which is produced following cleavage of collagen XVIII (O'Reilly et al, 1997). The migrating endothelial cells begin to form a lumen as they organize into tube-structures. Once the vessels are formed, a new basement membrane is produced and pericytes re-associate with the endothelial cells to stabilize the vessels. The angiopoeitins, Ang1 and Ang2, appear to be implicated in the stabilization of capillaries and along with platelet-derived growth factor (PDGF) may aid in facilitating the communication between endothelial

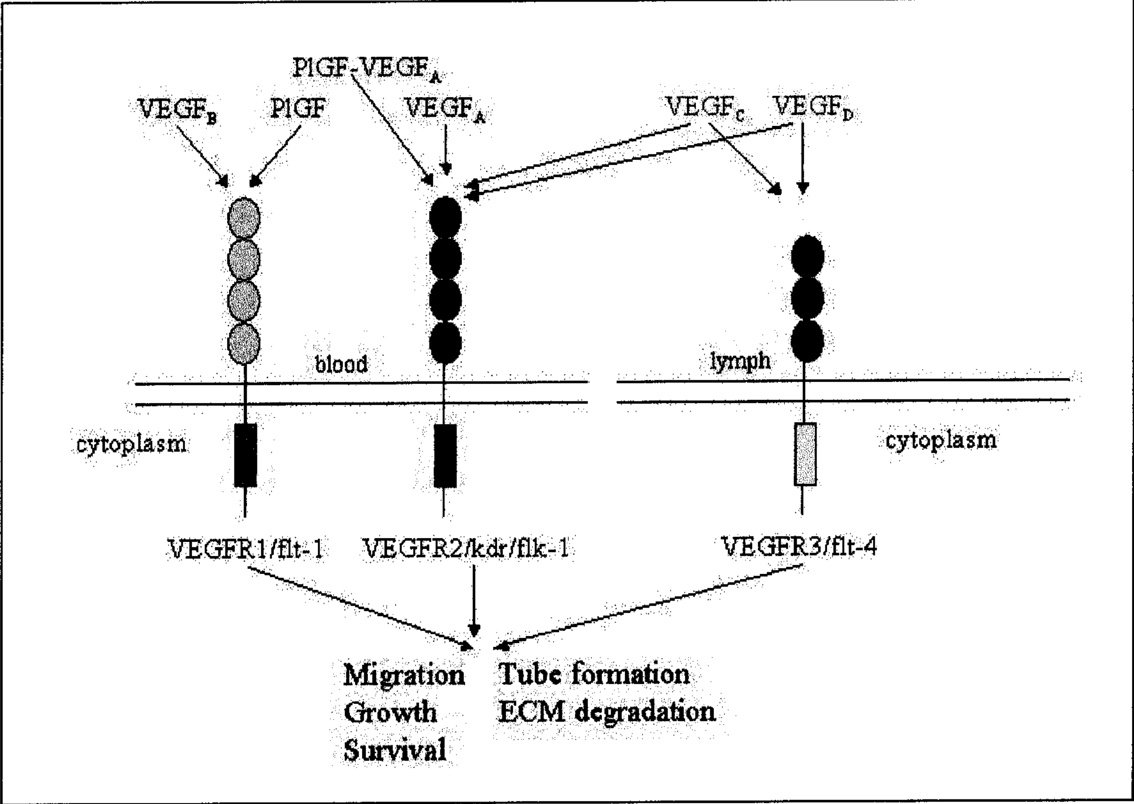
cells and pericytes (Uemura, A. 2002; Loughna (review) 2001). In tumour vasculature, the sequence of events is disrupted and leads to the prolonged existence of immature vessels that function improperly and may decrease the effectiveness of therapeutic drug delivery.

For the endothelial cells of a capillary to migrate towards a growth factor it must first degrade the existing basement membrane and surrounding ECM. This is accomplished using matrix metallo-proteinases (MMP's) and other proteases such as elastase and cathepsins produced by endothelial cells, pericytes and tumour cells (Zucker et al, 1998; Wang and Keiser, 1998; Sato et al, 1992). The basement membrane surrounding a resting capillary is composed primarily of collagen IV and laminin with lesser amounts collagen XV and XVIII. These normal matrices have a growth inhibiting function and serve to hold the capillary in its resting state. When a growth factor is introduced, this normal matrix is degraded and a provisional matrix is deposited. The provisional matrix is composed of ECM components such as fibronectin, collagen I, vitronectin and tenascin-c, which are normally found in a developing embryo, and provide a stimulating environment that increases the proliferative and survival capacity for endothelial cells (Madri and Williams, 1983). This temporary, provisional matrix will eventually be replaced with collagen IV and laminin forming a basal lamina once the vessel is complete and the angiogenic growth factor stimulus is removed. The provisional matrix is produced by endothelial cells that make up the capillary and by fibroblasts/stromal/tumour cells in the tumour microenvironment in response to growth factors. As long as the tumour and stromal cells continue to produce growth factors, more provisional matrix will be supplied and the capillaries will continue to grow and

feed the tumour. The sustained expression of growth factors inhibits a normal basement membrane from being laid down, decreases endothelial/pericyte association and leads to the leaky, disorganized vessels that are characteristic of tumours.

Several proteins can induce an angiogenic switch in tumours such as bFGF, transforming growth factor-β, and angiopoeitins, but the most important factors in controlling the angiogenic response are the VEGF proteins. The VEGF family of proteins (also known as vascular permeability factors) act as potent inducers of angiogenesis and consist of VEGFA-D, the placental growth factor (PlGF) and the orf virus VEGF (VEGFE). The VEGF family proteins form dimers that act as ligands for three receptor tyrosine kinases termed VEGFR1/flt-1, VEGFR2/kdr/flk-1 and VEGFR3/flt-4 that influence growth, survival, and migration of endothelial cells (see figure 1.). VEGFR1 and 2 are expressed on cells of the blood vasculature while VEGFR3 is found on endothelial cells of the lymphatic system. The most common isoform of the VEGF ligands is VEGFA, which acts as a potent activator of blood vessel angiogenesis through its major mitogenic receptor, VEGFR2. VEGFA is found as several splice variants including $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, or $VEGF_{206}$ named after the number of amino acids each contains (Houck et al, 1991; Poltorak et al, 1997). $VEGF_{121}$ is freely diffusible while $VEGF_{189}$ and $VEGF_{206}$ are highly basic and become tightly bound by extracellular heparin-containing proteoglycans. $VEGF_{145}$ is the most rare VEGF isoform and little is know about its activity. $VEGF_{165}$ has properties in between $VEGF_{121}$ and $VEGF_{189}$/ $VEGF_{206}$ and is the most active and abundant of the isoforms. VEGFB is less abundant and is specific to VEGFR1. VEGFC and VEGFD bind VEGFR3 and influence endothelial cells of the lymphatic vascular system but can

Figure 1. The VEGF family of proteins and their receptors.



PlGF-VEGF$_A$
VEGF$_B$
PlGF
VEGF$_A$
VEGF$_C$
VEGF$_D$
blood
lymph
cytoplasm
cytoplasm
VEGFR1/flt-1
VEGFR2/kdr/flk-1
VEGFR3/flt-4
Migration
Growth
Survival
Tube formation
ECM degradation

also impact the blood vasculature by binding to VEGFR2. PlGF binds to VEGFR1 but is capable of forming a heterodimer with VEGFA that can then bind VEGFR2 although it is less mitogenic than the VEGFA homodimer. The activity of VEGF on endothelial cells controls ECM degradation, migration, tube formation, growth and survival of endothelial cells once an angiogenic switch is initiated. Growth factors and cytokines such as epidermal growth factor, transforming growth factor-beta1 and interleukin-1β can induce transcription of VEGF mRNA (Frank et al, 1995; Li et al, 1995). Hypoxia can also upregulate VEGF expression by increasing the transcription and stabilization of VEGF mRNA (Shweiki et al, 1992). Some oncogenes, for example ras and erbB2, are capable of increasing VEGF production as well (Rak et al, 1995). VEGF is both necessary and sufficient for the induction and maintenance of angiogenesis. Notably, many of the factors that up-regulate VEGF expression are induced by tumours such as inflammatory mediators, hypoxia and oncogene activation making VEGF an important tumour angiogenic factor.

In addition to angiogenic factors that promote tumour angiogenesis, there are a number of endogenous inhibitors that may also regulate this process. Endostatin is a 20 kDa C-terminal fragment of collagen XVIII that has been shown to act as an inhibitor of angiogenesis in vivo and appears to work almost exclusively on endothelial cells (O'Reilly et al, 1997). Endostatin is a cleavage product of the non-collagenase (NC) domain of collagen XVIII. Cleavage of collagen XVIII by matrix metalloproteinase 9 (MMP9), elastase, and cathepsin L at a protease sensitive hinge releases endostatin, which accumulates in normal human tissue extracts and blood at concentrations of 20-35ng/ml (Wen et al, 1999). Endostatin may function by affecting migration, attachment,

proliferation or survival of endothelial cells through the binding of molecules such as heparan sulfate proteoglycans, VEGFR2 or integrins ($\alpha5\beta1$), although the signaling mechanism for endostatin leading to its inhibitory effects on endothelial cells is still not clear (Kreuger et al, 2002; Kim et al, 2002; Sudhakar et al, 2003). Endostatin reportedly binds and inactivates matrix metalloproteinases (MMPs), which would aid in decreasing migration and invasion of endothelial cells (Lee at al, 2002). The binding of endostatin to integrins could affect one or all of these processes and lead to inhibition of angiogenesis. The extracellular matrix protein laminin has been observed to bind endostatin and regulate its anti-angiogenic activity by sequestering it and holding it away from cells (as reviewed by Kalluri, 2003). Recombinant human endostatin went through phase 1 trials with limited success but is currently being tested in phase 2/3 clinical trials for cancer therapy.

Current literature on the mechanism of angiostatic action of endostatin has generated many new insights and some contradictory findings since its discovery. Several molecules have been speculated to be a receptor for endostatin such as the $\alpha5\beta1$ integrin, heparan sulfate proteoglycans, caveolin-1 and VEGFR2 but no internal signaling pathways have been clearly identified to corroborate these observations (Sasaki et al, 1999; Kim et al, 2002; Wickstrom et al, 2002). Interestingly, the pre-incubation of endostatin with endothelial cells seems to confer resistance to FAK tyrosine phosphorylation by VEGF stimulation (Kim et al, 2002). This is of note as FAK phosphorylation is necessary for cytoskeletal changes and cell migration that are required during the angiogenic process (Romer et al, 1994). The effect of endostatin on the apoptotic pathway seems to be of some discussion. Several publications have shown that

endostatin can induce cell death while another suggests that endostatin does not play a role in apoptosis. Phase I clinical trials of endostatin and other anti-angiogenic therapies have shown that this type of treatment is remarkably nontoxic. In fact, endostatin may help in protecting endothelial cells from injury (Ren et al, 2002). One situation had noted that upon addition of the angiogenic inhibitor the vessels that were present in the tumour took on a more structured appearance that lasted several days before a marked inhibition of angiogenesis was observed. Since the discovery of endostatin many other cryptic matrix proteins and peptides have been added to the array of molecules that act as angiostatic molecules.

Cells communicate with the surrounding ECM through a family of membrane bound proteins called integrins. Integrins are hetero-dimeric transmembrane glycoproteins responsible for cellular attachment by physically linking the ECM to the cytoskeleton. Integrins also relay signals to the cell from the ECM through cytosolic kinases such as focal adhesion kinase (FAK) and integrin-linked kinase (ILK), in a process known as outside-in signaling. These kinases in turn interact with other molecules such as Src, PI3-K, Grb2, Grb7, Cas, and paxillin to name a few (as reviewed in Liu et al, 2002). There are currently 18 known alpha subunits and 8 known beta subunits and approximately 24 known combinations of integrin hetero-dimers. Each arrangement can have one or more recognition sites and may bind multiple ECM components (see table 1.). Integrins attach to the matrix in clusters forming a focal adhesion that leads to the congregation of signaling molecules and adaptor proteins inside the cell to affect migration, proliferation, differentiation and survival. Integrins induce the activation of small Rho-like GTPases such as Rho, Rac and Cdc42 that control the

Table 1. Integrin receptors and their cognate ECM ligands.

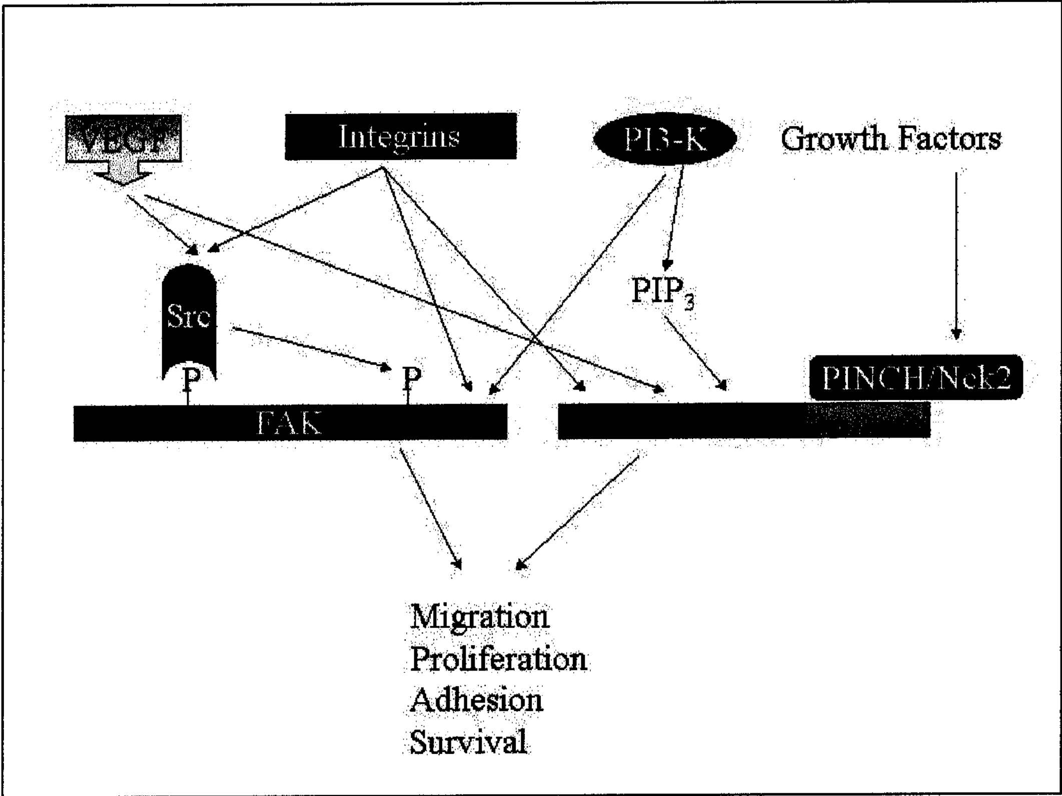
Integrin	ECM Ligands
$\alpha 1\beta 1$	collagen I, collagen IV, laminin
$\alpha 2\beta 1$	collagen I, laminin, fibronectin
$\alpha 5\beta 1$	Fibronectin
$\alpha 6\beta 1$	Laminin
$\alpha 8\beta 1$	fibronectin, tenascin
$\alpha v\beta 3$	fibronectin, vitronectin, fibrinogen, vWF, TSP-1,
$\alpha v\beta 5$	vitronectin
$\alpha v\beta 6$	fibronectin, tenascin

complex procedure of migration. Rac regulates the polymerization of actin at the leading edge of the cell. Rho appears to act at the rear of the cell where it directs the contractile actin:myosin filaments and the disassembly of focal contacts that pull the rear of the cell forward. Cdc42 is not necessary for migration but is important in giving the cell polarity and directing its migratory path. Under the control of these GTPases, integrin clusters gather at the leading edge of the cell and detach at the rear allowing the cell to migrate in the direction of a stimulus such as VEGF in a process known as inside-out signaling. Inside-out signaling involves conformational changes in the integrin extracellular domains that alter their affinity for the ECM to allow for matrix attachment or detachment (Sims et al, 1991). Integrin engagement also sends signals to the nucleus through MAP-Kinase pathways such as extracellular-signal regulated kinase (ERK), so that the cell can continue to proliferate or differentiate. A single cell can express several different combinations of integrin hetero-dimers and a cell with an unattached integrin will recognize that it is incorrectly located and can induce apoptosis (Stupack et al, 2001). There are currently some activity-blocking integrin antibodies and small molecule inhibitors of integrins involved in clinical trials for cancer therapy. An anti-$\alpha v\beta 3$ antibody has been shown to inhibit endothelial-specific integrin survival signaling and a small peptide antagonist of $\alpha 5\beta 1$ has shown anti-tumour activity by blocking migration of endothelial cells and invasion of tumour cells on fibronectin (Stoeltzing et al, 2003). These results highlight the importance of integrin and ECM interaction in tumour-angiogenesis.

A key transducer of integrin signaling is the non-receptor tyrosine kinase called focal adhesion kinase (FAK), which has been shown to be activated by beta integrin chains

following their binding to ECM. The activity of FAK itself is regulated by tyrosine and serine phosphorylation. Cellular attachment to the ECM induces integrin-dependent FAK autophosphorylation at tyrosine 397 (Y397), which subsequently recruits and activates Src that binds to FAK Y397 through its SH2 domain (see figure 2). Upon binding to FAK, a conformational change in Src allows Src's negative regulatory tyrosine (Y527) to become dephosphorylated, which then allows further activation via autophosphorylation at Y416 of Src (Xu et al, 1999). Src may then phosphorylate other tyrosines on FAK such as tyrosine 861 (Y861), which is phosphorylated upon VEGF stimulation of Src by an unknown mechanism (Abu-Ghazaleh et al, 2001; Calalb et al, 1996). Other sites of Src-dependent tyrosine phosphorylation on FAK include Y407, Y576, Y577, and Y925. Phosphorylation of tyrosine 925, in the focal adhesion targeting (FAT) domain, is required for Grb2 binding through its SH2 domain and links integrin stimulation of FAK to the ERK pathway (Schlaepfer et al, 1994). Tyrosines 576 and 577 are located in the kinase domain and phosphorylation at these sites appears to be necessary for maximal catalytic activity of FAK (Calalb et al, 1995). Less is known about the role of phosphorylated Y407 of FAK and no interactions of cellular signaling molecules have been observed with Y407 or Y861. Alternatively, when entering the cell cycle, FAK becomes phosphorylated on serines (S722, S843, S845 and S922) with a concomitant dephosphorylation of tyrosines that causes integrins to detach from the ECM so the cell may proceed through the cell cycle and divide (Ma et al, 2001). FAK also contains two proline-rich regions capable of binding proteins with an SH3 domain such as p130 Cas that connects FAK to the Rho GTPase/migration pathway (Kiyokawa et al, 1998). The c-terminal FAT domain of FAK has been shown to bind the adaptor proteins talin and paxillin and helps FAK localize to

Figure 2. The focal adhesion proteins FAK and ILK can be activated by several of the same proteins.


Integrins
PI3-K
Growth Factors
Src
PIP_3
P
P
PINCH/Nck2
FAK
Migration
Proliferation
Adhesion
Survival

focal adhesions. With numerous binding sites and regulatory domains, FAK is central to the downstream signaling cascade from integrins.

Another kinase and adaptor protein that is central to integrin signaling is integrin linked kinase (ILK). ILK is a 59 kDa cytoplasmic serine/threonine protein kinase with multiple binding domains including a pleckstrin homology motif and four ankyrin repeats. ILK is thought to phosphorylate Akt, which leads to Akt's activation and inhibition of suspension-induced cell death, termed anoikis. Other proteins phosphorylated by ILK include integrin β1, GSK-3 and affixin (Hannigan et al, 1996, Delcommenne et al, 1998, Yamaji et al, 2001). ILK's interaction with and phosphorylation of the β1 integrin leads to the localization of the integrin with focal adhesions (Mulrooney et al, 2000). The activation of integrin β1 is induced by growth factor stimulation and adhesion to ECM such as fibronectin. Phosphorylation of GSK-3 leads to its inhibition and allows for the stabilization of β-catenin and activation of the transcription factor AP-1 (Troussard et al, 1999). β-catenin stabilization induces the up-regulation of cyclin-D1 which results in progression through the cell cycle, while the transcription factor AP-1 can induce expression of MMP-9, which results in an ILK-induced increase in tumour migration and invasion making a more aggressive tumour. Affixin is an actin interacting protein that when phosphorylated by ILK reduces cell spreading. ILK binds other actin interacting proteins such as paxillin and the calponin-homology-ILK binding protein (CH-ILKBP) that allow for a direct link between integrins and the actin cytoskeleton through ILK. The interaction of ILK by its N-terminal ankyrin repeat to the LIM-only domain adaptor protein PINCH links ILK to Nck-2 and the growth factor receptor pathway as Nck-2 has been shown to interact with EGFR and PDGFR (Tu et al, 1999, Tu et al, 1998). Further, ILK

activation increases with the binding of the PI3-K product phosphoinositide 3,4,5,triphosphate (PIP3) to the pleckstrin homology motif implicating PI3-K in ILK's activation. The tumour-suppressor protein PTEN, which is capable of dephosphorylating PIP3, can decrease ILK's activity. Recently, an association between VEGF and ILK has been established. An increase in the expression of VEGF was observed in an ILK-Akt-HIF-1α dependent manner and inhibition of ILK expression blocked VEGF-induced endothelial migration and tube formation (Tan et al, 2004, Kaneko et al, 2004). Hence, ILK's many binding partners and signaling pathways are crucial to cell homeostasis and accordingly ILK has been observed to be upregulated in certain tumours and its constitutive activation, mostly by the suppression of PTEN, can induce oncogenic transformation of cells. ILK is also involved in tumour angiogenesis through interaction with integrins and regulation of and by VEGF in endothelial cells making ILK a likely candidate for endostatin's anti-angiogenic activity.

Other proteins that may be affected by endostatin-induced integrin signaling are phospho-inositide 3' kinase (PI3-K) and protein kinase B (Akt/PKB). PI3-K was first thought to be a pro-survival protein with its main effector being Akt. Recently it has been shown that PI3-K can influence cell cycle progression as well by stabilizing cyclin D1 mRNA, making it an attractive target as a chemotherapeutic (Dufourny et al, 2000). Two subunits make up the PI3-K protein: a regulatory subunit (p85), which is controlled by a GTPase-responsive domain and serine phosphorylation, and a catalytic subunit (p110) (Carpenter et al, 1990). PI3-K, as its name suggests, phosphorylates phosphoinositide 4,5 diphosphate (PIP2) to phosphoinositide 3,4,5 triphosphate (PIP3) upon growth factor stimulation, VEGF for example, or integrin engagement (Plopper et al, 1995, Guo et al,

1995). PIP3 binds to pleckstrin homology domains on proteins such as PDK-1, ILK and Akt aiding in their activation by recruiting them to the plasma membrane where they interact with other proteins (Alessi et al, 1997; Stokoe et al, 1997; Delcommenne et al, 1998). The lipid products of the PI3-K pathway regulate Akt by phosphorylation of two amino acid sites, T308 and S473 which both need to be phosphorylated for the protein to be active (Scheid and Woodgett, 2003). Akt is a pro-survival protein that has many downstream effectors including Bad, pro-caspase 9, I-κB kinase (IKK), the forkhead family of transcription factors (FKHR, AFX, FOX), GSK-3, $p21^{cip1}$, and Raf (Chang et al, 2003). Some of these protein targets become inactivated when phosphorylated by Akt such as Bad, caspase-9 and GSK-3 resulting in the inhibition of apoptosis (Datta et al, 1997; Cross et al, 1995). As mentioned above, Akt is involved in inducing the expression of VEGF and in turn, VEGF can increase the activity of Akt leading to enhanced endothelial cell survival (Mazure et al, 1997; Gerber et al, 1998).

As there are growth factors that induce angiogenesis there are also proteins that inhibit angiogenesis such as endostatin, thrombospondin, angiostatin and tumstatin. Inhibition of angiogenesis has recently become the focus for some novel therapies but there has been limited success of these agents in clinical trials although they appear to be surprisingly nontoxic. More than likely this was due to the testing of these agents in patients with end-stage cancer in early clinical trials. Using a Rip1-Tag2 mouse model for multi-stage disease, several angiogenic inhibitors including endostatin were tested for their efficacy on different stages in the progression of pancreatic islet cancer (Bergers et al, 1999). It was discovered that endostatin had its greatest effects on prevention of the angiogenic switch before the initiation of solid tumours and on the regression of small

tumours. Endostatin did not induce the regression of end-stage tumours and only slightly delayed growth of these tumours. The composition of the ECM surrounding the tumour may also play a role in blocking the anti-angiogenic proteins by providing a growth and survival-inducing environment. For example, the basement membrane components collagen IV and laminin were found to be decreased in breast, cervical, oral and colorectal tumours and the provisional matrices collagen I, fibronectin, vitronectin and tenascin-c were elevated in breast cancer (Lochter and Bissell, 1995; Noel et al, 1999; Zeng et al, 1999; Nair et al, 1997; Kannan et al, 1994; Tosios et al, 1998). Fibronectin was also increased in cervical and oral carcinomas while tenascin-c was higher in squamous carcinoma and melanoma (Kannan et al, 1994; Ramos et al, 1998; Tuominen and Kallioinen, 1994). Vitronectin was observed to be increased in progressing but decreased in regressing hemangiomas and inhibits microvascular endothelial cell apoptosis (Jang et al, 1998; Isik et al, 1998). Tenascin-C has also been shown to increase endothelial cell sprouting in response to angiogenic factors (Canfield and Schor, 1995). It is clearly evident that the ECM will play a crucial role in altering the effects of angiogenic inhibitors. However, since the mechanism of action of most angiostatic molecules is not known, it is difficult to understand why they are not effective clinically. The mechanism of endostatin in the inhibition of angiogenesis and the effects of tumour-associated ECM on this process were investigated to determine if tumour-associated ECM might be contributing to the failure of these inhibitors in the clinical environment.

The objectives of this project were to investigate the mechanism for inhibition of angiogenesis by the angiostatic molecule endostatin and the effect of tumour associated ECM on these mechanisms by addressing the following questions.

1. What are the effects of endostatin on adhesion of endothelial cells on different ECM's?
2. How is the proliferation of endothelial cells affected on different matrices in the presence or absence of endostatin, with and without VEGF stimulation?
3. What is the role of endostatin in the survival of endothelial cells on different ECM's?
4. What are endostatin's effects on the migration of endothelial cells on tumour and normal matrices?
5. What changes occur in the intra-cellular signaling pathways following treatment with endostatin?

Hypothesis

Endostatin binds directly to integrins and inhibits adhesion, proliferation, migration and survival of endothelial cells by decreasing expression or activity of downstream signaling molecules such as FAK, ILK and Akt and that these responses are altered by the presence of tumour-associated ECM.

Materials and Methods

Cell Culture. Human dermal microvascular endothelial cells (HDMEC), obtained from Cambrex (East Rutherford, NJ), used from passage 4-8, were grown using EGM-2MV (Cambrex) at 37°C with 5% CO_2. Cells are passaged by trypsinization except for the adhesion assays where cells are removed by incubation with ice-cold 5mM EDTA in PBS. Trypsinization was performed following washes in Hanks balanced salt solution, HBSS, (Gibco, Grand Island, NY) using a 0.05% solution of trypsin (Sigma, St. Louis, MO) diluted in HBSS by incubation with endothelial cells at 37°C for approximately 3 minutes or until the cells were lifted off the dish. Cells were then collected and the trypsin was neutralized by addition of a 20% FBS in HBSS solution. A 10ul sample of cells from the suspension was diluted 1:2 in a 4% trypan blue solution and counted on a heamocytometer to calculate the number of cells (cell count/4 x 2 x 10,000 x volume of suspension). HDMEC were centrifuged (Beckman, Fullerton, CA) at 1100 rpm at 4°C for 5 minutes prior to resuspension in EGM-2MV and seeding on new culture ware (VWR, Mississauga, ON).

Antibodies and recombinant proteins. Several antibodies were used in this project. Anti-FAK, anti-phosphoFAK Y397, anti-ILK, and anti-PI3-K were from BD Transduction Laboratories (Palo Alto, CA). Anti-phosphoFAK Y861 and the 2° HRP-conjugated anti-mouse and anti-rabbit antibodies were from Sigma (St. Louis, MO). Anti-Akt, anti-phosphoAkt S473 and rabbit anti-ILK were from Upstate (Lake Placid, NY). The adhesion blocking β1 integrin antibody was from Chemicon (Temecula, CA). The anti-tubulin antibody (E7) was produced by a hybridoma cell line that was given to

the lab by Dr. D.L. Brown. Recombinant $VEGF_{165}$ was purchased from R&D Systems (Minneapolis, MN). Recombinant human endostatin was purchased from Calbiochem San Diego, CA).

ECM coating. Collagen I was used as a fibrillar form that retains its triple alpha helical chain conformation and as a monomeric form that is denatured and only has a single alpha helical chain.

Fibrillar Collagen: 5 parts Collagen, Vitrogen 100 (Cohesion Technologies, Palo Alto, CA) was added to 1 part 10X PBS, 3.4 parts sterile water and 0.6 parts 0.1M NaOH. The collagen solution was adjusted to pH 7.4 ± 0.2 with 0.1M NaOH or 0.1M HCl. The solution was added to dishes or wells and incubated for at least 2 hours at 37°C to allow a collagen gel to form. The gel was washed with HBSS for 5 minutes. The collagen was allowed to equilibrate to EGM-2MV for 2 hours at 37°C followed by seeding with HDMEC.

Monomeric Collagen I: Collagen (Vitrogen 100, Cohesion Technologies, Palo Alto, CA) was diluted in 0.01N HCl to a concentration that resulted in $5ug/cm^2$ of collagen added to each well or dish. Wells were allowed to air-dry overnight in the fume hood and were ready to be seeded the next day.

Tenascin-c: Tenascin-c (Chemicon, Temecula, CA) was diluted to a concentration of 0.1ug/mL in PBS and added to wells or dishes to cover the bottom. Plates were then incubated overnight at 4°C. Wells were blocked the next day with 0.1% casein for 1 hour followed by three washes in PBS prior to seeding with HDMEC.

Fibronectin and Collagen IV: Fibronectin (Invitrogen, Burlington, ON) or Collagen IV (Rockland, Gilbertsville, PA) was diluted to a concentration that resulted in $1ug/cm^2$ of

the matrix added to each dish or well. Plates were incubated at room temperature for at least one hour. Following incubation, the plates were washed once with sterile distilled water before seeding with HDMEC.

Laminin: Laminin (Sigma, St. Louis, MO) was diluted to 1ug/cm^2 of laminin in PBS and added to each dish or well followed by air-drying overnight in a fume hood. HDMEC were seeded the next day.

Vitronectin: Vitronectin (Sigma, St. Louis, MO) was diluted in PBS to 100ng/cm^2 and added to each dish or well. Plates were then incubated for 1-2 hours at 37°C. Plates were washed with PBS and seeded with HDMEC.

Chemotaxis membranes (Neuro Probe, Gaithersburg, MD) were incubated in an acetic acid solution for 2 hours prior to coating with 1% gelatin in PBS. After 24 hours in gelatin, membranes were dried between pieces of Whatman filter paper until ready to be used.

Western blotting. Following seeding and stimulation for various periods of time, HDMEC were scraped and lysed in FRAK's buffer (10 mM tris pH 7.5, 150mM NaCl, 5mM EDTA, 1% Triton X-100) with protease and phosphatase inhibitors (0.2mM sodium orthovanadate, 2mM NaF, 2mM sodium pyrophosphate, 0.2mM phenylmethylsulfonylfluoride, 500uM ammonium vanadate, 2ug/ml aprotinin, 5ug/ml leupeptin). Lysates were collected in microfuge tubes and passed through a 26-gauge needle several times followed by centrifugation to remove insoluble material. Protein concentration was determined using the BCA (bicinchoninic acid) kit according to the manufacturer's directions (Pierce, Rockford, IL). 8-20 ug of total protein was diluted in SDS-PAGE loading buffer (125mM tris, 4% SDS, 30% glycerol, 2% β-mercaptoethanol)

and loaded on 10% poly-acrylamide gels and subjected to electrophoresis at 120V followed by transfer to nitrocellulose membranes (Bio-Rad, Hercules, CA) at 35V overnight at 4°C. Membranes were blocked for 1 hour using 5% milk in TBST (10mM tris, 150mM NaCl, 0.75% Tween-20, 5% milk). Primary antibodies were diluted in 5% milk in TBST for 1 hour to overnight. Membranes were washed 5 times for 5 minutes each time with TBST prior to incubation with secondary antibody. Secondary antibodies were incubated for one-two hours followed by washing 5 times by 5 minutes each time with TBST. Membranes were incubated with an enhanced chemiluminescence solution (Supersignal, Pierce, Rockford, IL) for 5 min and exposed to Kodak X-Omat film.

Immunoprecipitation. HDMEC were lysed and collected as above. 100ug of total protein was diluted up to 500 ul in FRAK's buffer and incubated with a primary antibody for 2 hours to overnight at 4°C with rotation. 20ul of a 50% slurry of protein G coated sepharose beads (Amersham, Uppsala, Sweden), diluted in FRAK's buffer, was added to the protein/antibody solution and rotated at 4°C for an additional 1-2 hours. Beads were then spun down and washed with FRAK's buffer 4 times. The beads were resuspended in 20 ul of SDS-PAGE loading buffer and loaded on a 10% polyacrylamide gel and subjected to western blot analysis as described above.

Adhesion Assay. HDMEC were removed by incubation with ice-cold 5mM EDTA in PBS so that membrane bound proteins (integrins) remain undisturbed. Cells were centrifuged at 1,200 rpm in a Beckman Avanti centrifuge and resuspended at 80,000 cells/ml in MCDB 131 + 1% FBS with either 100, 500 or 2500 ng/ml of endostatin (Calbiochem, San Diego, CA) for 1 hour. 40,000 cells were then seeded on 24 well plates coated with different ECM's. After 45 minutes incubating at 37°C, unattached

cells were washed off by rinsing two times with HBSS. Attached cells were then collected by trypsinization and diluted in an isotonic solution (150mM NaCl, 3.0mM KCl, 15mM Na_2HPO_4) and counted using a Coulter Counter.

Proliferation Assay. 15,000 HDMEC per well were seeded in EGM-2MV in 24 well plates coated with different matrices. After overnight adherence, cells were washed 2 times with PBS (137mM NaCl, 8mM Na_2PO_4, 1.5mM KH_2PO_4, 2.7mM KCl), starved in 1% FBS in MCDB 131 overnight and then stimulated with different doses of endostatin (100, 500, 2500ng/ml) in the presence or absence of 50ng/ml VEGF in MCDB 131 + 5% FBS. After incubation at 37°C in 5% CO_2 for 72 hours, dead cells were removed by washing twice in PBS. Attached cells were collected by trypsinization and diluted in an isotonic solution (as above) followed by counting using a Coulter Counter.

Apoptosis Assay. HDMEC were seeded in EGM-2MV in 60 mm dishes coated with a variety of matrices. Cells were allowed to adhere overnight followed by two washes with HBSS and starving overnight in MCDB 131 + 1% FBS. HDMEC were then stimulated with different concentrations of endostatin (100, 500, 2500ng/ml) with or without 50ng/ml VEGF in MCDB 131 + 5% FBS for 62 hours. After 62 hours the media with floating cells was collected along with two washes and the attached cells were trypsinized and pooled with the media and washes. Cells were kept on ice at all stages. Samples were centrifuged at 1,100rpm for 5 minutes followed by two washes of the pellet with ice-cold PBS. Pellets were resuspended in 70% ethanol and incubated a minimum of 20 hours at -20°C to permeabilize the cells. Cells were then washed 2 times with PBS and resuspended in PBS containing 100ug/mL propidium iodide and 40ug/ml RNAse A, followed by incubation at 4°C for 30 minutes. Samples were then read using a

BD LSR Flow Cytometer on the FL-2 laser and the sub-G1 population was recorded. The sub-G1 population is defined by having less DNA (ie, fluouresence) than a diploid cell and is characteristic of fragmented DNA as seen during apoptosis. Unstimulated cells were analyzed to determine the basal level of apoptosis and the percentage of sub-G1 cells in each sample was compared to the percentage of sub-G1 cells in this population.

Chemotaxis Assay. HDMEC were starved in 1% FBS in MCDB 131 for 2 hours prior to trypsinization and collection as described above. Cells were then diluted in MCDB 131 + 1% FBS at a concentration of $1x10^6$ cells/ml and 28ul of the cell suspension was loaded into the bottom well of a modified 48 well Boyden chemotaxis chamber (Neuro Probe, Gaithersburg, MD). A gelatin coated filter membrane (pore size of 5μm) was placed on the bottom chamber over the cells. A gasket was placed over the membrane and the top of the chamber was fastened on. The chamber was then inverted and incubated at 37°C for 3 hours to allow for cellular attachment to the underside of the membrane. After attachment the chamber was righted and endostatin (100, 500 or 2500 ng/ml), with or without 50ng/ml VEGF, was added to the upper portion of the chamber followed by incubation for another 3 hours at 37°C to allow for migration across the filter. The chamber was then dismantled and cells were scraped off the underside of the membrane leaving only the cells that migrated to the other side. Cells were fixed to the membrane, stained using Difquick (Fisher, Fairlawn, NJ) and dried overnight. The next day ten random fields of view were counted for each condition at 200X magnification.

Wounding Assay. HDMEC were grown to confluency in six well plates on glass coverslips in EGM-2MV. Using a P1000 pipette tip, a uniform scrape was made through

the cell monolayer. Scraped cells were washed off using HBSS and 500ng/ml endostatin with or without 50ng/ml VEGF in MCDB 131 + 5% FBS was added. Cells were washed with PBS and fixed with 4% paraformaldehyde (Sigma, St. Louis, MO) in PBS for 10 minutes at time points ranging from 8 hours to 72 hours. After fixation, cells were washed twice with PBS and stored at 4°C until ready to be stained. Staining was performed with Hoescht (bis-benzamide) and TRITC-conjugated phalloidin (both from Sigma, St. Louis, MO) to visualize the nucleus and actin cytoskeleton, respectively, of the migrated cells under a fluorescent microscope. Staining was performed as follows: Following fixation, nonspecific protein binding sites were then saturated with 1% bovine serum albumin (BSA) plus 0.5% tween-20 in PBS for 20 min. The cells were then washed with PBS, and incubated with a 1:1000 dilution of TRITC-conjugated phalloidin and a 1:20,000 dilution of Hoescht. The coverslips were then washed with PBS 2 times for 3 minutes each time and mounted on glass slides using fluorescent mounting media (KPL, Columbia, MD). The fluorescent images were obtained using an epifluorescent microscope.

Statistical Analysis. Statistical analysis was performed using a paired t-test with SigmaPlot 2000

Results

For all experiments, human dermal microvascular endothelial cells (HDMEC) that were isolated from dermal capillaries were used. HDMEC are a primary cell line and have a population doubling time of approximately 32 hours in endothelial growth media (according to the manufacturer's product information). Their normal turnover in the body is hundreds of days, however, it can increase to approximately every five days during active remodeling of vasculature (Folkman and D'Amore, 1996). These cells will more closely imitate the endothelial cells that are recruited by the tumour in its normal environment. Other labs use human umbilical vein endothelial cells (HUVEC) however these are large vessel cells that are probably never recruited by a tumour and are lacking an arterial supply of endothelial cells. Furthermore, biological differences have been noted in the responses of HUVEC and HDMEC (Salcedo et al, 2000). Thus microvascular cells are a more representative cell type that mimic the range of capillary venous and arterial endothelial cells and would be recruited by a tumour in humans. We have thus used these cells as a model system to investigate the role of the ECM in the response of endothelial cells to the angiostatic molecule endostatin. As angiogenesis is a multistep process we have focused our studies on the effects of ECM and endostatin on different steps of this process including adhesion, proliferation, survival and migration of HDMEC. Additionally we have attempted to ascertain the effects of endostatin and ECM on the potential signal transduction pathways activated in cells during angiogenesis and have focused on the effects on the integrin signaling pathway.

1. Effects of endostatin on adhesion of endothelial cells

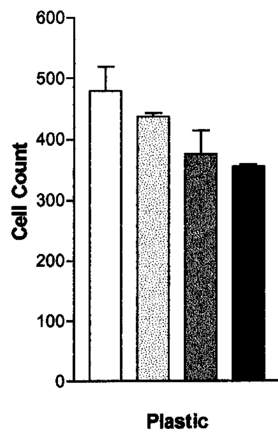
Adhesion of endothelial cells onto ECM requires the binding of cells via integrins. Once bound, the integrins congregate into focal adhesions and signal to the cells that there is a secure attachment. As endostatin has been shown to bind the $\alpha5\beta1$ and $\alpha v\beta3$ integrins, it is possible that its anti-angiogenic activity may be due in part to a decrease in endothelial cell adhesion (Rehn et al, 2001). In fact, endothelial cells are known to die by anoikis (suspension induced apoptosis) following prevention of attachment (Stupack et al, 2001). The effect of endostatin on adhesion of endothelial cells was examined by pre-incubating a suspension of HDMEC (generated with removal by EDTA to maintain surface expression of integrins) with endostatin prior to seeding on a variety of matrices, such as fibrillar collagen I, fibronectin, vitronectin, laminin, tenascin-c, and plastic (no ECM). Fig.3 shows that in the absence of pre-incubation with endostatin, unstimulated control HDMEC showed rates of adhesion on fibronectin, tenascin-c, laminin and collagen I that were all similar to that observed on plastic tissue culture dishes. Interestingly, on vitronectin alone (in unstimulated condition) HDMEC adhesion was reduced by approximately 50% as compared to what was observed on laminin and collagen I, both of which had the most adhesion. Pre-incubation of HDMEC with endostatin decreased the adhesion of endothelial cells on all the matrices tested as compared to control cells incubated in the absence of endostatin with the greatest effects seen on fibronectin and laminin with 18% and 17% decreases, respectively, and the smallest effect noted on collagen-I (10%) (fig.3C, E, F). Endostatin inhibited adhesion by 13% on both of the provisional matrices, vitronectin and tenascin-c (fig.3B, D). The

positive control β1 integrin adhesion-blocking antibody was able to decrease adhesion by 36% on collagen-I, which is known to bind numerous β1 containing integrins (fig.3F). The endostatin induced decrease in adhesion may be due to a direct interference with integrin binding although an internal signaling pathway that modulates integrins through inside-out signaling may also be affected.

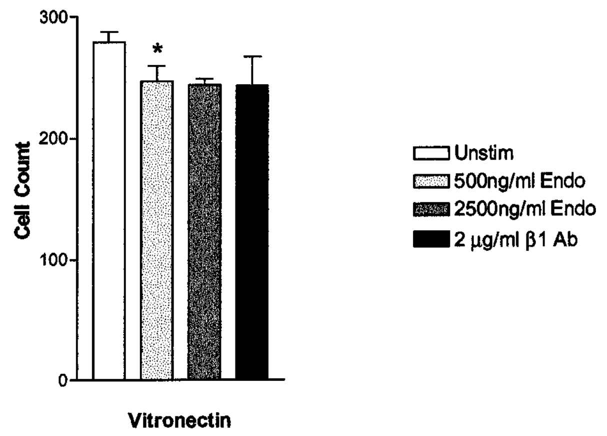
Figure 3. ECM adhesion response of endothelial cells to endostatin. A suspension of HDMEC was incubated with endostatin for 1 hour prior to seeding onto ECM. Cells were allowed 45 min. for attachment after which a Coulter Counter was used to count 4 samples of each condition (n=3). Graphs represent the mean and standard error of triplicate wells. The experiment was performed three independent times with similar results. * represents p-value < 0.05.

Figure 3. Adhesion Assay

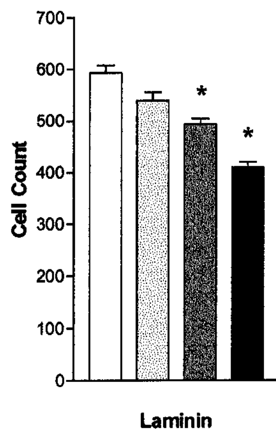
A



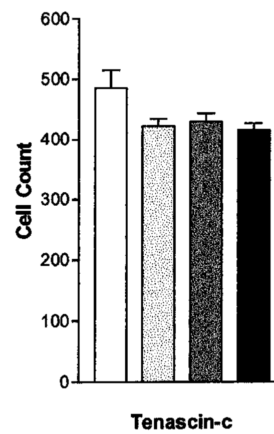
B



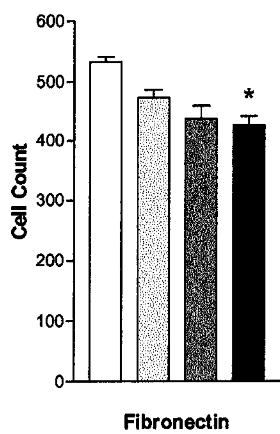
C



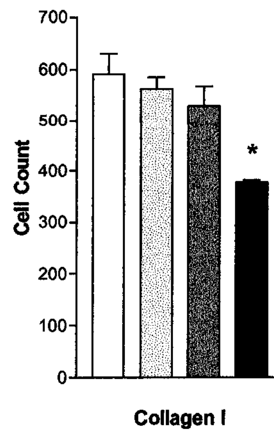
D



E



F



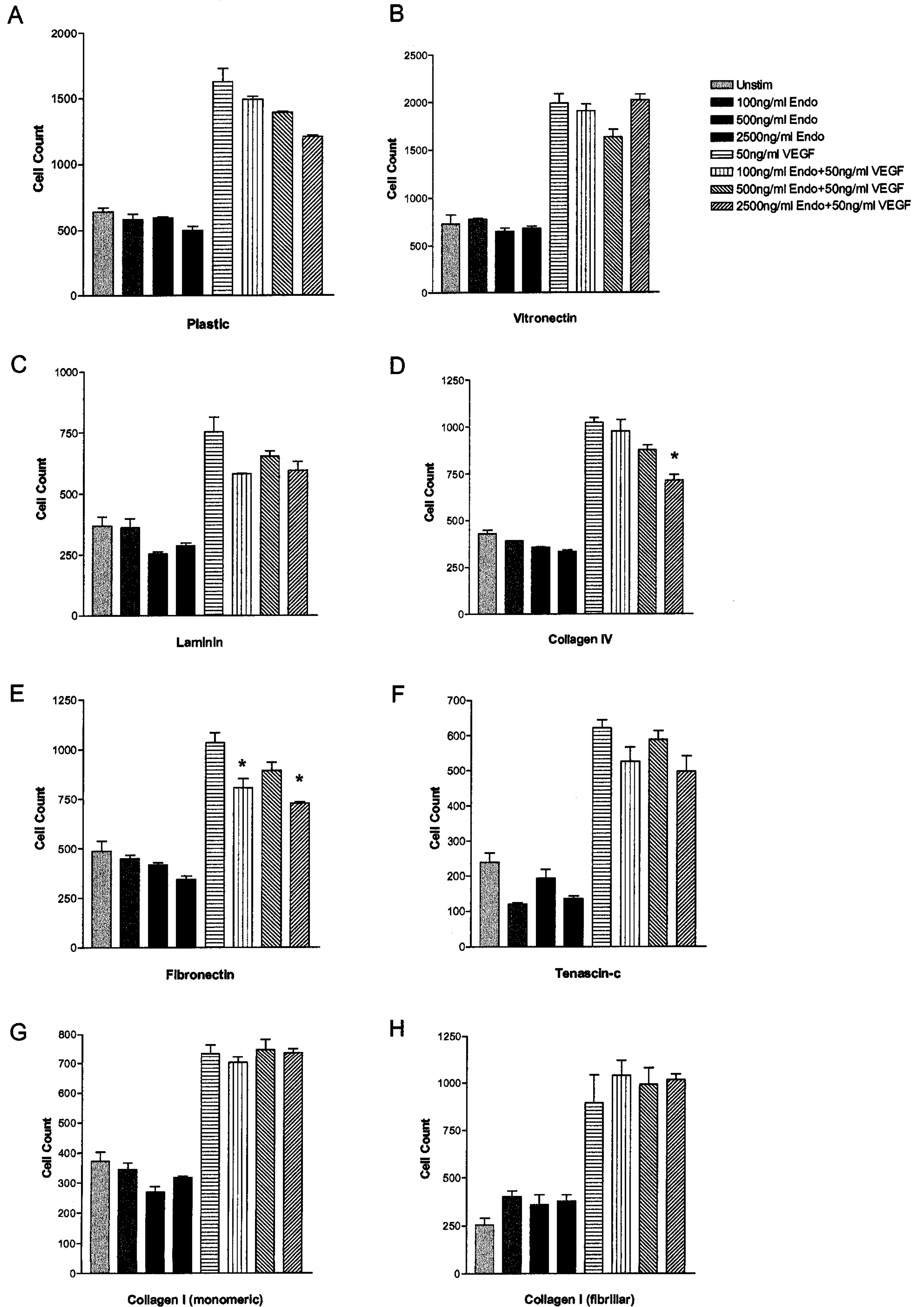
2. Effect of endostatin on proliferation of HDMEC

As endostatin has previously been shown to decrease proliferation of endothelial cells and certain ECM proteins can increase endothelial cell proliferation, we chose to examine whether inhibition of proliferation of endothelial cells by endostatin is altered by endothelial cell/ECM interactions. To test the ability to inhibit proliferation of endothelial cells on different matrices in response to endostatin, HDMEC were seeded subconfluently in 24 well plates coated with various ECM's (monomeric and fibrillar collagen I, collagen IV, fibronectin, laminin, vitronectin, tenascin-c and plastic) to ensure they do not become contact inhibited during the assay. HDMEC were then starved overnight and treated with endostatin, alone or in the presence of 50ng/ml VEGF, for 72 hours. At this time cells were harvested by trypsinization and counted using a Coulter Counter. VEGF-induced proliferation on uncoated plastic dishes had the second highest increase with a 2.53 fold increase between unstimulated and VEGF controls (Fig.4). In contrast to the adhesion data, vitronectin had the highest baseline levels of VEGF-induced proliferation while tenascin-c had the lowest, although these matrices had the second and third highest fold increase from unstimulated to VEGF controls, 2.73 and 2.60 fold respectively. Fibrillar collagen I demonstrated the highest fold increase between unstimulated and VEGF alone. This suggests that these matrices may act in concert with VEGF to increase proliferation of endothelial cells. The basement membrane component laminin had the lowest fold increase from unstimulated to VEGF (2.03 fold). Endostatin inhibited proliferation of HDMEC on all of the matrices except for collagen I. Strong inhibition of VEGF-induced proliferation by endostatin was

observed when HDMEC were cultured on the basement membrane components collagen IV and laminin with decreases of 30% and 23% respectively (fig.4C, D). Higher doses of endostatin were required for collagen IV inhibition then for laminin (100ng/mL endostatin for laminin, 2500ng/mL for collagen IV). Interestingly, VEGF-induced proliferation of HDMEC on fibronectin was reduced by endostatin by 30% as compared to control levels, which was as much as was observed on collagen IV (fig.4E). Endostatin showed 18% inhibition on vitronectin and 16% inhibition on plastic for VEGF-induced proliferation. Endostatin had the weakest effect as an inhibitor when HDMEC were cultured on collagen I. Both the monomeric and fibrillar forms of collagen I were used in this experiment and both types were able to inhibit endostatin's effects on VEGF-induced proliferation. Collagen IV and laminin are matrices found in the basement membrane of normal vessels and relay signals to reduce endothelial cell proliferation. In contrast, collagen I, fibronectin, vitronectin and tenascin-c are upregulated during tumourigenesis and can induce endothelial cell proliferation. Our results would support the contention that the signals from some of these tumour-associated matrices may be able to oppose the inhibitory effects of endostatin on endothelial cell proliferation.

Figure 4. The role of ECM in the proliferation of endothelial cells treated with endostatin and VEGF. HDMEC were plated on a variety of matrices and allowed to proliferate for 72 hours. Detached, floating cells were washed away and attached cells were removed by trypsinization and counted on a Coulter Counter (4 counts per condition, n=3). Graphs represent the mean number of cells in 500ul and standard error of triplicate wells. The experiment was performed three independent times with similar results observed. * represents $p\text{-value} < 0.05$.

Figure 4. Proliferation Assay



3. Effect of endostatin on the survival of HDMEC

Survival of endothelial cells is based on the balance between enhancers of endothelial cell proliferation and inducers of apoptosis. Molecules like tumour necrosis factor and angiostatin are known to induce apoptosis of endothelial cells while proteins such as VEGF and bFGF increase their survival. It has been reported that VEGF induced survival of endothelial cells occurs through the PI3-K/Akt pathway. The effect of endostatin on HDMEC survival, levels of PI3-K and on the activation of Akt was investigated. HDMEC were seeded on laminin, fibronectin, vitronectin, collagen I or plastic and treated with endostatin and VEGF for 62 hours. Previous lab data showed that apoptosis of HDMEC induced by endostatin was maximal at this time point. Both floating and adherent cells were collected and stained with propidium iodide. The sub-G1 population of cells was determined by FACS analysis and is representative of the fragmented DNA found in apoptotic cells. Endostatin did not appear to have any effect on the survival of VEGF treated HDMEC and failed to induce apoptosis on any of the matrices tested (fig.5A-D).

In parallel we examined the effects of endostatin on the PI3-K and Akt proteins as this pathway has been shown to be a critical modulator of apoptosis (Kauffmann-Zeh et al, 1997). PI3-K and Akt protein levels were unchanged in endostatin treated HDMEC based on western blotting analysis at several time points tested (fig.6). However, protein levels do not need to change to affect the activity of these proteins. Phosphorylation of proteins by kinases can act to increase or decrease the activity of certain proteins. Akt is one such protein and therefore its activation was measured by western blot analysis using

Figure 5. The effect of endostatin on VEGF-induced survival of endothelial cells cultured on different matrix components. HDMEC were plated on several matrices and treated with VEGF with or without varying doses of endostatin for 62 hours. All cells were then collected, permeabilized and stained with propidium iodide. The propidium iodide bound DNA was then measured and samples were assayed for apoptosis by measuring the sub-G1 population reflecting fragmented DNA on a flow cytometer (n=3). Graph is representative of the mean and standard error from triplicate dishes. The experiment was performed three independent times with similar results. * represents p-value < 0.05.

Figure 5. Apoptosis Assay

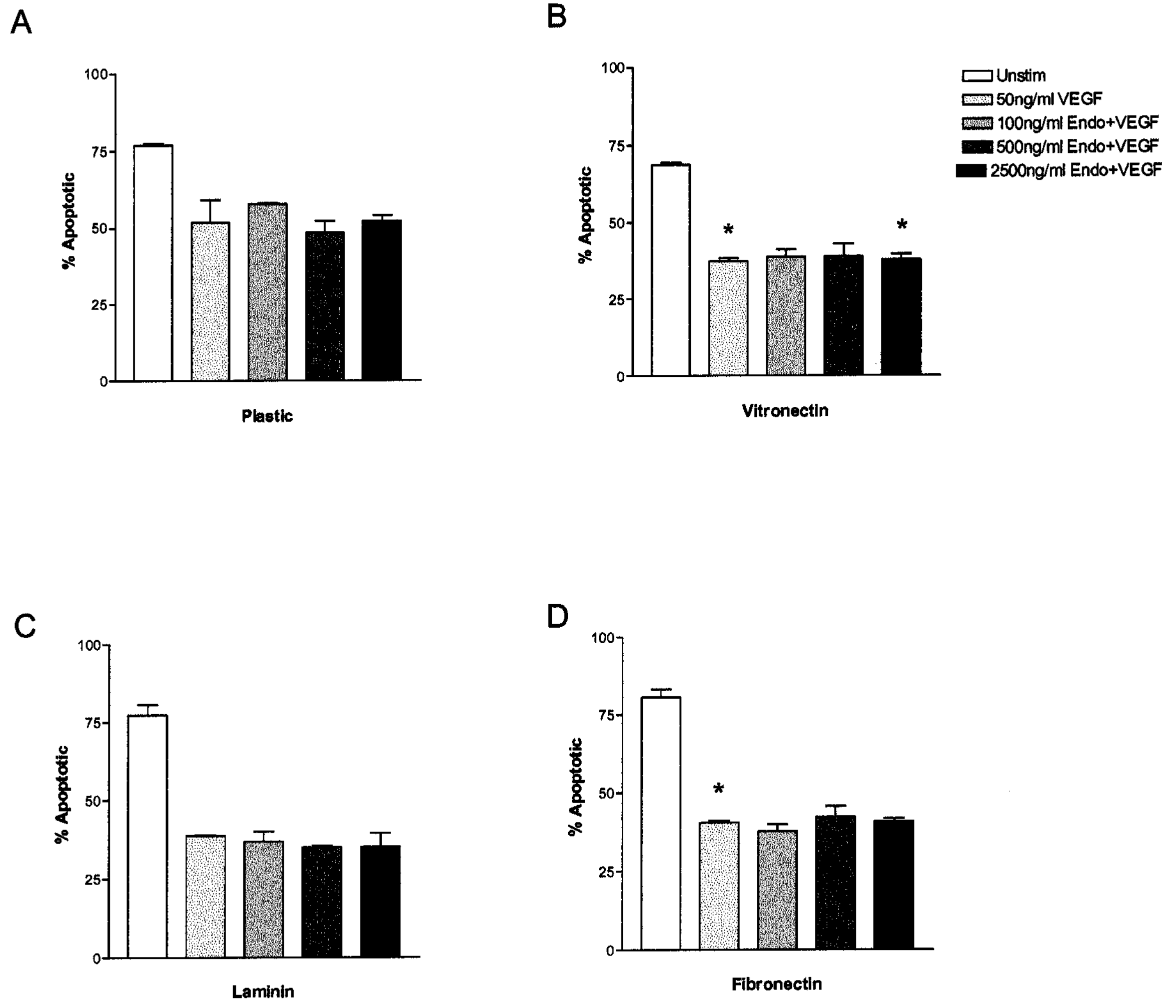
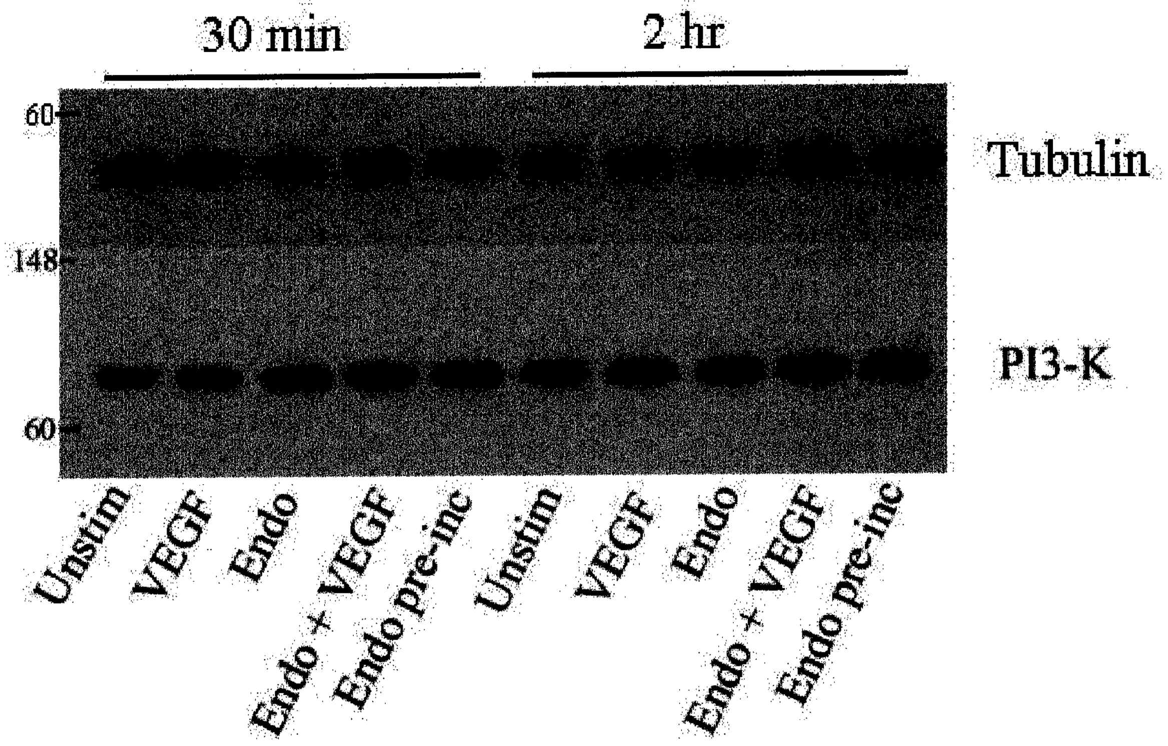


Figure 6. Effect of endostatin on PI3-K. HDMEC were treated with 100ng/ml, 500ng/ml, and 2500ng/ml of endostatin alone or in the presence of 50ng/ml VEGF for 30min. or 4 hours. Cells were lysed followed by western blot analysis to observe any changes in PI3-K protein levels. Anti-PI3-K antibody was diluted 1:1000 in 5% milk in TBST and used to probe nitrocellulose membranes for the protein of interest. Anti-tubulin was diluted 1:500 in 5% milk in TBST and used as a loading control for protein level. No change was observed in PI3-K levels.

Fig.6 Survival Protein analysis



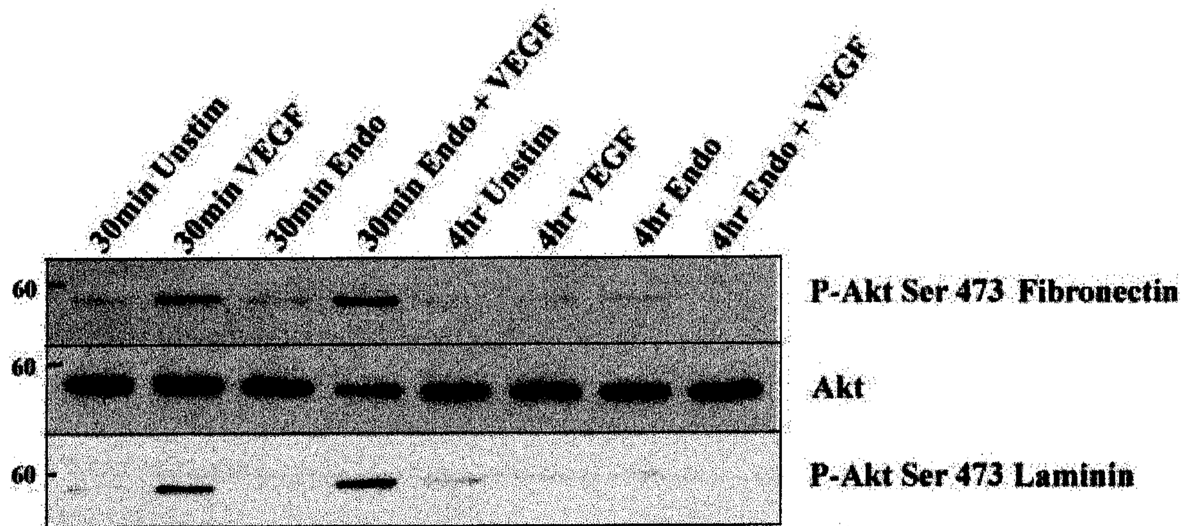
a phospho-ser-473 specific Akt antibody that only recognizes Akt in its activated state. VEGF strongly induced phosphorylation of Akt by 30 minutes but endostatin had no effect on this phosphorylation, either alone or in the presence of VEGF-induced activation (fig.7). As VEGF has been previously shown to increase the survival of endothelial cells and was observed to also reduce the percentage of cells in the sub-G1 population of cells in our experiments (fig.5), the increase in activated Akt following VEGF stimulation was expected and likely contributes to the increase in VEGF-mediated cell survival. The failure of endostatin to modulate the activity of Akt in HDMEC under these conditions suggests that apoptosis is not a primary mechanism for the inhibition of endothelial cells by endostatin although some published literature has previously described an induction of endothelial cell apoptosis by endostatin. It remains possible however, that by inhibiting adhesion, the cells may detach from the ECM and proceed to undergo apoptosis through anoikis, which is suspension induced cell death, and may be independent of Akt. In fact, previous results suggest that this type of apoptosis is a caspase-8 dependent phenomenon in endothelial cells (Stupack et al, 2001).

4. Effect of endostatin on migration of endothelial cells

Blood vessel formation involves several steps including ECM degradation, endothelial cell proliferation and migration of endothelial cells. Migration of endothelial cells occurs in response to increased concentration gradients of growth factors such as VEGF and bFGF. HDMEC migration was assessed by observing cell migration across a wound made in a confluent monolayer of cells by a 1000ul pipette tip. Endostatin and

Figure 7. Effect of endostatin on activation of the endothelial survival protein, Akt. HDMEC were stimulated in low serum with 100ng/ml, 500ng/ml, and 2500ng/ml of endostatin alone and in the presence of 50ng/ml VEGF for 30min. and 2 hours. Cell lysis was performed in FRAK's buffer with protease and phosphatase inhibitors to maintain phosphorylation status followed by assaying by western blot analysis to test endostatin's effect on activation of Akt. Anti-Akt and Anti-phospho-Akt-S473 were diluted 1:1000 in 5% milk in TBST prior to probing nitrocellulose membranes for Akt protein levels and Akt phosphorylation. Endostatin showed no effect on levels of Akt or Akt phosphorylation.

Fig.7 Survival Protein analysis

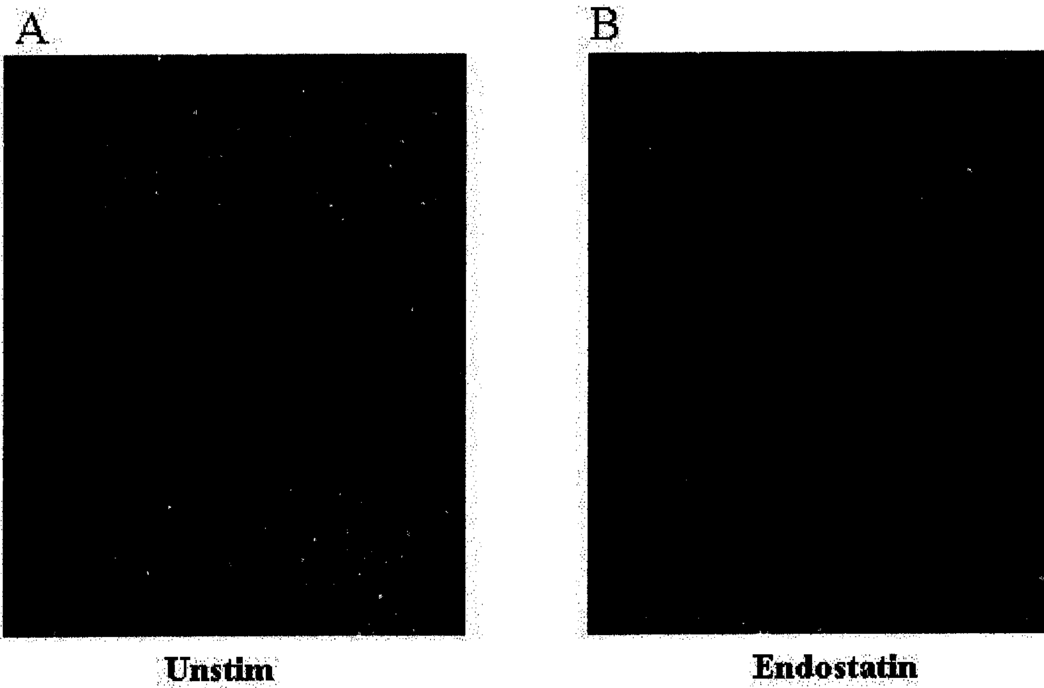


VEGF alone or in combination were added to the cells immediately following wounding. Cells were fixed at 8, 24 and 48 hours after stimulation to observe the degree of wound closure. There was negligible migration and closure at 8 hours in all cases (data not shown). By 24 hours VEGF had closed the wound by more than 50% and this closure was delayed in the presence of endostatin (fig.8C, D). However, VEGF fully induced migration and closure of the wound within 48 hours (data not shown).

Migration of HDMEC was also examined using a modified Boyden chamber with gelatin-coated filters. Cells were incubated on one side of a filter and allowed to attach. VEGF with or without endostatin was placed on the other side of the filter and HDMEC were allowed to migrate toward the growth factor stimulus. Cells were then scraped off the attachment side leaving only cells that have migrated through the filter towards the stimulus for assessment. VEGF induced migration of endothelial cells was inhibited by endostatin by 48%, to levels as low as endostatin alone or untreated without VEGF (fig.9). Interestingly, the lowest dose (100ng/ml) of endostatin was as efficient at inhibiting migration as the highest dose (2500ng/ml), while the middle dose (500ng/ml) was the least efficient. Gelatin is produced by heating animal collagen and is composed of denatured collagen type I primarily although it does not retain the same structure as collagen I (Bailey and Light, 1989). Although the remaining ECM proteins have yet to be tested, our preliminary results suggest that inhibition of endothelial cell migration may be one of the primary mechanisms of the inhibition of angiogenesis by endostatin.

Figure 8. Endothelial cell migration following endostatin and VEGF treatment of cells. HDMEC were grown to confluency on glass coverslips, wounded with a tip and stimulated with endostatin and VEGF alone or in combination. At 24 hrs cells were fixed with 4% paraformaldehyde and stained for f-actin and the nucleus. Endostatin appeared to slow the migration of VEGF-stimulated HDMEC.

Figure 8. Wounding Assay-24hr

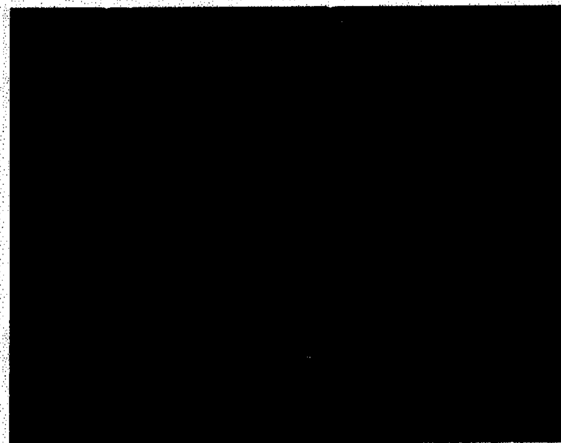


Wounding Assay-24hr

C

VEGF

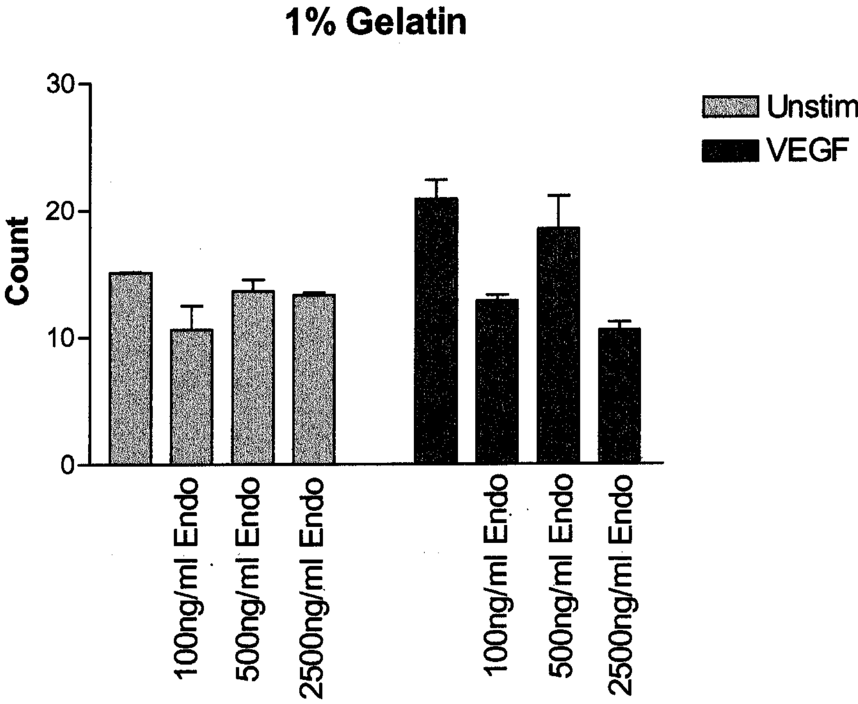
D



Endo + VEGF

Figure 9. Endothelial cell migration following endostatin and VEGF treatment of cells. HDMEC were added to the bottom of a gelatin-coated membrane (pore size 5μm) in a chemotaxis chamber and allowed to adhere to the membrane. Endostatin and VEGF were placed on the opposite side of the membrane and HDMEC were allowed to migrate through the membrane to the other side. Migrated cells were counted in ten random fields of view in duplicate wells and plotted on a graph (n=20). Endostatin inhibited the migration of VEGF-stimulated HDMEC across the gelatin-coated membranes by 48%.

Figure 9. Chemotaxis Assay



5. Intra-cellular effects of endostatin on focal adhesion signaling in endothelial cells

To determine the effect of ECM on the mechanism of the inhibition of adhesion, migration and proliferation by endostatin, the intra-cellular levels of the β1 integrin, FAK and ILK were assessed as well as the phosphorylation status of FAK as an indication of its activity in cells. There were no observable changes in the intra-cellular levels of the β1 integrin (fig.10) thus endostatin does not inhibit angiogenesis via downregulation of expression of the β1 integrin. It remains possible that endostatin may alter the conformation or phosphorylation state of the β1 integrin, however attempts to assess the phosphorylation of the β1 integrin were unsuccessful (data not shown).

We hypothesized that endostatin may be directly binding to and interfering with integrin signaling therefore we next examined one of the major focal adhesion signaling molecules that could potentially be involved in endostatin mediated anti-angiogenic activity, FAK. Following treatment with endostatin for various periods of time (5 minutes to 4 hours), the total protein level of FAK also remained unchanged in HDMEC (fig.11) indicating that the mechanism of inhibition by endostatin treatment does not involve a reduction of the level of FAK in cells. There are however numerous sites of tyrosine and serine phosphorylation on FAK that are critically involved in the regulation of its activity and these may be potential sites for regulation by endostatin (fig.12). By performing western blot analysis using phosphospecific antibodies to phosphorylated tyrosines on FAK, it was observed that endostatin appears to affect the phosphorylation status of FAK and results in a reduction of the tyrosine phosphorylation of sites Y397 and

Figure 10. Effect of endostatin on integrin β1 protein levels. HDMEC were treated with endostatin and VEGF in low serum followed by lysis in FRAK's buffer. Anti-β1 integrin antibody was diluted 1:2500 in 5% milk in TBST and used to probe for β1 integrin protein levels. Anti-tubulin was diluted 1:500 in 5% milk in TBST and used as a loading control for protein level. No change was observed on the level of β1 integrin.

Figure 10. Integrin analysis

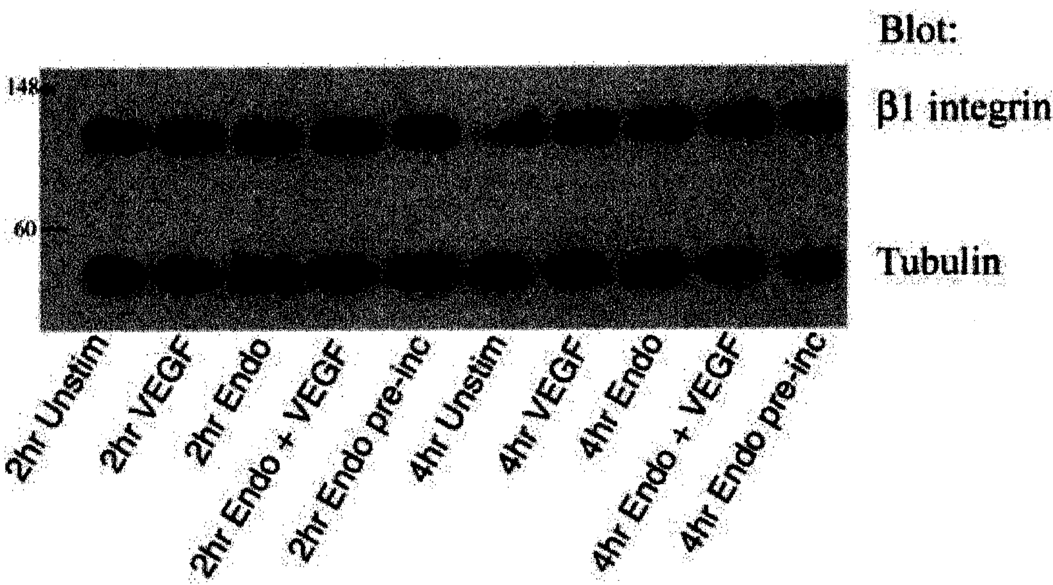


Figure 11. Effect of endostatin on focal adhesion kinase levels. HDMEC were treated with endostatin and VEGF in low serum followed by lysis in FRAK's buffer. Anti-FAK antibody was diluted 1:2500 in 5% milk in TBST and used to probe for FAK protein levels. Anti-tubulin was diluted 1:500 in 5% milk in TBST and used as a loading control for protein level. No change was observed on the level of FAK.

Figure 11. FAK protein level analysis

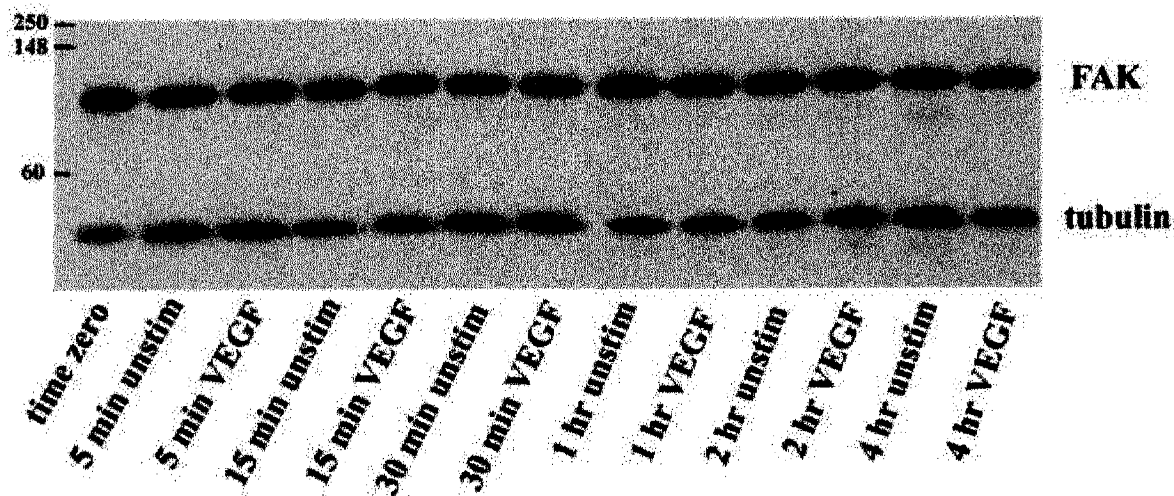
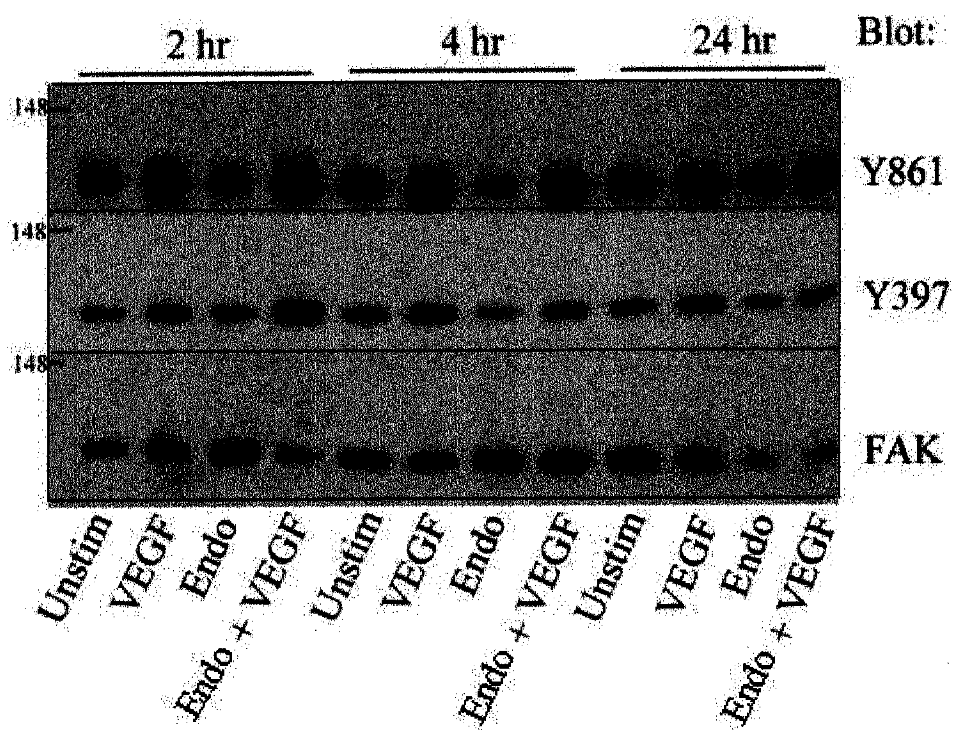


Figure 12. Effect of endostatin on FAK Y397 and Y861 phosphorylation on uncoated, plastic dishes. HDMEC were treated with endostatin and VEGF in low serum followed by lysis in FRAK's buffer with protease and phosphatase inhibitors to preserve protein status. Anti-phospho-FAK-Y397 was diluted 1:1000 and anti-phospho-FAK-Y861 was diluted 1:750 in 5% milk in TBST and used to observe the phosphorylation of FAK at specific sites of tyrosine phosphorylation. Endostatin alone appeared to decrease both sites of phosphorylation but this effect was not seen with the addition of VEGF.

Figure 12. FAK phosphorylation analysis on plastic



Y861 particularly after 4 hours of incubation with endostatin (endostatin alone compared to unstimulated HDMEC, fig.12). Interestingly, in the presence of VEGF, treatment with endostatin did not show the same reduction in Y397 and Y861 phosphorylation suggesting that the ability of endostatin to inhibit endothelial cell proliferation and migration may not be a result of changes in FAK activity. Also of note is the fact that the reduction of FAK phosphorylation induced by endostatin appears to vary with respect to which matrix the endothelial cells are in contact with. On tumour matrices such as collagen I and fibronectin, endostatin decreases the phosphorylation of Y861 in response to VEGF but does not affect FAK's autophosphorylation at Y397 (fig.13 and 14). On laminin and collagen IV, which are regular components of endothelial cell basement membranes, there is little or no effect of endostatin on the phosphorylation of FAK at Y861 or Y397 regardless of the presence or absence of VEGF (fig.13 and 14). On another tumour matrix, tenascin-c, endostatin did not decrease VEGF induced FAK phosphorylation at Y861 but did inhibit Y397 phosphorylation slightly (fig.13 and 14). The pre-incubation of FAK was also examined as it was previously shown to have an effect on the phosphorylation status of FAK (Kim et al, 2002). Notably, the pre-incubation of endothelial cells with endostatin prior to addition of VEGF did result in a decrease in VEGF-induced FAK Y861 phosphorylation as compared to the simultaneous addition of the two proteins (fig.15). Although we could replicate these results, the likelihood that this is a main mechanism of inhibition in vivo is slim as the addition of endostatin prior to the appearance of VEGF in a tumor microenvironment would not be possible in a therapeutic situation.

Figure 13. Effect of endostatin on FAK Y397 phosphorylation on several ECM. HDMEC were treated with endostatin and VEGF in low serum followed by lysis in FRAK's buffer with protease and phosphatase inhibitors to preserve protein status. Anti-phospho-FAK-Y397 was diluted 1:1000 in 5% milk in TBST and used to observe the phosphorylation of FAK at Y397. Endostatin only affected FAK Y397 phosphorylation on tenascin-c.

Figure 13. Phospho-FAK Y397 analysis on different ECM

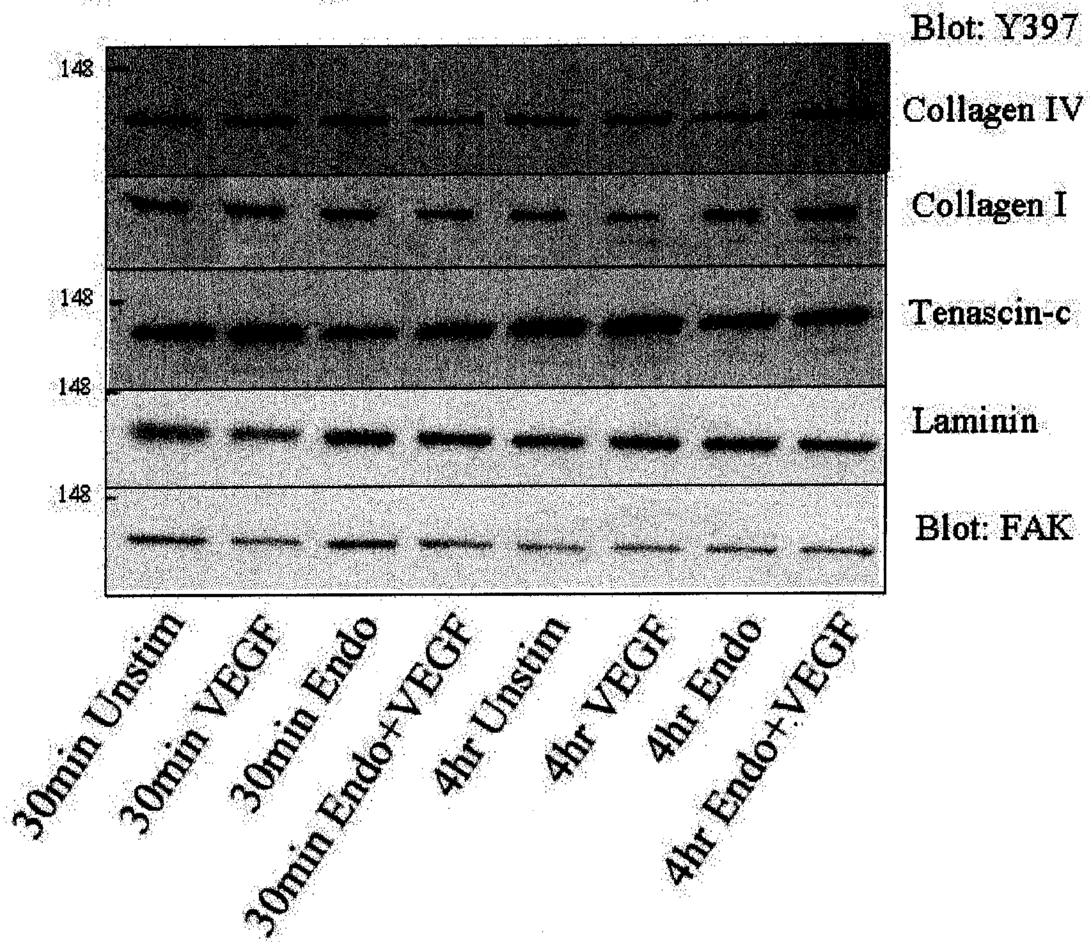


Figure 14. Effect of endostatin on FAK Y861 phosphorylation on several ECM. HDMEC were treated with endostatin and VEGF in low serum followed by lysis in FRAK's buffer with protease and phosphatase inhibitors to preserve protein status. Anti-phospho-FAK-Y861 was diluted 1:750 in 5% milk in TBST and used to observe the phosphorylation of FAK at Y861. Endostatin decreased VEGF-induced FAK Y861 phosphorylation on collagen I and fibronectin but not tenascin-c or the two basement membrane components, laminin and collagen IV.

Figure 14. Phospho-FAK Y861 analysis on different ECM

148

Blot: Y861

Collagen IV

148

Collagen I

148

Tenascin-c

148

Laminin

148

Fibronectin

148

Blot: FAK

30min Unstim

30min VEGF

30min Endo

30min Endo+VEGF

4hr Unstim

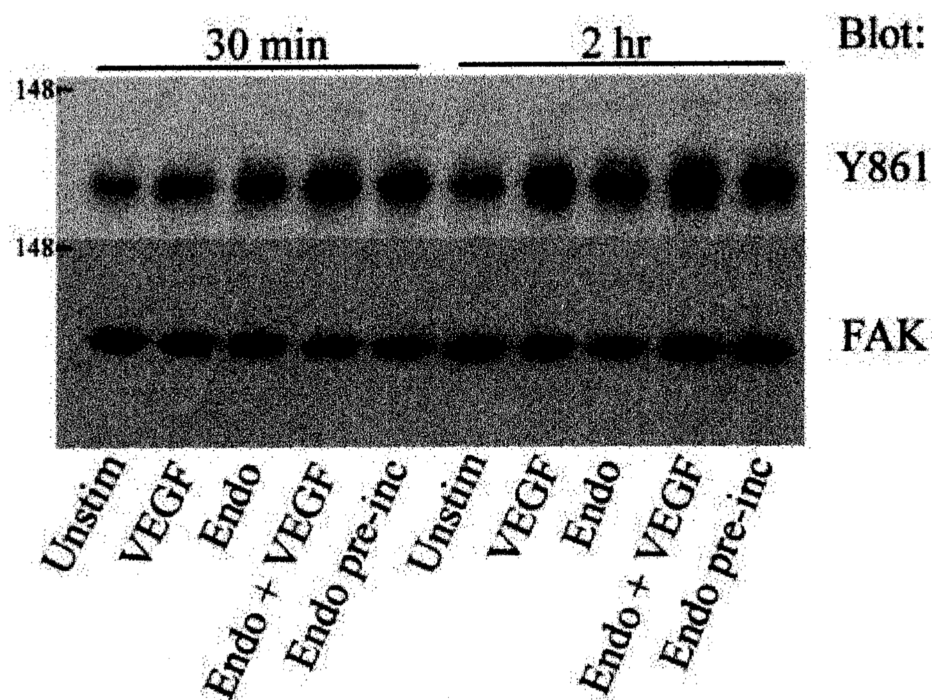
4hr VEGF

4hr Endo

4hr Endo+VEGF

Figure 15. Effect of pre-incubation of endostatin on VEGF-treated HDMEC on plastic. HDMEC were treated with endostatin and VEGF in low serum followed by lysis in FRAK's buffer with protease and phosphatase inhibitors to preserve protein status. Anti-phospho-FAK-Y861 was diluted 1:750 in 5% milk in TBST and used to observe the phosphorylation of FAK at Y861. Endostatin was pre-incubated with the HDMEC for 30 minutes prior to the addition of VEGF. Pre-incubation of endostatin was shown to decrease VEGF-induced FAK Y861 phosphorylation as compared to simultaneous addition of the two factors.

Figure 15. Effect of pre-incubation of endostatin on VEGF-stimulated HDMEC



Another integrin signaling molecule that could potentially be involved in endostatin-induced inhibition of angiogenesis is ILK. As mentioned above ILK is capable of phosphorylating the β1 integrin leading to its activation. The intra-cellular levels of the signaling/adaptor protein ILK were also investigated for any endostatin-induced changes. Similarly to FAK, there were no observable modifications to the protein level of ILK in endostatin treated cells (fig.16). Tests to ascertain the phosphorylation status of ILK were unsuccessful due to antibody interference from the immunoprecipitation on the western analysis (fig.17) and that there are no commercially available phospho-specific antibodies to ILK. Although ILK is thought to autophosphorylate itself there is a lack of information on ILK regulation, which lead to an inability to determine if endostatin had any effect on ILK activity (Persad et al, 2001).

Figure 16. Effect of endostatin on integrin linked kinase levels. HDMEC were treated with endostatin and VEGF in low serum followed by lysis in FRAK's buffer. Anti-ILK antibody was diluted 1:2500 in 5% milk in TBST and used to probe for ILK protein levels. Anti-tubulin was diluted 1:500 in 5% milk in TBST and used as a loading control for protein level. No change was observed on the level of ILK.

Figure 16. ILK protein level analysis

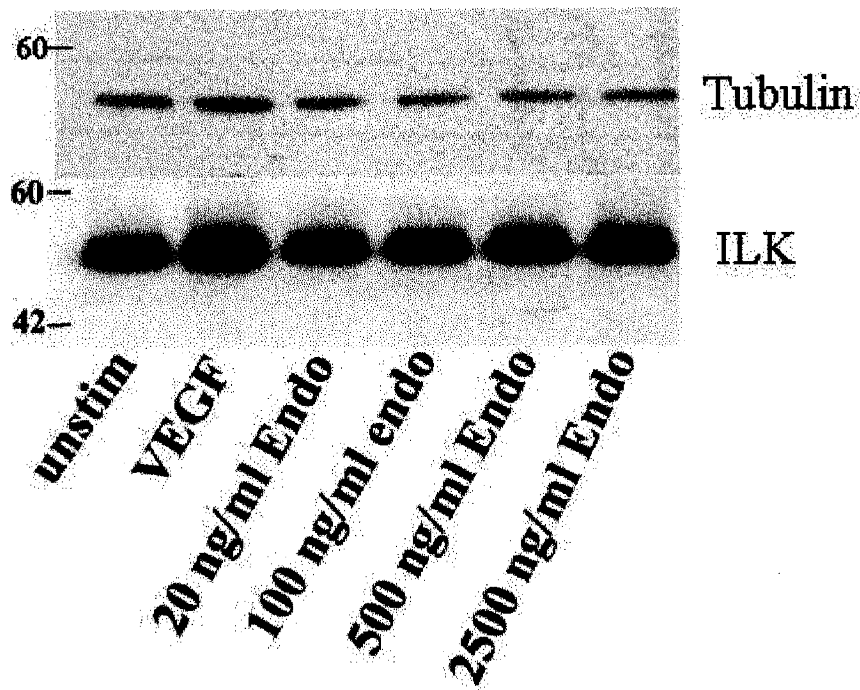
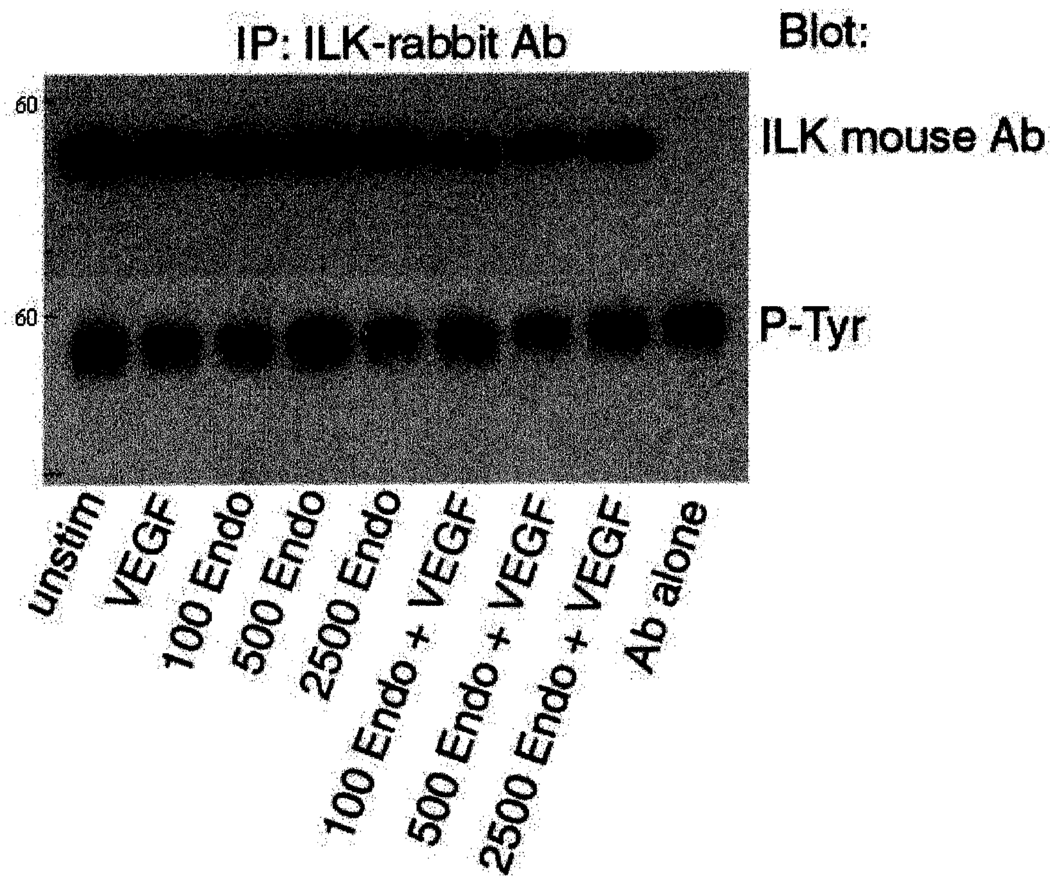


Figure 17. Endostatin's effect on the phosphorylation of ILK. HDMEC were treated with endostatin and VEGF in low serum followed by lysis in FRAK's buffer with protease and phosphatase inhibitors to preserve protein status. ILK was immunoprecipitated with 4ul a rabbit anti-ILK antibody on protein G sepharose beads followed by western blot analysis of the immunoprecipitated complex. Mouse anti-ILK antibody was diluted 1:2500 in 5% milk in TBST and used to probe for the level of ILK that was immunoprecipitated. A PY20 anti-phospho-tyrosine antibody was diluted 1:1000 in 5% milk in TBST and used to assess the phosphorylation status of the immunoprecipitated ILK. Interference from the antibody used to capture ILK during the immunoprecipitation inhibited us from observing the phosphorylation of ILK.

Figure 17. ILK phosphorylation analysis



Discussion

This project was designed to investigate the effects of different ECM proteins on endostatin's inhibition of endothelial cells, as we know that the tumour environment contains matrices that are conducive to endothelial cell growth and survival. To test these effects we grew HDMEC on a variety of normal and tumour-associated matrices and looked for differences in adhesion, proliferation, survival and migration of endothelial cells as well as changes in intracellular signaling molecules that are potentially involved in regulating these processes. Our results suggest that in this model system the mechanism for the inhibition of angiogenesis by endostatin involves changes in adhesion, proliferation and migration, but not survival of endothelial cells, and these changes are altered depending on the ECM in the surrounding environment.

Adhesion of endothelial cells to the ECM occurs through the clustering of integrins into complexes known as focal adhesions. Engagement of integrins to the ECM and the formation of focal adhesions can relay signals to the cell that control many activities such as growth, survival, migration and differentiation. By interrupting attachment, endostatin may be able to inhibit any or all of these processes. The effects that were observed on adhesion are likely to be a result of endostatin binding to and interfering directly with one or more integrins. Endothelial adhesion was not drastically altered with addition of endostatin but this may represent the fact that endostatin may bind to a specific integrin and block only its binding to the ECM. Endothelial cells have several different combinations of integrins and it is unlikely that endostatin binds to all of them thus allowing for attachment of endothelial cells to ECM via unaffected integrins

that do not associate with endostatin. In addition we tested certain doses of endostatin that had been shown to inhibit endothelial cell activities such as proliferation or migration. It is possible that the doses of endostatin used in these assays are not high enough to saturate all of the potential integrin binding sites available to endostatin thus using higher concentrations of endostatin could further reduce the endothelial cell adhesion observed in these studies. The fact that we observed dose dependent decreases in adhesion (Fig.3) suggests that this is a strong possibility. Alternatively, endostatin could possibly alter the inside-out signaling of integrins, which would result in a decrease in the adhesion of the endothelial cells to the matrix by inducing a conformational change in the integrin extracellular domain. This could result, for example, from endostatin binding the $\alpha5\beta1$ integrin, inhibiting its adhesion to fibronectin, and in turn sending a signal to other integrins to undergo a conformational change. The tumour matrices that are normally associated with leaky membranes (collagen I, tenascin-c, and vitronectin) showed greater adhesion while the basement membrane component laminin and the provisional matrix fibronectin proved to have the most disruption of adhesion following endostatin treatment. It is not surprising that fibronectin adhesion was blocked the most as endostatin has been shown to interact with the $\alpha5\beta1$ integrin that is a known binding molecule for fibronectin. Sudhakar et al (2003) demonstrated the ability of human umbilical vein endothelial cells (HUVECs) to adhere to endostatin coated plates and this binding was subsequently blocked by soluble $\alpha5\beta1$ integrin protein indicating an ability of endostatin to bind endothelial cells primarily via $\alpha5\beta1$. As we observed no decreases in the level of expression of $\beta1$ and modest effects on endothelial cell adhesion following endostatin treatment, these results suggest that endostatin may act primarily on internal

signaling pathways that affect the signaling from the $\alpha5\beta1$ integrin. In support of this we did observe changes in the phosphorylation status (as an indication of activity) of FAK, which could lead to dramatic effects on cell migration. Moreover, the decrease in endothelial cell adhesion by endostatin may be a result of an upset in the turnover of focal adhesion dynamics that control migration. Alternatively, endothelial cell detachment from the matrix occurs when cells divide and this may also be responsible for the decrease that is observed in adhesion. The increased level of cell adherence to tumour matrices may be explained by some early evidence that endostatin acts initially to aid in vessel maturation where it reduces the proliferative cues from the provisional matrix so that vessels begin to mature and form networks and associations with pericytes prior to an inhibition of angiogenesis (Ergun et al, 2002). And by correcting vessel damage from these provisional matrices, endostatin may aid in the delivery of other chemotherapeutic drugs to a tumour.

Endothelial cells are some of the most stable cells in the body and rarely proliferate. However these cells can be induced to grow quickly during wound healing and angiogenesis. The ECM plays a crucial role in regulating the growth of endothelial cells as described above. We found that endostatin was able to decrease the growth of endothelial cells on almost all matrices tested, except for monomeric and fibrillar collagen I. As mentioned previously, collagen I is rarely expressed in normal tissue but is increased in tumor microenvironments. The ability of collagen I to suppress the inhibitory effects of endostatin on endothelial cell proliferation would suggest that its anti-angiogenic activity may be reduced in tumors with high levels of this ECM. Similar to the adhesion data, endostatin inhibited the VEGF-induced proliferation of endothelial

cells on the basement membrane associated matrices (collagen IV and laminin) most significantly and this is likely as a consequence of the intrinsic nature of these matrices that have previously been shown to reduce endothelial cell proliferation (Madri and Williams, 1983). VEGF-induced proliferation of endothelial cells on tenascin-c and vitronectin was also decreased in response to endostatin but not to a significant level suggesting that these provisional matrices are also capable of reducing any inhibitory effects endostatin has on proliferation. Unexpectedly, when endothelial cells were cultured on the $\alpha5\beta1$ integrin binding matrix fibronectin, the VEGF-induced proliferation was inhibited by endostatin as efficiently as was observed on the basement membrane component collagen IV suggesting that endostatin does indeed signal or inhibit the signaling through the $\alpha5\beta1$ integrin as this integrin primarily binds to fibronectin. The endostatin-induced inhibition of proliferation could be due to an interruption in any number of growth promoting signaling pathways but most likely involves the ERK or p38 MAPK pathways as inhibition of these molecules has been previously shown to decrease proliferation of endothelial cells (Sudhakar et al, 2003). The $\alpha5\beta1$ integrin has been shown to bind the RGD sequence motif of fibronectin and endostatin is able to abrogate this interaction however endostatin itself does not contain an RGD peptide and endostatin's integrin binding domain is still unknown (Ruoslahti and Pierschbacher, 1987; Rehn et al, 2001). Our data suggests that collagen I appears to prevent endostatin from inhibiting VEGF-induced proliferation, as collagen I has been shown to increase endothelial cell proliferation where collagen IV does not (Madri and Williams, 1983). This may be a result of collagen I-induced proliferation masking endostatin's inhibition or from collagen I being able to sequester endostatin away from the cell by binding to

endostatin directly and preventing its association with integrins. In our model system the ability of endostatin to inhibit endothelial cell proliferation was modest on the majority of provisional matrices and this may indicate that this inhibition of endothelial cell proliferation, along with inhibition of adhesion, is not the primary mechanism for endostatin's inhibition of angiogenesis.

Apoptosis is a programmed cellular mechanism that is responsible for the removal of damaged, infected or unnecessary cells. We looked to see if endothelial cell apoptosis was a mechanism for the inhibition of angiogenesis induced by endostatin in our model system. Interestingly, in our system endostatin did not appear to have any effect on the regulation of cell death, which is in contrast to previously, published literature (Dhanabal et al, 1999). The addition of VEGF significantly reduced the apoptosis of endothelial cells on all matrices tested (as has been previously observed) and endostatin was not able to reverse this protection. VEGF has been shown to induce expression of the anti-apoptotic protein Bcl-2 in endothelial cells and thus increase their survival (Nor et al, 1999). Other observations in the laboratory suggest that endostatin had no effect on VEGF-induced levels of Bcl-2 in HDMEC (unpublished data, C. Addison, personal communication). With administration of endostatin we observed no change in the level of PI3-K or Akt along with no decrease in the VEGF-induced activation of Akt which is in agreement with the observation that endostatin did not induce the apoptosis of endothelial cells in the presence of VEGF in our studies. Early work proposed that endostatin was able to induce apoptosis of endothelial cells (Dhanabal et al., 1999) but recent publications (Sudhakar et al, 2003) have contradicted this finding and suggested that endostatin does not induce endothelial cell apoptosis. This is possibly a major

contributing factor to why there was limited success in early clinical trials with end-stage cancer patients. Namely, endostatin was unable to induce regression of the vessels that were present in those tumours due to enhanced endothelial cell survival in the presence of angiogenic factors and tumor-associated ECM.

The migration of endothelial cells is one of the most important steps in the formation of new vasculature. It also appears to be the most drastically affected pathway in endothelial cells by endostatin. Initial observations from chemotaxis data demonstrated that endostatin could reduce VEGF stimulated migration of HDMEC up to 50% on collagen I coated filters which was almost equal to endostatin alone control levels. Shichiri and Hirata (2001) found that several genes downregulated by endostatin primarily affected cell migration. They also observed that endostatin did not restore proliferating endothelial cells to their resting state nor could it induce apoptosis. Furthermore, they observed that these effects only occurred in exponentially growing endothelial cells and not quiescent ones. The c-myc gene was shown to be downregulated by endostatin and the over-expression of c-myc abrogated the ability of endostatin to inhibit migration. This downregulation is important as c-myc can promote vascular development by functioning as a regulator of angiogenic factors such as VEGF (Baudino et al, 2002). In light of our observations that the effects of endostatin can be modulated by ECM components, it will be important to determine the effects of tumor-associated ECM on migration of endothelial cells and how endostatin can inhibit this process in the presence of various ECM molecules. Delineating the intracellular migratory signals that are interrupted by endostatin will also be key to understanding its mechanism of action.

Phosphorylation signals regulate many of the pathways involved in angiogenesis, including proliferation, gene regulation, certain components of migration and many other processes. Therefore we decided to test the intracellular kinases and signaling proteins that may be affected by endostatin and contribute to its mechanism of action. Although endostatin did not affect the levels of the FAK autophosphorylation site, Y397, endostatin was shown to decrease the tyrosine phosphorylation of FAK specifically at Y861, the VEGF responsive tyrosine phosphorylation site. However, the decreased phosphorylation of FAK does not appear to be directly linked to events in adhesion or proliferation suggesting that changes in FAK phosphorylation are not the primary cause for the inhibition of these pathways. This is supported by the observations that when endothelial cell adhesion and proliferation are inhibited by endostatin, such as on laminin and collagen IV, there is no change in the phosphorylation status of FAK Y861. Conversely, on vitronectin, tenascin-c and collagen I when Y861 phosphorylation is reduced, there are only small decreases in proliferation and adhesion. There was a correlation between adhesion or proliferation and decreased FAK phosphorylation when HDMEC were cultured on fibronectin however the significance of this has yet to be determined. It is our contention that perhaps the endostatin-induced decrease in FAK phosphorylation will prove to be linked to the inhibition of migration of endothelial cells on tumour-associated matrices or may assist in directing integrin conformational changes. Recently, a paper was published showing decreased migration of NIH 3T3 cells with an Y861F mutation in FAK that inhibits phosphorylation at this site suggesting that this tyrosine is involved in regulating cell migration (Lim et al, 2004). FAK also has other tyrosines that may be affected by endostatin such as Y576 and Y577 that are necessary

for maximal catalytic activity and Y925 that is located in FAK's focal adhesion targeting domain. Analysis of the phosphorylation status of these sites in FAK has yet to be determined on the various matrices used in our model system however it remains possible that changes at these sites may correlate with the decreases in adhesion or proliferation observed following endostatin treatment of endothelial cells cultured on different matrices. Another question that remains unanswered is whether the endostatin dependent decrease in FAK phosphorylation observed in our studies is a result of its phosphorylation being blocked or from the induction of a phosphatase that actively dephosphorylates FAK. One published paper demonstrated a phosphatase-dependent activation of Src by endostatin although a link to FAK was not addressed (Wickstrom et al, 2002).

The study of the function of ILK is a rapidly growing area of research and more and more pathways are being shown to have ILK involvement, such as migration, proliferation, survival and oncogenic transformation. Deciphering what controls the regulation of ILK may hold clues to the mechanism for numerous therapeutics including endostatin. Although our results demonstrated that endostatin did not affect the level of expression of ILK it remains entirely possible that endostatin treatment could decrease the activity of ILK or result in changes in its intracellular location. Kinase assays for ILK have been developed and could be used to ascertain if its activity is modulated by endostatin treatment of endothelial cells on the various matrices. Moreover the intracellular location of ILK can be assessed by immunocytochemical analysis of cultured cells.

Based on the results presented in this project, it appears that endostatin inhibits signals from the fibronectin binding α5β1 integrin most efficiently implying that this integrin is a receptor for endostatin as has been previously reported (Sudakar et al, 2003). However, endostatin still affects endothelial cells in the absence of fibronectin hence, binding the α5β1 integrin may allow endostatin to modulate signals coming from other matrices or alternatively endostatin may additionally function independently of binding to the α5β1 integrin. Endostatin may also be able to exert some of its effects through interactions with heparan sulfate proteoglycans, another proposed receptor for endostatin. Inhibiting signals from proteoglycans may lead to a more general inhibitory mechanism while inhibition through the fibronectin receptor is specific for blocking signals from that provisional matrix. Sulfated glycosaminoglycans on the cell surface have been implicated in modulating cytokine activation and cell adhesion and thus may regulate certain aspects of endostatin signaling (Rusnati and Presta, 1996; Stamatoglou and Keller, 1983). Another proposed receptor for endostatin is the VEGFR2, but if endostatin were to bind the VEGFR2 receptor as has been published, it would be expected that there would be a decrease in VEGF induced survival as well as other VEGF activated pathways (Kim et al, 2002). However, our data does not support this finding. Other data in this paper showed an inhibition of FAK phosphorylation that was observed with the pre-incubation of 10μg/mL of endostatin prior to VEGF stimulation. It appears that endostatin is able to protect cells in this manner where the simultaneous addition of the two proteins does not decrease FAK phosphorylation. Although this may indeed occur the ability to administer endostatin before the appearance of VEGF in a tumor microenvironment would be highly unlikely. Additionally this group used a

concentration of endostatin that was 4 times higher then the maximal concentration used in our studies. It is possible that the VEGFR2 binding only occurs at very high concentrations of endostatin and not at the levels used in our experiments. Perhaps an anti-migratory signal from the $\alpha5\beta1$ integrin is capable of inhibiting migration on other matrices, as our preliminary chemotaxis data may suggest. It has been demonstrated that integrins can relay inhibitory signals. For example, the $\alpha v\beta3$ integrin induces apoptosis when it is not bound by its cognate matrix and even does so while other integrins are bound and the cell is attached to the ECM (Stupack et al, 2001). Thus, the $\alpha5\beta1$ integrin may be a key receptor that can regulate the activity of endothelial cells and tumours that produce fibronectin may be more receptive to endostatin therapy while those that have a predominance of collagen I may be relatively resistance to the anti-angiogenic effects of endostatin.

Endostatin's effects on endothelial cell adhesion, proliferation and FAK phosphorylation seem rather modest although it may be capable of increasing the efficacy of other therapies if taken in conjunction. Through inhibiting migration alone endostatin may be able to decrease or stop excessive angiogenesis. By rescuing immature vessels endostatin can increase the normal flow of vasculature to aid in the proper delivery of chemotherapeutics to the tumour. As well, by inhibiting new vessels that are forming, endostatin can starve a tumour of the oxygen and nutrients it needs to grow and survive thus limiting tumour progression and metastases. Endostatin has been shown to be extremely nontoxic and this may reflect that endostatin may not function without an angiogenic switch being initiated or an induction of growth or motility by a growth factor such as VEGF. In other words, endostatin may not affect quiescent or dormant

endothelial cells but only activated endothelial cells. Clearly the mechanism of anti-angiogenic activity by endostatin has yet to be elucidated and further studies are warranted. Understanding the mechanism for the inhibition of angiogenesis by endostatin and the effects tumor-associated ECM have on this mechanism of inhibition will be critical if endostatin is to prove to be a useful tool in fighting tumour angiogenesis as well as other angiogenic pathologies.

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