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Characterization of the Anti-angiogenic Properties of KDR-Ig4-7 and its Variants as Potential Anti-cancer Therapeutics

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CHARACTERIZATION OF THE ANTI-ANGIOGENIC PROPERTIES OF KDR-Ig4-7 AND ITS VARIANTS AS POTENTIAL ANTI-CANCER THERAPEUTICS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Microbiology

by
Jang Pyo Park
December 2007

Accepted by:
Dr. Wen Y. Chen, Committee Chair
Dr. Lyndon L. Larcom
Dr. Lesly Temesvari
Dr. Tzuen-Rong Tzeng
ABSTRACT

Beginning with the development of an embryo and throughout one’s adult life angiogenesis plays an essential role for organ growth and repair. The balancing of pro-angiogenic factors to anti-angiogenic factors is what regulates the process of angiogenesis. When this balance is disrupted in one direction or the other, numerous disorders can occur including ocular and inflammatory diseases and, in particular, tumor growth and metastasis. Therefore, inhibition of angiogenesis has become an effective therapy for arresting the growth of tumors.

A key regulator of angiogenesis involves vascular endothelial growth factor (VEGF) and its receptor, VEGFR-2 (KDR). The binding of VEGF to KDR activates the receptor which, in turn, initiates the signaling cascade leading to cell proliferation, migration, and survival. The extracellular portion of KDR consists of seven Ig-like domains (Ig1-7), in which Ig1-3 is believed to be responsible for the ligand binding and Ig4-7 for receptor dimerization. We hypothesized that a soluble fragment of the KDR extracellular domain (KDR-Ig4-7) that lacks a tyrosine kinase domain may serve as a KDR antagonist by binding to the membrane bound KDR to form a KDR dimer thus preventing VEGF induced signal transduction.

In this study various KDR proteins (KDR-Ig4-7, KDR-Ig4-6, KDR-Ig5-7 and KDR-Ig5-6) were produced, purified and characterized for their potential anti-angiogenic ability. We demonstrated that KDR-Ig4-7 was able to specifically bind to human umbilical vein endothelial cells (HUVEC), and accumulated specifically in tumors indicating its ability to target endothelial cells. Furthermore, KDR-Ig4-7 was able to
inhibit HUVEC (and various breast and leukemic cancer cells) proliferation, tube formation and migration. An apoptosis assay conducted using T47-D cells revealed that the induction of apoptosis was the likely mechanism for the inhibition of cell proliferation by KDR-Ig4-7. The inhibitory effect of KDR-Ig-4-7 was at least, in part, through the blocking of ERK phosphorylation. Among all the variants tested, KDR-Ig-4-7 was the most potent inhibitor, which suggested the involvement of domains Ig4 and Ig7 in the receptor dimerization process. Moreover, we found that the inhibitory activity of KDR-Ig4-7 was enhanced in the presence of VEGF, which suggested that VEGF might induce a conformational change which favors the interaction between KDR-Ig4-7 and KDR. In our 4T1 xenograft animal studies, however, KDR-Ig4-7 treatment was not effective. In summary, our results suggest that KDR-Ig4-7 is a functional antagonist to KDR.
DEDICATION

It would like to dedicate this dissertation to my father and mother. During my time here at Clemson University of working towards my degree, I have for the first time in my life genuinely come to realize the meaning of and appreciate the word “family”. My father and mother but also my sister and brother have always given their unconditional love and support throughout my life and in particular during my studies at Clemson University. As I struggled through some difficult moments during my study and seriously contemplated giving up, their love, encouragements and belief in me more than anything gave me the strength and confidence to continue and complete my studies. For that I whole heartedly want to thank them. I sincerely wish this dissertation brings as much joy and satisfaction to them as it does to me because they rightfully deserve it for their many years of sacrifice and perseverance.
ACKNOWLEDGMENTS

I would like to convey my sincerest and deepest appreciation to my advisor and mentor Dr. Wen Y. Chen for all of his tireless efforts in teaching, guidance, support and most of all patience during my graduate studies in his lab. Furthermore I do not believe this work would have been completed without his help that went far and beyond what any advisor would provide and encouragements and friendship during the difficult periods. To have been given the opportunity to get to know him and graduate from his lab was a joy and great privilege. I would also like to thank my committee members Dr. Lyndon Larcom, Dr. Lesly Temesvari and Dr. Tzuen-Rong Tzeng for their support and guidance that has made getting this degree possible.

I would also like to give many thanks to friends and colleagues that have given me support and great memories. John Langenheim for his friendship and humor, Seth Tomblyn for delicious dinners and friendship, Dr. Alison Springs for her warm heart, Dr. Michele Scotti for lending her ear as good friends do and Dr. Isabelle Jacquemart for being a good friend and my favorite tennis partner. And also to Dr. Mike Beck, Dr. Nian Y. Chen, Dr. Susan Peirce and Dr Karl Franek for their invaluable help.

Lastly but not least, I would like to say how much I love and appreciate my wonderful family for giving me their love and support throughout my study. I am very fortunate and grateful to have such a family back home in Korea. My sister, Yun Young, and her husband, Jong Tae Song with my nieces Hyunji and Sooji, for their love and support which I dearly appreciate, my brother Jae Pyo for his frequent phone calls and gifts of moral support, and aunt Bok-In Nam for her caring thoughts and words.
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<tr>
<td>Akt</td>
<td>protein kinase B</td>
</tr>
<tr>
<td>AMD</td>
<td>age-related macular degeneration</td>
</tr>
<tr>
<td>Ang-2</td>
<td>angiopoietin-2</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl-2 associated death promoter</td>
</tr>
<tr>
<td>BS₃</td>
<td>[Bis(Sulfosuccinimidyl) suberate]</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic Fibroblast growth factor</td>
</tr>
<tr>
<td>CDC</td>
<td>Center for Disease Control</td>
</tr>
<tr>
<td>CSS</td>
<td>charcoal-stripped serum</td>
</tr>
<tr>
<td>Da</td>
<td>dalton</td>
</tr>
<tr>
<td>DAG</td>
<td>1, 2-diacylglycerol</td>
</tr>
<tr>
<td>DATS</td>
<td>diallyl trisulfide</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>Flk-1</td>
<td>fetal liver kinase-1</td>
</tr>
<tr>
<td>Flt-1</td>
<td>fms-like tyrosine kinase</td>
</tr>
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- g, gravity
- G3PDH, Glyceraldehyde 3-phosphate dehydrogenase
- HAAE-1, human aortic endothelial cell
- HER2/Neu human epidermal growth factor receptor-2
- HIF, hypoxia inducible factor
- HUVEC, human umbilical vein endothelial cell
- IAP, inhibitors of apoptosis
- IGF-1 insulin-like growth factor-1
- IL-3, interleukin-3
- IL-6, interleukin-6
- IL-8, interleukin-8
- IP3, inositol 1, 4, 5-triphosphate
- KDR, kinase domain region
- LSEC, liver sinusoidal endothelial cell
- MAPK, mitogen-activating protein kinase
- MEK, MAPK/ERK kinase
- MMPs, matrix metalloproteinases
- MMTV, mouse mammary tumor virus
- mRNA, messenger ribonucleic acid
- MT, metallothionein
### Abbreviations (Continued)

<table>
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<tr>
<td>NO,</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NP-1,</td>
<td>neuropilin-1</td>
</tr>
<tr>
<td>NP-2,</td>
<td>neuropilin-2</td>
</tr>
<tr>
<td>PBS,</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PDGF,</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PDGF-BB,</td>
<td>PDGF containing two 109 amino acid residue B chain monomers</td>
</tr>
<tr>
<td>PI₃,</td>
<td>phosphoinositide-3 kinase</td>
</tr>
<tr>
<td>PI-3 K,</td>
<td>phosphotidylinositol-3 kinase</td>
</tr>
<tr>
<td>PIP₂,</td>
<td>phosphatidylinositol 4, 5-bisphosphate</td>
</tr>
<tr>
<td>PKB,</td>
<td>phosphokinase B</td>
</tr>
<tr>
<td>P1GF,</td>
<td>placenta-like growth factor</td>
</tr>
<tr>
<td>PLCγ₁,</td>
<td>phospholipase C gamma-1</td>
</tr>
<tr>
<td>PMSF,</td>
<td>phenylmethylsulphonyl fluoride</td>
</tr>
<tr>
<td>pRb,</td>
<td>retinoblastoma protein</td>
</tr>
<tr>
<td>PRL,</td>
<td>prolactin</td>
</tr>
<tr>
<td>PRLR,</td>
<td>prolactin receptor</td>
</tr>
<tr>
<td>pTEN,</td>
<td>phosphatase and tensin homolog</td>
</tr>
<tr>
<td>RA,</td>
<td>rheumatoid arthritis</td>
</tr>
<tr>
<td>RTK,</td>
<td>receptor tyrosine kinase</td>
</tr>
<tr>
<td>RTKI,</td>
<td>receptor tyrosine kinase inhibitor</td>
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### Abbreviations (Continued)

<table>
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<tr>
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<tbody>
<tr>
<td>RT-PCR</td>
<td>reverse transcription-polymerase chain reaction</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sFlt-1</td>
<td>soluble Flt-1</td>
</tr>
<tr>
<td>STAT</td>
<td>signal transducers and activators of transcription</td>
</tr>
<tr>
<td>TBS-T</td>
<td>tris-buffer saline tween-20</td>
</tr>
<tr>
<td>Tie</td>
<td>angiopoietin receptor</td>
</tr>
<tr>
<td>TGF-α</td>
<td>tumor growth factor alpha</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor beta</td>
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<tr>
<td>TIMPs</td>
<td>tissue inhibitors of metalloproteinases</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
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<td>TSP-1</td>
<td>thrombospondin-1</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<td>vascular endothelial growth factor receptor-1</td>
</tr>
<tr>
<td>VEGFR-2</td>
<td>vascular endothelial growth factor receptor-2</td>
</tr>
<tr>
<td>VHL</td>
<td>von Hippel-Landau</td>
</tr>
<tr>
<td>XIAP</td>
<td>X-linked inhibitor of apoptosis protein</td>
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1. INTRODUCTION

1.1. Angiogenesis

The study of angiogenesis has become an important field not only for developmental biologist but also for scientists studying cancer and other diseases. For higher organisms to develop from an embryo to an adult form, it requires the development of a complicated but essential blood and lymphatic vasculature. This development requires two essential processes, vasculogenesis and angiogenesis. Vasculogenesis is defined as *de novo* formation of vessels from endothelial cell precursors and angiogenesis is defined as the formation of new blood vessels from existing vessels (Ferrara, 2002; Ferrara et al., 2003; Nagy et al., 2002; Olsson et al., 2006; Risau, 1997). Vasculature development initiates with vasculogenesis where progenitor cells are assembled and grown into a primitive vascular network followed by angiogenesis that takes place with the help of pericytes and smooth muscle cells expanding the vascular network (Carmeliet, 2005). This expansion is aided by growth factors that stimulate the progenitor cells to migrate, divide, differentiate and give rise to endothelial cells that eventually form the blood and lymphatic vessels (Cebe-Suarez et al., 2006; Jain, 2003). This development is an essential process because it delivers oxygen, nutrients and other molecules to the tissues in our body for the purpose of organ development during embryogenesis and wound healing in adulthood (Jain, 2003; Otrock et al., 2007).
There are a number of different signal transduction systems involved in vasculogenesis/angiogenesis including vascular endothelial growth factor (VEGF)/VEGF receptor, angiopoietin/Tie receptor, platelet-derived growth factor (PDGF)/PDGF receptor and EprinB2/EphB4 receptor (Alitalo and Carmeliet, 2002; Davis et al., 1996; Ferrara and Davis-Smyth, 1997; Heldin and Westermark, 1999; Olsson et al., 2006; Shibuya et al., 1999; Wang et al., 1998). Studies have demonstrated that among the signal transduction systems listed above the VEGF and its receptor are considered to be the key regulators of angiogenesis (Carmeliet, 2005; Ferrara and Davis-Smyth, 1997; Jain, 2003; Nagy et al., 2002; Olsson et al., 2006; Otrock et al., 2007; Shibuya and Claesson-Welsh, 2006). Experiments with mice have elucidated the importance of VEGF in that inactivation of VEGF to any degree resulted in embryonic lethality or abnormal development such as underdeveloped forebrain, rudimentary dorsal aorta, and defects in vasculature of placenta and nervous system (Carmeliet et al., 1996; Ferrara et al., 1996; Ferrara and Davis-Smyth, 1997; Theiler, 1989). Any disruption of normal expression of wild-type VEGFR in mice also resulted in in utero death, abnormal vasculature channels or failed development of blood islands (Ferrara and Davis-Smyth, 1997; Fong et al., 1995). The Tie receptors are involved in stabilization of the initial endothelial sprout and its interaction with subendothelial cells. And the EprinB2/EphB4 receptors are thought to be involved in arterial-venous specification and in invasive angiogenesis (Adams et al., 1999; Eklund and Olsen, 2006; Fuller et al., 2003; Heroult et al., 2006; Hofer and Schweighofer, 2007; Wang et al., 1998). Additionally, the expression of PDGF/PDFGR on early endothelial precursors helps with the
differentiation of endothelial cells and regulation of vascular/hematopoietic development (Rolny et al., 2006).

In normal human adults angiogenesis is usually kept in a quiescent state by keeping the pro-angiogenic to anti-angiogenic factors in balance. However, when angiogenesis is utilized in either normal physiological process i.e. wound healing or pathological process i.e. cancer, the balance is tilted in either direction that results in blood vessel formation or inhibition of blood vessel formation (Carmeliet, 2005; Ferrara and Davis-Smyth, 1997; Jain, 2003; Olsson et al., 2006). Angiogenesis requires many components to form the blood vessel, like endothelial cells, mural cells and the matrix (Ferrara and Davis-Smyth, 1997; Jain, 2003; Nagy et al., 2002; Olsson et al., 2006; Otrock et al., 2007). The initiation of angiogenesis requires the expression of many pro-angiogenic proteins, such as VEGF, basic fibroblast growth factor (bFGF), Interleukin-8 (Il-8), placenta-like growth factor (P1GF), transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), pleiotrophin and others (Table 1). Among the plethora of factors involved in the multiple-step process required for angiogenesis, VEGF plays an important role not only in initiating vessel formation but also in starting the molecular and cellular events that ultimately form the mature vascular network (Carmeliet, 2005; Cebe-Suarez et al., 2006; Ferrara et al., 2003; Nagy et al., 2002). The cellular and molecular events result in the proliferation, migration and survival of endothelial cells that make up the blood vessels (Ferrara and Davis-Smyth, 1997; Otrock et al., 2007; Shibuya et al., 1999). There are many factors in addition to VEGF that are involved in angiogenesis.
Table 1. Endogenous activators of angiogenesis

<table>
<thead>
<tr>
<th>Proangiogenic Factors</th>
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<tbody>
<tr>
<td>VEGF</td>
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<tr>
<td>α-5 integrins</td>
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<tr>
<td>Angiogenin</td>
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<tr>
<td>Angiopoietin-1 and Angiopoietin-2</td>
</tr>
<tr>
<td>Cyclooxygenase-2</td>
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<tr>
<td>Del-1</td>
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<tr>
<td>bFGF and acidic FGF</td>
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<tr>
<td>Follistatin</td>
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<tr>
<td>G-CSF</td>
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<tr>
<td>Hapatocyte growth factor/scatter factor</td>
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<tr>
<td>IL-8</td>
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<tr>
<td>Leptin</td>
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<tr>
<td>MMPs</td>
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<tr>
<td>Midkine</td>
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<tr>
<td>Nitric oxide</td>
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<tr>
<td>P1GF</td>
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<tr>
<td>Platelet-derived endothelial cell growth factor (PD-ECGF)</td>
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<tr>
<td>PDGF-BB</td>
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<tr>
<td>Pleiotrophin</td>
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<tr>
<td>Proliferin</td>
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<tr>
<td>Tie-2</td>
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<tr>
<td>TGF-α</td>
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<tr>
<td>TGF-β</td>
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<td>TNF-α</td>
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(Ferrara. *Oncologist*. 2004:2-10)
bFGF as a potent stimulator of angiogenesis *in vitro* and *in vivo* can induce proliferation, migration and tubular morphogenesis of the endothelial cells (Presta et al., 2005). Nitric oxide on the other hand is responsible for causing vessels to dilate and become leaky in response to VEGF (Jain, 2003). Angiopoietin-2 aids in sprout formation in the initial step of angiogenesis (Jain, 2003). Matrix metalloproteinases (MMPs), when active, can dissolve the basement membrane and extracellular matrix (ECM) to allow endothelial cells to migrate in the process of angiogenesis (Carmeliet, 2005; Jain, 2003). Angiopoietin-1 functions to recruit mural cells and ECM to stabilize nascent vessels that have formed (Carmeliet, 2005).

As shown in the Table 2 endogenous angiogenic inhibitors exist to keep the balance in favor of inhibiting angiogenesis when it is not needed most of the time. To describe a few of them, thrombospondin-1 (TSP-1), a protein of the extracellular matrix was found to inhibit endothelial cell proliferation, induction of Fas ligand that leads to endothelial cell apoptosis and vascular regression (Sato, 2006). Moreover TSP-1 inhibits various proteases that aid in angiogenesis (Dawson et al., 1997). P53 tumor suppressor has been implicated in suppressing angiogenesis by upregulating TSP-1 production as well (Dameron et al., 1994). Metalloproteinase inhibitors, like tissue inhibitors of metalloproteinases (TIMPs) are another class of angiogenic inhibitors that are able to stop MMPs from degrading the matrix (DeClerck and Imren, 1994; Moses et al., 1990; Murphy et al., 1993). TIMPs are found in variety of cells and tissues and can also inhibit tumor growth and metastasis (DeClerck et al., 1992; Imren et al., 1996; Khokha, 1994; Montgomery et al., 1994).
Table 2. Endogenous inhibitors of angiogenesis

<table>
<thead>
<tr>
<th>Anti-angiogenic Factors</th>
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<tr>
<td>Angiostatin</td>
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<tr>
<td>Calreticulin</td>
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<tr>
<td>Cartilage-derived inhibitor</td>
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<tr>
<td>Endostatin</td>
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<tr>
<td>Fibronectin fragment</td>
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<tr>
<td>Gro-β</td>
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<tr>
<td>Heparinases</td>
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<tr>
<td>Heparin hexasaccharide fragment</td>
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<tr>
<td>Human chorionic gonadotropin</td>
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<tr>
<td>Interferon α/β/γ</td>
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<tr>
<td>Interferon inducible protein-10</td>
</tr>
<tr>
<td>IL-4, IL-12, and IL-18</td>
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<tr>
<td>Kringle 5</td>
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<tr>
<td>Metalloproteinase inhibitors 2-Methoxyestradiol</td>
</tr>
<tr>
<td>Placental ribonuclease inhibitor</td>
</tr>
<tr>
<td>Plasminogen activator inhibitor</td>
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<tr>
<td>Platelet factor-4</td>
</tr>
<tr>
<td>Prolactin 16-kDa fragment</td>
</tr>
<tr>
<td>Proliferin-related protein</td>
</tr>
<tr>
<td>Retinoids</td>
</tr>
<tr>
<td>SPARC fragment</td>
</tr>
<tr>
<td>Tetrahydrocortisol-S</td>
</tr>
<tr>
<td>Thrombospondin-1</td>
</tr>
<tr>
<td>TGF-β</td>
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<tr>
<td>Vasculostatin</td>
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(Ferrara. Oncologist. 2004:2-10)
Another class of inhibitors is the tumor-derived inhibitors that include angiostatin and endostatin. First to be discovered, angiostatin is a proteolytic fragment of plasminogen that is able to inhibit endothelial proliferation and migration (Gately et al., 1996; O'Reilly et al., 1994). Endostatin is a proteolytic fragment of collagen XVIII that is able to inhibit microvascular endothelial cell proliferation as well as primary tumor and metastasis (Kim et al., 2002; O'Reilly et al., 1997; Zetter, 1998). It was revealed that endostatin is able to directly bind VEGFR-2 and prevent VEGF binding thus inhibiting tyrosine phosphorylation and activation of MAPK (Kim et al., 2002).

Angiogenesis is a complex multiple step process with various proteins to coordinate and aid in the growth of a new blood vessel (Figure 1). In a review paper by Papetti and Herman (2002) the normal process of angiogenesis is described in great detail. It begins with the removal of pericytes from the existing vessel to destabilize the endothelium by angiopoietin-2. This causes the endothelial cells to shift from a stable, growth-arrested state to a plastic, proliferative state. Next, basement membrane and extracellular matrix is degraded and then remodeled by proteases such as MMPs. At the same time VEGF causes hyperpermeability of the vessels to allow local extravasation of proteases and matrix components. The new matrix and growth factors induce endothelial cells to migrate and proliferate to form tube-like structures composed of monolayer of endothelial cells for blood to flow. Integrins play an important function, especially, $\alpha_1\beta_1$ and $\alpha_2\beta_1$ in promoting cell migration and matrix reorganization (Senger et al., 1997). Finally, mesenchymal cells proliferate and migrate along the new vessel and differentiate.
Figure 1. **Schematic representation of steps in angiogenesis.** A. Multi-step process in the formation of new blood vessels from existing blood vessel. B. Balance between angiogenesis inhibitors and activators determine the fate of blood vessel formation. In general the balance favors increased inhibitors to maintain angiogenesis “off”.

(from: [http://www.angio.org/understanding/understanding.html](http://www.angio.org/understanding/understanding.html))
into mature pericytes followed by the stabilization of the vessel (Moses, 1997; Papetti and Herman, 2002; Senger et al., 1997).

One of the events that causes new vessel formation is a condition called hypoxia. Hypoxia is usually defined by physiologists and clinicians as a condition of reduced O₂ availability or decreased O₂ partial pressures below critical thresholds, thereby limiting or putting an end to the function of organs, tissues or cells (Zander and Vaupel, 1985; Hockel and Vaupel, 2001). This threshold of O₂ partial pressure in the blood at the venous end of capillaries is found to be around 45-50 mmHg (Hockel and Vaupel, 2001). Having tissues and cells under hypoxia causes upregulation of the expression of many genes involved in vessel formation (Hockel and Vaupel, 2001; Marti and Risau, 1998). A significant experiment was carried out in the early 1990’s to show that hypoxia could induce PDGF and VEGF mRNA expression in cultured cells (Kourembanas et al., 1990; Shweiki et al, 1992). In addition to genes of VEGF and PDGF, hypoxia also regulates angiopoietin-2 and nitric oxide synthase (Petrova et al., 1999). Moreover, hypoxia within the tumor is an important stimulator of tumor angiogenesis (Zetter, 1998).

Angiogenesis plays a crucial role in a number of physiological processes in addition to embryonic development including normal growth and development of vessels, wound healing, reproductive cycling and ocular maturation (Ferrara and Davis-Smyth, 1997; Jain, 2003). As VEGF is the major regulator of physiological angiogenesis, its expression in kidney glomerulus, pituitary, heart, lung, and brain suggest not only proliferative role but also maintenance role of differentiated state of blood vessels (Ferrara and Davis-Smyth, 1997; Ferrara et al., 1992; Hollenberg et al., 1977; Monacci et
al., 1993). In cases of cutaneous wounds, VEGF and angiopoietin-2 expression is increased until the vascular network is established and then expression is down-regulated (Jain, 2003).

1.2. Lymphangiogenesis

The development of new lymph vessels is an important part of the circulatory system that is closely integrated with angiogenesis. The lymphatic vasculature is involved in a network of vessels that drains interstitial fluid from the tissues and returns it to the blood (Alitalo et al., 2005). As part of the larger lymphatic system, that encompasses the bone marrow, spleen, thymus and lymph nodes, the lymphatic vessels function to remove parts of microbes or dead cells that are broken down by our body’s immune system. The protein-rich plasma that is leaked from the capillaries is composed of extravasated leukocytes, and activated antigen presenting cells that are returned to the blood circulation through the lymphatico-venous junctions (Alitalo et al., 2005). Lymphatic vasculature system functions as a secondary vasculature system for the maintenance of balanced fluids (Alitalo et al., 2005). Therefore, the lymphatic vessel is an essential part of the body’s immune system and plays a crucial part in pathogenesis such as cancer.

The formation of new lymph vessels requires VEGF-C and D activators and their respective receptor, the VEGFR-3 (Achen et al., 1998; Jeltsch et al., 1997; Joukov et al., 1996; Kaipainen et al., 1995; Makinen et al., 2001). During the stage of the embryo, VEGFR-3 is involved in blood vascular development but later its expression is mainly involved in sprouting of the lymphatic vessels (Oliver and Detmar, 2002). Experiments
conducted in mice with a homozygous deletion of $Vegfc$ resulted in complete absence of lymphatic vasculature and Vegfc-null mice exhibited incomplete formation of lymph sacs (Karkkainen et al., 2004). This demonstrated VEGF-C functions as an activator of lymphangiogenesis. The binding of VEGF-C to the receptor leads to tyrosine phosphorylation and signal transduction through various signaling molecules (i.e. Erk-1/2; Akt; JNK 1/2) to finally stimulate the lymphatic endothelial cell migration, proliferation and survival (Dixelius et al., 2003; Salameh et al., 2005). Further study is needed to elucidate the exact signal transduction pathway.

As angiogenesis is governed by maintaining the balance between pro-angiogenic factors to angiogenic inhibitors, it is vital to regulate the balance of factors to respond to the needs of the physiological signals. But when the balance is broken to favor one side or the other, it usually leads to a many types of disease. For instance, in cancer, diabetic eye disease and rheumatoid arthritis, over expression of angiogenic factors promote the disease and destroy healthy tissues. On the other hand with insufficient angiogenesis due to the presence of overwhelming angiogenic inhibitors, inadequate blood vessel growth can be the underlying reason for conditions such as heart disease, stroke, and delayed wound healing.

1.3. Factors in Angiogenesis

1.3.1. VEGFs

VEGF, as part of the PDGF family of growth factors, is one of the most crucial and specific pro-angiogenic factor that regulates angiogenesis with high specificity for vascular endothelial cells (Gupta and Zhang, 2005; Neufeld et al., 1999). It is a disulfide-
linked homodimeric glycoprotein with a molecular weight of 40,000-46,000 Da (Ferrara et al., 2003; Ferrara et al., 1992). The dimer forms an anti-parallel orientation with receptor binding site located at the poles of the dimer (Olsson et al., 2006). VEGF can be subdivided into seven members, VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F and placental growth factor (P1GF) (Otrock et al., 2007). Among the members, VEGF-A, with its VEGF Receptor-2, is considered to be the major mediator of mitogenesis and angiogenesis (Ferrara and Kerbel, 2005). VEGF is produced in many different cell types, mainly by hematopoietic, stromal and endothelial cells in response to hypoxia and also when specific growth factors or differentiation factors are encountered. These factors that increase VEGF production include FGF-4, PDGF, TNF-α, TGF-β, keratinocyte growth factor, IGF-1, IL-8β, IL-6 and nitric oxide (Neufeld et al., 1999). Transformed cells, like cancer cells are also able to produce VEGF. Upon binding of VEGF to the extracellular domain of the receptor, dimerization and autophosphorylation of the intracellular receptor tyrosine kinase activates the cascade of downstream signaling proteins (Otrock et al., 2007).

Initially VEGF-A was described as a vascular permeability factor secreted by tumor cells (Senger et al., 1997). Increased vascular permeability permits extravasation of plasma proteins and forms favorable matrix environment to allow migration of endothelial and stromal cells (Papetti and Herman, 2002). VEGF-A is known to play a role both in normal and tumor associated angiogenesis. VEGF-A found on the short arm of chromosome 6 is made up of eight exons, separated by seven introns (Ferrara and Davis-Smyth, 1997). VEGF-A can form six different size isoforms based on alternate
splicing of the gene to produce mature forms of VEGF121, VEGF145, VEGF165, VEGF183, VEGF189, and VEGF206 with numeric designation referring to the number of amino acids in the molecule (Figure 2). Of these, only VEGF121, VEGF145, and VEGF165 are found to induce proliferation of endothelial cells and angiogenesis. Among the isoforms VEGF165 is the most abundantly expressed form and found to be over-expressed in many solid tumors (Ferrara and Davis-Smyth, 1997; Otrock et al., 2007; Stimpfl et al., 2002). These different VEGF isoforms have differing heparin binding ability and binding specificity to cell surface receptors such as VEGFR-1, VEGFR-2, neuropilin-1 and neuropilin-2 and as to their biologic effects (Neufeld et al., 1999; Otrock et al., 2007).

Effects of VEGF-A have been investigated in much detail since its discovery. It is known to have the most potent angiogenic properties compared to other angiogenic factors such as bFGF, PLGF and PDGF that can induce proliferation, sprouting, migration and tube formation of endothelial cells (Otrock et al., 2007; Papetti and Herman, 2002). It can also induce endothelial cells to produce anti-apoptotic proteins to prolong survival. VEGF-A is also involved in the induction of endothelial nitric oxide synthase that leads to increased nitric oxide production (Otrock et al., 2007). In vivo, VEGF-A is a key protein in angiogenesis and vasculogenesis. As shown by Carmeliet et al. (1996) deletion of VEGF-A gene in mice resulted in vascular defects and death at embryonic day 9.5-10.5 (E9.5-10.5), mouse heart developmental stage specifically the blood progenitor development. A single VEGF allele loss also resulted in vascular defects and death at E11-12. Over-expression of VEGF-A in transgenic mice has lead to
Figure 2. VEGF isoforms. VEGF gene is located on the short arm of chromosome 6. It is composed of 8 exons and is differentially spliced to produce five different amino acid isoforms with varying lengths denoted with number of amino acids. VEGF 165 is the predominant isoform and is highly expressed in many solid tumors.
a high level of cutaneous angiogenesis and a psoriasis-like skin condition and tumor growth (Larcher et al., 1998; Xia et al., 2003). When VEGF-A was mutated it was responsible for delaying the process of wound healing (Rossiter et al., 2004). In pathological conditions, VEGF-A is found to be over-expressed in many solid tumors and in some hematological malignancies. This lead to the finding that the level of VEGF-A expression correlates with the disease progression and survival of certain cancers (Larcher et al., 1998).

VEGF-B is different in comparison to VEGF-A in that it only binds to VEGFR-1 and not VEGFR-2 or VEGFR-3 (Otrock et al., 2007). It contains eight exons and six introns and yields two isoforms by alternative splicing. It is also different in that it is not regulated by hypoxic conditions as seen with VEGF-A (Ferrara and Davis-Smyth, 1997; Neufeld et al., 1999). Based on the fact that VEGF-B binds VEGFR-1 exclusively and induces poor mitogenic effects on endothelial cells VEGF-B may be a weak mitogen, not essential for angiogenesis (Otrock et al., 2007; Papetti and Herman, 2002). In an experiment using a mice that were deficient in VEGF-B, the mice were found to be normal with minor cardiac defects (Papetti and Herman, 2002). The precise function of VEGF-B is still under investigation but it has been found to be able to regulate cell proliferation and vessel growth and inflammatory angiogenesis in vivo studies (Otrock et al., 2007).

VEGF-C/VEGF-D both act as ligands for VEGFR-2 and VEGFR-3 and both can induce angiogenesis of the lymphatic vessels (Neufeld et al., 1999). VEGF-C and D
form a homodimer by proteolytic activity in the extracellular space and are able to bind to either a homo or heterodimer of VEGFR-3 and VEGFR-2 receptor (Alitalo et al., 2005).

VEGF-E is another member of the VEGF family and shares only 20-25% amino acid identity with VEGF-A (Otrock et al., 2007). It was originally found in sheep and goats infected by the orf virus, a parapoxvirus (Otrock et al., 2007). It has a strong affinity to VEGFR-2 to induce autophosphorylation and rise in intracellular Ca2+ concentration. With its strong angiogenic properties it would be a good candidate for treatment in pro-angiogenic therapy.

VEGF-F has been identified most recently from snake venom (Suto et al., 2005). It shares 50% structural homology with VEGF165 and binds with VEGFR-2. P1GF, originally isolated from the placenta, is also expressed in heart and lungs. P1GF binds to VEGFR-1 and Neuropilin-1 and plays an important role in angiogenesis (Papetti and Herman, 2002). It has been shown that the loss of P1GF impaired angiogenesis, collateral growth during ischemia, wound healing, inflammation and cancer (Papetti and Herman, 2002).

1.3.2. VEGF Receptors

VEGF receptors belong to the type III receptor tyrosine kinase family of receptors and are closely related to the PDGF receptors. VEGF receptors can be divided into VEGFR-1/Flt-1, VEGFR-2/Flk-1/KDR, and VEGFR-3/Flt-4 (Matsumoto and Mugishima, 2006; Otrock et al., 2007). VEGFR-2 and 3 have about 80% homology to VEGFR-1 in the tyrosine kinase domain (Shibuya and Claesson-Welsh, 2006). All three receptors are known to produce different forms of the receptor by alternative splicing.
Both VEGFR-1 and VEGFR-2 are found in soluble forms and VEGFR-3 can form two isoforms with different C-terminal ends (Shibuya and Claesson-Welsh, 2006). They bind different but specific types of VEGF ligands as shown on Figure 3 (Otrock et al., 2007; Shibuya and Claesson-Welsh, 2006). With the findings that VEGFR mRNA is highly expressed in yolk sac, intraembryonic mesoderm, angioblasts, endocardium, and small and large vessel endothelium but down-regulated in adult endothelial cells strongly suggested an essential role for VEGFR in vasculogenesis and angiogenesis (Ferrara and Davis-Smyth, 1997). This also suggested VEGFR can be used as an early marker for endothelial cell precursors. VEGFRs are found mainly expressed on endothelial cells but also on hematopoietic cells, macrophages, malignant cells and vascular smooth muscle cells (Cebe-Suarez et al., 2006).

The structure of the receptor can be divided into three domains, the extracellular immunoglobulin (Ig)-like domain, transmembrane domain, tyrosine kinase domain with carboxy terminal region at the end as shown in Figure 4 (Otrock et al., 2007; Shibuya, 2006). The extracellular domain of VEGFRs is composed of seven Ig-like domains. Some of the domains are thought to be involved specifically in ligand binding and/or receptor dimerization. Similar to the mechanism of known RTKs, ligand binding induces VEGFR dimerization causing juxtaposition of cytoplasmic tyrosine kinase domain resulting in transphosphorylation of specific amino acids and finally signal transduction (Ferrara et al., 2003; Tao et al., 2001). It was found that tyrosine kinase activity of the VEGFR-2 is ten fold stronger than VEGFR-1, which strongly supported VEGFR-2 to be
Figure 3. Interactions of VEGF family of proteins with its receptors (VEGFR-1, -2, -3, co-receptors neuropilin -1, -2). Ligand binding domain of each receptor is shaded in grey. As shown in the diagram, activation of VEGFR-1 and -2 can result in blood vessel formation while activation of VEGFR-2 and -3 can result in lymphatic vessel formation. Co-receptors NP-1 and NP-2 act to facilitate binding of specific VEGF ligand to the specific VEGF receptor to initiate angiogenic properties.
Figure 4. Structure of VEGFR-2 (KDR, human; Flk-1, mouse). VEGFR-2 is a type III transmembrane kinase receptor. The extracellular portion is composed of seven immunological-like (Ig) domain of which Ig2 and Ig3 is involved in binding VEGF. It is suggested that Ig4 is involved in receptor dimerization. Intracellular portion contains two kinase domains of which five major tyrosine phosphorylation sites have been highlighted (Y951, Y1054, Y1059, Y1175, Y1214).
the main inducer of VEGF (Ferrara et al., 2003; Holmes et al., 2007). In addition to the VEGF-VEGFR interaction, there are other co-receptors that influence the signaling. Neuropilins, heparan sulfate, integrins and cell adhesion molecules such as vascular endothelial cadherin are known co-receptors (Shibuya and Claesson-Welsh, 2006).

VEGFR-1 consists of 1338 amino acids and has a molecular weight of 180 kDa (Ferrara et al., 2003). It is composed of seven extracellular Ig-like domains, a single transmembrane domain and a tyrosine kinase domain (Shibuya and Claesson-Welsh, 2006; Shibuya et al., 1999). It binds VEGF-A, B and P1GF with high affinity. VEGF-A is measured to bind with an order of magnitude higher affinity to VEGFR-1 (K_d = 10-30 pM) than to VEGFR-2 (K_d = 75-125 pM) (Gibbs, 2000). VEGFR-1 is expressed on endothelial cells, osteoblasts, monocytes/macrophages, placental trophoblasts, renal mesangial cells and some hematopoietic stem cells (Otrock et al., 2007). VEGFR-1 on endothelial cells functions to help develop normal blood vessels during embryogenesis but the expression on non-endothelial cells suggests it may play a role in cell survival (Shibuya and Claesson-Welsh, 2006). In terms of VEGFR-1 gene regulation, the gene is upregulated by hypoxic condition via HIF-1 dependent pathway and upon activation by macrophages (Shibuya and Claesson-Welsh, 2006). There is evidence that indicates VEGFR-1 plays significant roles in hematopoiesis, recruitment of monocytes and bone marrow-derived progenitor cells to promote angiogenesis and induction of matrix metalloproteinases. (Gerber et al., 2002; Hattori et al., 2002; Luttun et al., 2002). In an experiment with liver sinusoidal endothelial cells (LSEC), VEGFR-1 caused release of
hepatocyte growth factors, IL-6 that promoted proliferation of hepatocytes when co-cultured with LSECs (LeCouter et al., 2003). Tumor cells in some cancers have been shown to express VEGFR-1 which may mediate chemotactic signaling in growth of the cancer (Ferrara and Kerbel, 2005).

In addition, the study of VEGFR-1 has shown it to be more complex in regulating angiogenesis. It has been found that it can exist endogenously in the form of soluble Flt-1 (sFlt-1) that selectively inhibits VEGF by sequestering VEGF to prevent interactions with VEGFR-2 (Ferrara and Kerbel, 2005; Hasumi et al., 2002). It has been shown that sFlt-1 can suppress endothelial cell proliferation activity and angiogenesis in vivo of malignant ascites tumors in ovarian cancer cells (Hasumi et al., 2002). Soluble Flt-1 can also heterodimerize with the VEGFR-1 and VEGFR-2 receptors by binding to the ligand binding region and inhibiting downstream signal transduction (Kendall et al., 1996). Therefore it has some regulatory function that helps regulate VEGF-induced VEGFR-2 activation. Soluble VEGFR-1 has been implicated in the preeclampsia, a disorder that occurs during pregnancy (Smith et al., 2007).

VEGFR-2 is mostly expressed on endothelial cells in adults and highly expressed on vascular endothelial progenitors in early embryogenesis (Shibuya, 2006; Shibuya and Claesson-Welsh, 2006). VEGFR-2 is also expressed on hematopoietic cells that can later develop into endothelial cells and non-endothelial cell types such as neuronal cells, liver, colon, placenta, osteoblasts, pancreatic duct cells, retinal progenitor cells and megakaryocytes (Neufeld et al., 1999; Shibuya, 2006; Stewart et al., 2003). Phosphorylated VEGFR-2 was also detected on tumor cells including breast carcinomas,
colonic carcinomas and non-Hodgkin’s lymphomas while VEGFR-2 has been found expressed on various cancer cells (Stewart et al., 2003). VEGFR-2 is very similar to VEGFR-1 in that it has seven extracellular Ig-like ligand-binding domains, a transmembrane domain and tyrosine kinase domain. It is initially produced as a 150 kDa protein and then it is further glycosylated to produce a mature protein on the cell surface with a size of 230 kDa (Otrock et al., 2007). Recently, a soluble truncated form of the VEGFR-2 (sVEGFR-2) has been reported from mouse and human plasma but its function has not been yet fully elucidated (Gatto and Cavalli, 2006).

The human VEGFR-2 gene is located on chromosome 4q11-q12 and encodes 1356 amino acids length receptor. VEGFR-2 binds VEGF-A, C, D, E and F. Human and murine forms of VEGFR-2, share 85% of the amino acid sequence homology (Ferrara and Davis-Smyth, 1997). Experiments using receptor mutagenesis and in vivo mapping identified phosphorylation sites Y951, Y1054, Y1059, Y1175 and Y1214, with Y1175 site as the most crucial tyrosine for VEGF-dependent endothelial cell proliferation (Cebe-Suarez et al., 2006; Lamalice et al., 2006; Otrock et al., 2007; Shibuya, 2006). The importance of the tyrosine 1175 was shown in a study with knock-in mutant mice that replaced Y1175 with a phenylalanine in humans that resulted in lethality during embryogenesis by disrupting vasculogenesis/angiogenesis (Sakurai et al., 2005). Tyrosine 951 has been found to be associated with T-cell specific adapter that leads to actin stress fiber organization and migration of endothelial cells in response to VEGF-A (Shibuya and Claesson-Welsh, 2006). In addition, Src-dependent focal adhesion kinase (FAK), PI-3 kinase, PLCγ−1 regulates actin organization and cell migration (Cebe-
Suarez et al., 2006; Chou et al., 2002). Down-regulation of VEGFR-2 occurs through dephosphorylation, internalization into endocytic vesicles and finally degradation by lysosomes (Olsson et al., 2006). Dephosphorylation can be accomplished by direct association with phosphatases or indirectly by association with other receptors carrying SH-1 and -2 phosphatases (Olsson et al., 2006).

As VEGF is the most potent ligand for angiogenesis, its receptor, VEGFR-2 is the major signal transducer of physiological and pathological angiogenesis. It functions to regulate vascular endothelial cell migration, proliferation, differentiation, and survival with vessel permeability and dilation (Cebe-Suarez et al., 2006). The importance of the receptor has been seen in many experiments. When the gene for VEGFR-2 was inactivated, embryonic death occurred due to absence of vasculogenesis and poor hematopoietic development (Shalaby et al., 1995). In another experiment, the importance of VEGFR-2 in the formation of blood vessels was apparent when it was required for the differentiation of endothelial cells and the movement of precursor endothelial cells from the posterior primitive streak to the yolk sac (Shalaby et al., 1997). In tumor vasculature, the expression of VEGFR-2 is found to be 3 to 5 fold higher than in normal vasculature (Shibuya, 2006).

VEGFR-3 is the least well known among the VEGF receptors. During embryogenesis, it is involved in blood vascular development (Shibuya and Claesson-Welsh, 2006). By the time embryo becomes an adult it is virtually only expressed on lymphatic endothelial cells of the vein but not artery and considered to be involved in mediating lymphangiogenesis (Matsumoto and Mugishima, 2006). It has only six Ig-like
domains and preferentially binds to VEGF-C and D (Alitalo et al., 2005). These ligands and VEGFR-3 have been implicated in vascular tumors, solid tumors such as breast and prostate cancers and lymph node metastasis (Otrock et al., 2007). Thus this receptor is believed to play roles in both vascular development and lymphangiogenesis.

In addition to the three VEGF receptors there are neuropilin receptors that function as co-receptors of VEGF. As co-receptors of VEGF, neuropilin receptors function to aid in VEGFR-1 and VEGFR-2 receptor mediated angiogenesis. Neuropilin-1 (NP-1) was first discovered as a glycoprotein involved in the development of axon and neuron (Miao and Klagsbrun, 2000). It has now been shown to be involved in formation of capillaries and blood vessels by its expression on arterial endothelial cells and many organs (Miao and Klagsbrun, 2000; Otrock et al., 2007; Shibuya and Claesson-Welsh, 2006). NP-1 binds VEGF-A, B and P1GF and acts as a co-receptor enhancing VEGF-A-VEGFR-2 interaction (Miao and Klagsbrun, 2000; Otrock et al., 2007; Shibuya and Claesson-Welsh, 2006). Based on RNA interference-mediated silencing of NRP-1, NP-1 was shown to function in attaching human endothelial cells to extracellular matrix proteins (Murga et al., 2005). It also forms a complex with VEGFR-1 and increases tumor angiogenesis in vivo by enhancing VEGF165 binding to surrounding endothelial cells (Otrock et al., 2007; Soker et al., 1996). Neuropilin-2 (NP-2) binds VEGF-A, C and P1GF and is also linked to lymphatic vessel development (Miao and Klagsbrun, 2000; Otrock et al., 2007). It has also been found that neuropilin-2 acts as co-receptor to the activation of VEGFR-3, because mice deficient in neuropilin-2 develop lymphatic hypoplasia (Yuan et al., 2002).
1.4. Regulation of VEGF and VEGFR

We understand that beyond a certain size simple diffusion cannot provide adequate oxygen to the cells and tissues. This requires a metabolic regulatory system to detect low oxygen level and initiate various pathways to deliver oxygen. Among many cellular and systemic responses to hypoxia, angiogenesis is one that responds to hypoxia. Within angiogenesis lies a complex process that involves the expression of multiple gene products in multiple steps to establish a network of new blood vessels. An example is the upregulation of VEGF-A gene expression by the actions of transcription factor hypoxia-inducible factor (HIF) in concert with von Hippel-Landau (VHL) tumor suppressor gene under hypoxic condition (Namiki et al., 1995; Olsson et al., 2006). HIF binds to the cis elements in the VEGF promoter and increases the VEGF gene transcription (Papetti and Herman, 2002). VHL inactivation results in the increased expression of VEGF (Neufeld et al., 1999). Hypoxia can also have an effect on the increased expression of VEGF in other ways like increasing the stability of mRNA by binding proteins to specific sequences in the 3’ UTR and inducing the expression of VEGF receptors (Blechman et al., 1995; Stein et al., 1998). Under normoxic conditions, HIF is targeted for ubiquitination and ultimately degradation by VHL (Ferrara and Kerbel, 2005). In conditions of cell culture, hypoxia was shown to be able to induce endothelial cell migration and tube formation (Pugh and Ratcliffe, 2003).

In contrast to hypoxic condition, a hypoxia-independent pathway can also induce VEGF production. Cytokines or growth factors are able to upregulate VEGF mRNA expression and/or induce release of VEGF ligand. Furthermore, cell differentiation and
transformation has been shown to play a role in regulation of VEGF gene expression. Changing of 3T3 preadipocytes into adipocytes and exposure to luteotrophic hormone are instances of induction of VEGF expression (Ferrara and Davis-Smyth, 1997). In tumorigenic cells, inactivation of p53 tumor suppressor protein or amplification of \( \text{ras} \) or exogenous growth factors or hormones can activate VEGF production as well (Neufeld et al., 1999).

It is suggested that conformational changes in the Ig-like domain four of VEGF receptor is induced by ligand binding and this might promote receptor dimerization, either homo or hetero dimerization (Blechman et al., 1995; Tao et al., 2001). Based on electron microscopy, it was suggested that the binding of dimeric VEGF ligand to the Ig-like domain 2 and 3 of one receptor monomer, increases the chance for the second receptor to bind to the first ligand-receptor complex to form the receptor dimer (Ruch et al., 2007). Once the receptor dimerizes it is thought to lead to structural changes in the intracellular kinase domain. Based on studies with PDGF receptors, transmembrane domain of VEGF receptor is thought to be involved in regulating receptor kinase activity (Cebe-Suarez et al., 2006). In the case of VEGFR-2, VEGF-A binding to it can lead to different pathways that result in different angiogenic phenotype (Figure 5). As stated before VEGFR-2 activates the Ras-dependent signaling pathway involving Raf-MEK-ERK1/2 (Cebe-Suarez et al., 2006; Otrock et al., 2007; Rousseau et al., 1997; Shibuya, 2006). This is the pathway for induction of endothelial cell proliferation. Within this pathway, tyrosine 1175 plays an important role in VEGF induced stimulation of mitogenesis.
Figure 5. Schematic representation of VEGFR-2 signaling. Binding of the VEGF ligand induces dimerization of the receptor which in turn activates downstream signaling pathway that ultimately causes proliferation, migration, survival and increased permeability of the endothelial cells.
Once the phosphorylated Y1175 is bound by phospholipase-Cγ (PLCγ), PLCγ itself is tyrosine phosphorylated and activated to produce 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP₃) from hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) (Cebe-Suarez et al., 2006; Gille et al., 2001; Shibuya, 2006; Wu et al., 2000). Protein Kinase C is activated by DAG and leads to Raf-MEK-ERK1/2 pathway activation (Cebe-Suarez et al., 2006). Finally activating transcription factors, like MAP kinases, translocate to the nucleus and regulate gene expression (Cebe-Suarez et al., 2006).

Migration of endothelial cells is an important step in angiogenesis and involves a number of signaling pathways. Tyrosine residues 1175, 1214, and 951 have been implicated in p38 MAPK activation by VEGF induced VEGFR-2 activation to be phosphorylated in cell migration, permeability and survival (Gille et al., 2001; Lamalice et al., 2006; Rousseau et al., 1997; Zeng et al., 2001). A pathway different from Raf-MEK-ERK1/2 pathway involving VEGF induced activation of VEGFR-2 is the PI3-kinase-Akt/PKB pathway that is thought to be involved in vascular permeability and endothelial cell survival (Shibuya and Claesson-Welsh, 2006; Yu and Sato, 1999). Vascular permeability involves nitric oxide (NO) generated by endothelial nitric oxide synthase (eNOS), which in turn is activated by phosphorylation of serine 1179 of eNOS mediated through Akt/PKB pathway (Shibuya and Claesson-Welsh, 2006; Yu and Sato, 1999). PI3 kinase/Akt phosphorylates Bcl-2 associated death promoter (BAD) and caspase 9 to inhibit their apoptotic activity and prevent phosphorylation of p38 MAPK and reduce caspase-3 activity to promote survival (Holmes et al., 2007; Yilmaz et al., 2003). It was shown, apoptosis induced by serum starvation in cultured cells was
protected by VEGF. VEGF-A also induced expression of anti-apoptotic proteins, Bcl-2, A1 and inhibitors of apoptosis (IAP) family proteins, XIAP and surviving (Ferrara and Davis-Smyth, 1997; Yilmaz et al., 2003). Integrins αvβ3 also play a role in survival of the cells through interaction with VEGFR-2 (Holmes et al., 2007).

1.5. Cancer and Angiogenesis

As cancer is the second leading cause of death in U.S. after heart disease in 2004 it has taken the lives of 553,888 people as reported by CDC (Center for Disease Control). Among cancer patients, metastasis is the leading cause of death. It has been a long and difficult pursuit for many years by many doctors and scientists to find a permanent cure to treat cancer. But with great strides in extending the survival time of many cancer patients and increasing the quality of life for millions of cancer patients it still is ranked second in causes of death reported by CDC.

It is a disease that can be defined as a population of host’s cells that lose homeostasis and proliferate abnormally due to its defects in growth regulation. This loss of control is the result of accumulation of multiple genetic changes over time leading to the acquisition of growth advantages that transform normal cells into cancer cells (Weinberg, 2007). These genetic changes lead to sequential activation of oncogenes or loss of tumor suppressor genes. In a paper by Hanahan and Weinberg (2000), they describe a set of molecular, biochemical and cellular characteristics that are common to most if not all types of cancer. These are shown in Figure 6 with six essential changes
Figure 6. Hallmarks of Cancer. This describes the 6 different traits that are commonly found in all cancer cells. The traits can exist as single trait or in multiple traits, nonetheless they are found in all transformed cancer cells. One trait that pertains to my research is sustained angiogenesis. This trait allows for the tumor to grow and eventually metastasize to secondary location to invade and grow.

(Hanahan and Weinberg. Cell. 2000 100:57)
that a cell acquires within the cell’s physiology that ultimately results in transformation into cancer cell.

The first acquired trait of the cancer cell is its ability to generate growth signals to which they become responsive, overexpress growth signal receptors and reduce the dependency on exogenously produced signals. Examples are production of PDGF, Tumor growth factor α (TGFα) and HER2/neu receptor by glioblastomas, sarcomas and mammary carcinomas, respectively (Hanahan and Weinberg, 2000). The second acquired trait is the ability to ignore anti-growth signals. For cells to become quiescent or enter post-mitotic state, retinoblastoma protein (pRb) plays a major role, but TGFβ acts to block pRb actions in number of ways to allow cells to proliferate (Hanahan and Weinberg, 2000). Furthermore, to escape entering into state of terminal differentiation, overexpression of c-Myc oncoprotein has been known to prevent differentiation and promote growth. The third acquired trait is the avoidance of apoptosis (Hanahan and Weinberg, 2000). One of the most common ways to avoid apoptosis has been to inactivate p53 tumor suppressor gene that is seen in over 50% of human cancers. Other ways have included activation of survival signaling pathway by insulin-like growth factor (IGF-1/2) or Il-3, loss of the pTEN tumor suppressor and short circuit the FAS death signal (Zetter, 1998).

The fourth acquired trait is to have an infinite replicative potential or immortalization (Hanahan and Weinberg, 2000). Since all types of mammalian cells have an intrinsic characteristic of limited replication and eventually die due to the shortening of telomeres, cancer cells have found a way to maintain the length of
telomeres above the critical length to replicate without limit. The importance of telomeres in cells that become immortal has been shown by the expression of telomerase in a variety of normal cells that allow it to become immortal cells or late passage cells avoid cell death when telomerase is supplied to regenerate the shortened telomeres (Weinberg, 2007). The fifth acquired trait and one that is the main focus of my research is sustained angiogenesis (Hanahan and Weinberg, 2000). For tumors to grow and proliferate, tumor cells need to turn on the “angiogenic switch” or to tilt the balance toward positive angiogenic factors. This is accomplished by upregulating the VEGF and/or FGF genes and/or downregulating the expression of endogenous inhibitors, such as thrombospondin-1 or β-interferon. In studying the steps involved in tumorigenesis of transgenic mice, angiogenesis was found to be activated in mid-stage lesions, before developing into full-grown tumors. The sixth acquired trait is tissue invasion and metastasis (Hanahan and Weinberg, 2000). This causes 90% of human cancer deaths. The primary tumor is able to invade surrounding tissues and escape to distant sites to re-establish a new colony. For this to happen, there are factors like cell-cell adhesion molecules, E-cadherin and integrins and extracellular proteases that gets altered to facilitate tissue invasion and metastasis (Hanahan and Weinberg, 2000).

These traits that are listed above are acquired through changes in the genome of cancer cells but are rarely acquired as permanent traits because of cells’ own repair mechanisms (Weinberg, 2007). But cancers do occur and in increasing numbers and it is this genetic instability that may be the underlying event that leads to acquiring the traits. p53 tumor suppressor protein is responsible for DNA repair or apoptosis in cases of
irreparable damage malfunctions in most if not all human cancers (Hanahan and Weinberg, 2000). A correlation has been established between production of angiogenic factors and relapse, metastasis and poor prognosis in human cancer patients. VEGF-positive tumors exhibited worse prognosis than those of VEGF-negative tumors. Decreased survival was correlated with increased serum VEGF level in breast, ovarian, lung, gastric, and colon cancer patients (Gibbs, 2000). Thus angiogenesis can affect the outcome in human cancer. Angiogenic factors have been detected in tumors, body fluids such as serum, urine, and ocular fluids (Zetter, 1998). It has been shown in breast cancer patients that increased VEGF production correlates with early relapse (Toi et al., 1994). Increased vascular density, another characteristic of tumor angiogenesis has been shown to be directly correlated with increased metastasis and decreased survival in many cancers, such as breast, prostate, lung, stomach, cervix, ovary and squamous cell carcinoma of the head and neck (Zetter, 1998).

1.6. Tumor Angiogenesis

With the vast amount of new knowledge being acquired about cancer and its process, understanding the tumor microenvironment, such as the tumor vasculature has expanded our understanding of cancer as a disease of a closed system i.e. a single cell to a much bigger and dynamic system consisting of tissues, organs and the surrounding environment. With the better understanding of cancer as a much complex system, it has inspired novel ideas for developing new therapeutics to treat cancer.

As the tumor mass grows in size, the cells within the tumor are further removed from the nearest blood vessels and are deprived of oxygen. This results in hypoxic
pockets within the tumor. It was first hypothesized that for a tumor to grow beyond a certain size (1-2 mm) tumor angiogenesis was an essential requirement that was initiated by cancer cells in the early steps of tumorigenesis (Folkman, 1971). This was shown in an experiment where murine tumors implanted into canine thyroid glands remained viable but did not grow until transplanted into highly vascularized mice (Folkman, 1972). For many years following this experiment, numerous studies have shown that tumor growth is angiogenesis dependent (Cao, 2005; Folkman, 2003; Papetti and Herman, 2002; Zetter, 1998). This concept is now accepted as one of the hallmarks of cancer, as shown in Figure 6 (Hanahan and Weinberg, 2000). This also became the basic concept of anti-angiogenic therapy where inhibition of angiogenesis would cause tumors to regress and die or at least become dormant due to oxygen depravation and starvation. The realization that tumor angiogenesis is not only essential for growth of the tumor but also as an avenue for tumor cells to metastasize to distant sites was an important finding in cancer research. Furthermore lymph vessels are also implicated in providing tumor cells with an escape route to metastasize to distant sites (Webb and Vande Woude, 2000).

In the process of tumor progression, an “angiogenic switch” is used to describe a phenomenon where tumor cells take the first step towards tumor vascularization. This “switch” is the tilting of the balance of positive and negative angiogenic regulators towards more positive factors. These pro-angiogenic factors are released from tumor cells, fibroblasts, macrophages and infiltrating leukocytes to promote tumor angiogenesis (Webb and Vande Woude, 2000). Lin et al. (2006) showed in a mouse breast cancer model that tumor-associated macrophages release pro-angiogenic factors that promote
tumor angiogenesis and metastasis. In addition, repression of critical inhibitors of angiogenesis, like thrombospondin-1 is imperative for tumor angiogenesis to begin (Watnick et al., 2003). For tumor angiogenesis, conditions that favor increased gene expression of pro-angiogenic factors like VEGF depend on physiological stimuli hypoxia, which results from increased tumor mass, and by oncogene activation or tumor suppressor mutation and the effects of cytokines (Ferrara and Kerbel, 2005; Stimpfl et al., 2002).

Because solid tumors require neovascularization for continual proliferation and metastasis, understanding tumor angiogenesis is vital to the development of cancer therapeutics. One of the characteristics of a solid tumor environment is the formation of abnormal vasculature that is different from normal vasculature via the effects of VEGF and other angiogenic factors (Ruoslahti, 2002). Tumor vasculature is characterized as having a chaotic tumor vessel organization where hypoxic and avascular areas occur and structure of vessels have uneven vessel diameters, imperfect lining of the endothelial cells and increased density of immature, highly permeable blood vessels. (Carmeliet, 2005). This results in tumors having blood vessel networks that are unorganized and leaky. In addition, lymphatic vessels in tumor also develop abnormally both structurally and functionally. As with normal angiogenesis, tumor angiogenesis recruit many of the same molecules that used in normal angiogenesis. VEGF functions as a major factor for tumor angiogenesis to establish the tumor vasculature needed for tumor growth and survival. Therefore tumor cells themselves have shown they can produce VEGF. It has been detected in many cancer cells in vivo and in vitro to be highly upregulated as in the
Figure 7. Tumor angiogenesis. VEGF is secreted by tumor cells and binds to its VEGFR-2 and to neuropilin on endothelial cells. MMPs are released from tumor cells, but also by VEGF-stimulated endothelial cells. MMPs mobilize pro-angiogenic proteins from the stroma. Tumor cells secrete angiopoietin-2, which competes with angiopoietin-1 for binding to the endothelial TIE-2 receptor. Angiopoietin-2 increases the degradation of vascular basement membrane and migration of endothelial cells, therefore facilitating sprout formation. PDGF and bFGF are also secreted by tumors. Certain pro-angiogenic proteins upregulate endothelial integrins that is required for the migration towards the tumor. Bone-marrow-derived endothelial cells are also recruited. Some angiogenic regulatory proteins are scavenged by platelets, stored in alpha granules and seem to be released within the tumor vasculature.
cancers of lung, breast, ovary, kidney, gastrointestinal tract, bladder, endometrial carcinomas, glioblastomas, hemangioblastomas, intracranial tumors and capillary hemangioblastomas (Papetti and Herman, 2002). In addition to VEGF, other factors like FGF, hapananase, Ang-2, IL-8 and MMP-2 are involved to stimulate tumor angiogenesis. In human metastatic colon carcinoma both VEGF and VEGFR-2 were highly expressed. In a tumor environment, it is hypothesized that in most cases VEGF acts as a paracrine mediator (Ferrara and Davis-Smyth, 1997). This was shown by VEGF mRNA being expressed in tumor cells but not on endothelial cells and VEGFR mRNA being upregulated in endothelial cells associated with tumor but not on tumor cells (Ferrara and Davis-Smyth, 1997; Gatto and Cavalli, 2006). But there have been cases where both VEGF and VEGFR were expressed on tumor cells (i.e. leukemia cells, cancer cell lines of the breast, colon, and pancreas) suggesting VEGF can also act as an autocrine factor via an endothelial independent pathway (Ferrara and Davis-Smyth, 1997; Gatto and Cavalli, 2006). Recently more data are showing both VEGF and VEGFR are expressed on numerous cancer cells.

As the VEGF/VEGFR pathway is the major pathway utilized in tumor angiogenesis, VEGF-A expression can have important effects on tumor angiogenesis. HIF, which is upregulated in tumor angiogenesis, because of hypoxic condition, also induces the expression of VEGF-A in many cancers. During tumor angiogenesis HIF activation is not only induced by hypoxia but also by growth promoting stimuli like insulin, IGF-1, epidermal growth factor (EGF) and mutant Ras and Src kinase pathways (Pugh and Ratcliffe, 2003). But in cases of tumor suppressor mutations of PTEN, p53,
p14ARF and pVHL, HIF can also be activated (Pugh and Ratcliffe, 2003). Hypoxic condition, VHL mutation, oncogene upregulation (i.e. ras, erbB-2/her-2, activated EGFR), tumor suppressor gene downregulation or inactivation are all effects that can cause upregulation of the level of VEGF-A in tumor angiogenesis (Ferrara and Kerbel, 2005).

It is well known that metastatic tumors are spread through the blood and lymphatic vessels. More data are accumulating indicating tumor lymphatics as an important avenue of metastasis. Actually, experimental evidence has shown that tumor cells release VEGF-C and VEGF-D which regulates lymphatic growth and enhances lymphatic metastasis (Mandriota et al., 2001; Skobe et al., 2001; Stacker et al., 2001). Lymphatic vessels have been shown to grow into the tumor and to metastasize utilize the sentinel lymph node as the first lymph node in which to disperse (Alitalo et al., 2005; Ruoslahti, 2002).

1.7. Angiogenesis and Other Diseases

It is well documented that there are many diseases that are associated with angiogenesis such as tumor formation, tumor-dependent ascites formation, metastasis, inflammatory diseases such as rheumatoid arthritis (RA), and psoriasis, hyperthyroidism, and atherosclerosis (Alitalo and Carmeliet, 2002; Ferrara and Davis-Smyth, 1997; Shibuya et al., 1999). We know that hypoxia is closely related to angiogenesis in that it drives the expression of VEGF. When premature babies are placed in hyperoxygenated incubators due to underdeveloped lung capacity, this blocks the normal retinal angiogenesis due to decreased VEGF production. After babies are removed from the
incubator and the retina is put under hypoxic condition, a burst of VEGF expression leads to increased angiogenesis during retinal development resulting in blindness to the baby (Pierce et al., 1996). Neovascularization is also a major factor of blindness in age-related macular degeneration (AMD). With AMD, new vessels that are formed from choriocapillary exhibit leakage and bleeding that lead to damage to the macula and ultimate loss of vision in elderly patients (Ferrara and Davis-Smyth, 1997). In the case of RA inflammation of the joint is accompanied by progressive erosion of the cartilage and bone. VEGF has been linked to a highly vascularized tissue called pannus that invades and destroys the articular cartilage (Fassbender and Simmling-Annefeld, 1983; Fava et al., 1994; Koch et al., 1994). Psoriasis is a chronic skin disease that is characterized by thickening and scaling of the erythematous skin (Brown et al., 1995; Detmar et al., 1994). It was determined VEGF has high expression in psoriatic skin that is characterized by increased vascularity and permeability.

1.8. Current Status of Anti-angiogenic Therapy for Cancer

VEGF/VEGFR plays an essential role in the process of normal angiogenesis but it also has vital implications in pathological conditions such as cancer and diabetic retinopathy. Therefore the application of anti-angiogenesis therapy, more specifically targeted molecular therapies, is a promising approach to treating cancer and other vascular diseases. Compared to conventional chemotherapies used in treating cancer, anti-angiogenic therapies have some advantages over it. One, endothelial cells that are primary targets of anti-angiogenic therapy are generally genetically stable and homogeneous, therefore they are unlikely to develop drug resistance compared to the
cancer cells that are highly mutable. Second, they are readily accessible to anti-angiogenic drugs because the endothelial cells line the blood vessels that are used in delivering the drugs to the tumor. Third, based on experiments with animals, one of the advantages has been anti-angiogenic drug’s ability to cause regression of metastatic lesions and prevent spreading in addition to causing regression of the primary tumor (Zetter, 1998). Fourth, anti-angiogenic therapy was shown to have fewer side effects from toxicity than with chemotherapy. Some of the common side effects recorded with anti-angiogenic drugs have been hypertension, bleeding and perforations (Eskens and Verweij, 2006). However, recent data on anti-angiogenic drug treatments show they are considered to be manageable and safe (Eskens and Verweij, 2006).

There are some disadvantages or shortcomings of using anti-angiogenic therapy in treating cancers. One is the fact that anti-angiogenic drugs can only reduce the mass of the tumor to its avascular size but they are incapable of completely eradicating the tumor. It is true that anti-angiogenic drugs’ main focus is on the vasculature surrounding the tumor, but some anti-angiogenic agents alone have exhibited direct killing of tumors. Second is the fact that anti-angiogenic drugs might also cause drug resistance that is seen with conventional chemotherapy. It is less likely for endothelial cells to develop drug resistance and have fewer mutations occurring compared to growing tumor cells (Boehm et al., 1997). In a study with endostatin treatment of Lewis lung carcinoma in mice, the cancer regressed repeatedly during six treatment cycles. The rate of tumor regression was basically the same in the first or last treatment cycle, indicating absence of drug resistance in the treated mice (Boehm et al., 1997). However, there lies the possibility
that anti-angiogenic drugs that target one or two angiogenic factors can be circumvented by tumors expressing other angiogenic proteins as a compensatory response (Folkman, 2007).

Since the findings that tumor growth is dependent on neovasculature, it was hypothesized that disruption of angiogenesis would be an effective strategy for treatment of cancer. This has led to a search of inhibitors of angiogenesis. By mid 1990’s various drugs with anti-angiogenic activity were being tested in clinical trials for various diseases and some received FDA approval (Table 3). As of now 10 new anti-angiogenic drugs have been approved for use in treatment of cancer and other diseases and many more are in clinical trials awaiting approval. A major breakthrough was first achieved with bevacizumab (Avastin) which was the first drug developed solely as an angiogenesis inhibitor. It received FDA approval in 2004. Bevacizumab is a humanized anti-VEGF, monoclonal antibody that binds and inhibits the biological activity of all known VEGF-A isoforms (Ferrara and Kerbel, 2005; Folkman, 2007). In vitro experiments with human umbilical vein endothelial cells (HUVEC) showed that bevacizumab was able to inhibit VEGF-induced cell proliferation, migration and survival (Wang et al., 1998). Bevacizumab is now used for treating metastatic colorectal cancer in combination with 5-fluorouracil (Gatto and Cavalli, 2006). Clinical studies of bevacizumab with chemotherapy as a combination was shown to prolong the survival of metastatic colorectal cancer and advanced non-squamous, non-small cell lung cancer patients and also prolong the onset of recurrent breast cancer patients (National Cancer Institute). Other clinical studies are ongoing with cancers of the pancreas, metastatic non-small cell
Table 3. Anti-angiogenic drugs approved for clinical use and phase of clinical trials for other indications.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Approved*</th>
<th>Phase III</th>
<th>Phase II</th>
<th>Phase I</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bortezomib (Velcade; Millennium Pharmaceuticals)</td>
<td>Multiple myeloma (2003)</td>
<td>NSCLC, multiple myeloma, NHL</td>
<td>Multiple myeloma, NHL, NSCLC, lymphoma, gliomas, melanoma, Waldenstrom’s macroglobulinaemia, prostate, head and neck, breast, liver, nasopharyngeal, gastric, pancreatic, colorectal, cervical/vaginal cancer, and others</td>
<td>Lymphoma, myelodysplasia, multiple myeloma, NHL, solid tumours, head and neck, cervical, colorectal, ovarian, prostate cancer, and others</td>
</tr>
<tr>
<td>Thalidomide (Thalomid; Celgene Corporation)</td>
<td>Multiple myeloma (2003)</td>
<td>Multiple myeloma, brain metastases, SCLC, NSCLC, prostate, kidney, ovarian, hepato/pancreatic cancer</td>
<td>Glioma, glioblastomas, paediatric neuroblastoma, NSCLC, NHL, paediatric solid tumours, myelofibrosis, myelodysplastic syndrome, AML, CML, SCLC, Hodgkin’s disease, paediatric brain stem, liver, colorectal, kidney, neuroendocrine, endometrial, thyroid, uterine, ovarian cancer, and others</td>
<td>Solid tumours, glioma</td>
</tr>
<tr>
<td>Erlotinib (Tarceva; OSI Pharmaceuticals, Roche)</td>
<td>Lung cancer (2004)</td>
<td>NSCLC, colorectal, pancreatic, ovarian, head and neck, oral cancer</td>
<td>NSCLC, mesothelioma, glioblastoma, glioma, gall bladder, GIST, biliary tumours, bladder cancer prevention, malignant peripheral nerve sheath tumours, endometrial, colorectal, pancreatic, breast, renal cell, prostate, ovarian, head and neck, gastric/oesophageal, liver cancer, and others</td>
<td>NSCLC, glioblastoma, solid tumours, colorectal, pancreatic, head and neck cancer</td>
</tr>
<tr>
<td>Pegaptanib (Macugen; OSI Pharmaceuticals)</td>
<td>Age-related macular degeneration (2004)</td>
<td>Kidney, melanoma, hepato/pancreatic cancer</td>
<td>Melanoma, glioblastoma, GIST, SCLC, thyroid, neuroendocrine, mesothelioma, soft tissue sarcoma, NSCLC, CML, multiple myeloma, cholangiocarcinoma, NHL, kidney, colorectal, prostate, ovarian, peritoneal, pancreatic, breast, gastric, head and neck, uterine, gall bladder, bladder cancer, and others</td>
<td>Solid tumours, melanoma, glioblastoma, NHL, glioma, multiple myeloma, Kaposi’s sarcoma, ALL, CML, MDS</td>
</tr>
<tr>
<td>Sorafenib (Nexavar; Onyx Pharmaceuticals)</td>
<td>Kidney cancer (2005)</td>
<td>Kidney, melanoma, hepato/pancreatic cancer</td>
<td>Multiple myeloma, NHL, multiple myeloma, CML, myelofibrosis, myelodysplastic syndrome, glioblastoma, ocular melanoma, AML, mantle-cell lymphoma, Waldenstrom’s macroglobulinaemia, ovarian/ peritoneal, thyroid, prostate cancer</td>
<td>Multiple myeloma, prostate cancer, melanoma, myelodysplastic syndrome, solid tumours, paediatric CNS tumours</td>
</tr>
<tr>
<td>Lenalidomide (Revlimid, Celgene Corporation)</td>
<td>Myelodysplastic syndrome (2005)</td>
<td>Multiple myeloma, myelodysplastic syndrome</td>
<td>NSCLC, NHL, multiple myeloma, CML, myelofibrosis, myelodysplastic syndrome, glioblastoma, ocular melanoma, AML, mantle-cell lymphoma, Waldenstrom’s macroglobulinaemia, ovarian/ peritoneal, thyroid, prostate cancer</td>
<td>Melanoma, VHL/solid tumour, NSCLC, GIST, hepatocellular, colorectal, prostatic, breast, renal cell, gastric, neuroendocrine cancer, and others</td>
</tr>
<tr>
<td>Sunitinib (Sutent; Pfizer)</td>
<td>GIST, kidney cancer (2006)</td>
<td>Renal cell cancer, GIST</td>
<td>Melanoma, VHL/solid tumour, NSCLC, GIST, hepatocellular, colorectal, prostatic, breast, renal cell, gastric, neuroendocrine cancer, and others</td>
<td>Melanoma, solid tumours, colorectal, breast cancer</td>
</tr>
<tr>
<td>Ranibizumab (Lucentis; Genentech)</td>
<td>Age-related macular degeneration (2006)</td>
<td></td>
<td></td>
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</tbody>
</table>

*Year of first approval by the US Food and Drug Administration, unless stated otherwise. ‡Australia, approved by US Food and Drug Administration in 2006. §China State Food and Drug Administration. ALS, amyotrophic lateral sclerosis (or Lou Gehrig’s disease); ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CML, chronic myeloid leukaemia; CNS, central nervous system; GIST, gastrointestinal stromal tumour; MDS, myelodysplastic syndromes; NSCLC, non-small-cell lung cancer; NHL, non-Hodgkin’s lymphoma; SCLC, small-cell lung cancer; VHL, von Hippel Lindau.

lung, ovary, breast, prostate, non-Hodgkins lymphoma, multiple myeloma, myelodysplastic syndrome and renal cell carcinoma in combination with conventional chemotherapy (Gatto and Cavalli, 2006; Gibbs, 2000; Olsson et al., 2006). The suggested underlying explanation for this efficacy is the normalization of the tumor blood vasculature and subsequent efficient delivery of the chemotherapeutic drugs (Cao, 2005).

Another FDA approved drug is pegaptinib (Macugen), a single-stranded RNA aptamer with pegylation that binds and inhibits VEGF activity (Ferrara and Kerbel, 2005). An aptamer is a nucleic acid macromolecule that can bind tightly to a specific molecular target. It is currently approved for use in treatment of age-related macular degeneration (AMD) and in clinical trials for diabetic macular edema and retinal vein occlusion (Folkman, 2007; Gatto and Cavalli, 2006). Another anti-angiogenic drug of interest in relation to this research is the VEGF-Trap. VEGF-Trap combines VEGF binding Ig-like domain 2 of VEGFR-1 with Ig-like domain 3 of VEGFR-2 and links it to the Fc portion of human IgG1 (Gatto and Cavalli, 2006). This produces an agent with the highest affinity for VEGF (Kd ~ 1 pM), which is better than the wildtype receptors (Gatto and Cavalli, 2006). VEGF-Trap is currently in clinical trials for treatment of ovarian cancer (phase III), kidney, age-related macular degeneration, non-small cell lung cancer (phase II) (Folkman, 2007). This agent bears a resemblance to our proposed KDR antagonist protein in that it utilizes soluble fragments of the VEGF receptor to generate a recombinant soluble receptor attached to an immunoglobulin. Targeting VEGF receptor has been an area of great interest. It has been shown that inactivation of VEGFR-2 not only inhibits angiogenesis but also tumor invasion. In vivo studies using anti-VEGFR2
antibody (DC101) injected into mice with highly malignant and metastasizing keratinocytes resulted in inhibition of both angiogenesis and tumor invasion (Skobe et al., 1997). There was also a change in phenotype of the tumor cells from highly malignant to pre-malignant and non-invasive tumor with the inhibition of VEGFR-2 (Skobe et al., 1997). Others have also shown success with an antibody directed at VEGFR-2 that neutralized VEGF activities (Miao et al., 2006).

Another method utilized was the injections of viruses encoding soluble forms of Flk-1 and Flt-1 into preexisting murine and human tumor models that resulted in ~80% inhibition of pancreatic tumor growth (Tseng et al., 2002). These recombinant adenoviruses were used as a delivery vehicle encoding the ligand binding ectodomains of both proteins (Tseng et al., 2002). The use of DNA vaccine against VEGFR-2 may provide an alternative method of targeting tumor vasculature (Niethammer et al., 2002). Niethammer et al. (2002) reported that DNA vaccines encoding autologous mouse VEGFR-2 delivered by attenuated strain of *S. typhimurium* was able to protect the mice from melanoma, colon carcinoma and lung carcinoma and inhibited metastasis (Niethammer et al., 2002).

In developing an anti-angiogenic therapy the classic target has been the endothelial cells. They were targeted because they formed the blood vessels that provided the necessary oxygen and nutrients to the tumor cells and also due to their inherent genetic stability that renders them less likely to develop drug resistance to anti-angiogenic therapy (Carmeliet, 2005). The actions of anti-angiogenic agents have shown they are able to inhibit endothelial cell proliferation, vessel growth and increase cell
death. In addition, as abnormal vasculature of the tumor is the result of imbalance between pro and anti-angiogenic factors, restoring the balance might normalize the vasculature to a certain degree which would facilitate delivery of therapeutics to the site of action (Folkman, 2007; Jain, 2003). Therefore continuous application of anti-angiogenic agents may increase the efficacy of the conventional drugs. Anti-angiogenic drugs were able to show three distinct mechanisms of sensitizing the tumor cells to chemotherapy (Kerbel, 2006). The treatment of tumors with anti-angiogenic drugs has been able to normalize the disorganized tumor vasculature in such way to increase the efficacy of chemotherapy by improving the delivery of the drugs. Anti-angiogenic drugs are also thought to be able to prevent the rapid repopulation of tumor cells after stoppage of chemotherapy since they can be administered more frequently than conventional chemotherapy. Anti-angiogenic drugs may also be able to augment the antivascular effects of chemotherapy. When certain anti-angiogenic drugs neutralize VEGF, this can then allow chemotherapy to have its cytotoxic effects on endothelial cells or endothelial progenitor cells. Another way to increase efficacy of treatment with chemotherapeutic drugs was seen with change in dosing schedule. When cyclophasamide was administered more frequently at a lower dose, tumors were inhibited. The optimization of chemotherapy to treat vascular endothelium in the tumor was termed anti-angiogenic chemotherapy or ‘metronomic’ therapy (Kerbel, 2006). This method is being tested in brain and other tumors when conventional chemotherapy is refractory (Folkman, 2007).

To inhibit the growth of the vascular endothelial cell of tumors, anti-angiogenic drugs have targeted the VEGF/VEGFR pathway. This can be accomplished in different
ways. As seen in Figure 8, monoclonal antibodies targeting VEGF-A (a), or VEGF receptors (c, d), chimeric soluble molecules or aptamers targeting VEGF/VEGFR (d, e), and small molecule receptor tyrosine kinase inhibitors (RTKI) that target intracellular autophosphorylation sites (Ferrara and Kerbel, 2005). In addition, newer methods of using antisense RNA and siRNA have been explored to target VEGF/VEGFR signaling. These are able to interfere with mRNA in way to silence gene expression of proteins. As examples of current drugs being developed in addition to the ones approved by FDA are two RTKIs, Sorafenib (BAY 43-9006), and Sunitinib (SU11248). They are being tested in phase III clinical trials for treatment of metastatic kidney cancer and gastrointestinal stromal tumors, respectively (Olsson et al., 2006). As with many anti-tumor drugs, these anti-angiogenic agents also face difficulties in in vivo delivery to the targeted tissues for drugs to take effect. One of the benefits of using anti-angiogenic therapy to reduce the primary tumor’s vasculature was that it also decreased tumor metastasis. Many of the anti-angiogenic inhibitors including angiostatic steroids, thalidomide, fumagillin analog, TNP-470, thrombospondin, angiostatin, and endostatin have inhibited tumor metastasis (Zetter, 1998).

Alternative targets for anti-angiogenesis have been to target mural, stromal, haematopoietic or tumor cells. Mural and stromal cells are involved in vessel stabilization and actively contribute to tumor progression. By inhibiting PDGF-BB that is involved in recruitment of mural cells, fewer pericytes are recruited and it increases the anti-angiogenic effects (Carmeliet, 2005). In response to VEGF and P1GF haematopoietic cells and endothelial progenitor cells are recruited to avascular sites of
Figure 8. Ways of inhibiting VEGF signaling through VEGFR. Monoclonal antibodies targeting VEGF (a) or VEGF receptors (b,c). Recombinant soluble receptors targeting VEGF (d). Aptamers (e) that bind and inhibit heparin-binding domain of VEGF and small molecule inhibitors(e) that inhibit ligand dependent receptor autophosphorylation of VEGFR-1 and -2. Additional strategies include antisense and siRNA.

tumors and they themselves release VEGF, P1GF and angiopoietin-2 to stimulate resident vessels. For instance, by inhibiting P1GF one can prevent recruitment of these cells to reduce tumor angiogenesis. Finally, the ultimate goal of cancer treatment is to kill tumor cells. It is known that tumor cells express angiogenic factors as well as its corresponding receptors on the cell surface (Carmeliet, 2005). Therefore, anti-angiogenic drugs could potentially inhibit tumor cells directly by targeting the receptors of angiogenic factors.

An important aspect of tumor cells, the genetic background, can also influence the outcome of the treatment with anti-angiogenic therapy (Yu et al., 2002). It was reported that in mice with tumor derived from HCT116 human colorectal cancer cells deficient in p53 was less responsive to anti-angiogenic combination therapy than mice with normal p53 (Yu et al., 2002). The tumor cells with mutant p53 were less susceptible to apoptosis under hypoxic condition compared to wildtype p53 bearing tumor cells suggesting p53 mutant cells are less reliant on vascular supply and ultimately on anti-angiogenic therapy (Yu et al., 2002). Since p53 mutation is seen in majority of human cancers, it is an important aspect that warrants investigation to mitigate the effects and improve anti-angiogenic treatment.

As we search for newer and better drugs to cure cancer, more anti-angiogenic drugs will be available to cancer patients and by combining with chemotherapy will provide increased efficacy compared to either treatments alone. With anti-angiogenic therapy targeting just one of the hallmarks of cancer, it seems most logical to target multiple hallmarks of cancer simultaneously in combination with other conventional cytotoxic agents to bring forth an additive or even better synergistic anti-tumor effects.
One example would be to develop a combination therapy that can simultaneously stop angiogenesis and tumor cell invasion for treating metastatic tumors (Steeg, 2003). The application of combining different anti-angiogenic agents with various chemotherapies should be investigated more vigorously. As tumor angiogenesis plays an essential but also a detrimental part in the manifestation of cancer in terms of stimulating growth but also allowing for metastasis, research in to developing anti-angiogenic therapy is very promising.
2. HYPOTHESIS AND OBJECTIVES

2.1 Hypothesis

Tumors depend on formation of new blood vessels for continued growth. We hypothesized that a segment of the extracellular VEGF receptor that possesses the ability to participate in receptor dimerization could be utilized as a VEGFR antagonist to prevent VEGF induced VEGFR activation (as illustrated in Figure 9).

Figure 9. Schematic representation of the proposed mechanism of inhibition of VEGF induced VEGFR activation by VEGFR “antagonist”. The diagram on the left illustrates the normal VEGF binding to the ligand binding Ig-like domains of the receptor that causes receptor dimerization and eventually signal transduction leading to angiogenesis. The diagram on the right illustrates the proposed inhibitory mechanism of the VEGFR extracellular fragment that would bind to a VEGFR, therefore, preventing it from forming VEGFR dimers.
2.2 Objectives

There are three objectives for this study.

1) Cloning and production of segments of the extracellular domains of KDR proteins as test KDR proteins.

2) Characterization of the KDR variants by various *in vitro* assays including radio-receptor binding, cell proliferation, ERK phosphorylation, tube formation, endothelial cell migration and apoptosis assay.

3) Evaluation of the therapeutic value of the lead candidate of VEGFR antagonist in multiple cancer cells and in mice.
3. MATERIALS AND METHODS

3.1 Cell Lines, Reagents and Antibodies

The human umbilical vein endothelial cell line HUVEC, human breast cancer cell lines, T-47D, BT-474, MDA-MB 231, SKBR-3, human leukemia cell lines HEL and HL-60, mouse mammary gland tumor cell line 4T1 and mouse L-cells (CCL-1.4) were purchased from the American Type Culture Collection (Manassas, VA). McNeu, an epithelial origin was isolated from a tumor of a MMTV-Neu transgenic mouse. They were all maintained in their respective medium containing 10µg/ml gentamicin at 37°C with humidity and 5% CO₂ with the exception of MDA-MB 231 cells that were incubated at 37°C in the absence of CO₂ and McNeu was maintained in DMEM medium supplemented with 10% FBS, 10 µg/ml gentamicin and 1 mM sodium pyruvate. All media and supplements were purchased from Invitrogen (Carlsbad, CA) unless otherwise mentioned. HUVEC were maintained in Medium-199 supplemented with 10% fetal bovine serum (FBS) and EGM-2 Singlequot (Cambrex, East Rutherford, NJ). T-47D, MCF-7, HEL and HL-60 cells were maintained in RPMI Medium 1640 supplemented with 10% FBS. BT-474 cells were maintained in RPMI 1640 medium and supplemented with 10% FBS, 1.0 mM sodium pyruvate, and 10 µg /ml insulin (Sigma, St. Louis, MO). MDA-MB 231 cell lines were maintained in Leibovitz’s L-15 medium supplemented with 10% FBS. 4T1 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate. Mouse L-cells were maintained in DMEM with 10% FBS. Positive
transfected L-cells were selected and maintained in DMEM with 10% FBS containing 500 μg/ml G418 sulfate (Invitrogen).

The following primary antibodies were used at the following dilutions: 1:1000 dilution of anti-human VEGFR-2 (R&D Systems, Minneapolis, MN) for cell lysate; 1:5000 dilution of anti-phospho-ERK (Santa Cruz Biotechnology); 1:1000 dilution of anti-ERK-1 (Santa Cruz Biotechnology); 1:10,000 anti-β-Actin (Sigma); 1:1000 dilution of anti-STAT5 (Santa Cruz Biotechnology). Anti-phospho-Erk1/2 and were obtained from Santa Cruz Biotechnology and used at 1:1000. The secondary antibodies, rabbit anti-goat IgG-, goat anti-mouse IgG- and goat anti-rabbit IgG-horseradish peroxidase-conjugates were obtained from Bio-Rad Laboratories (Hercules, CA) and used at 1:2000.

3.2 Construction of Plasmids Encoding KDR-Ig1-3, KDR-Ig4-7, KDR-Ig4-6, KDR-Ig5-7, KDR-Ig5-6, and Fusion Proteins KDR-Ig4-7-VEGF, VEGF-KDR-Ig4-7, KDR-Ig4-7-G129R Constructs

KDR-Ig1-3, KDR-Ig4-7, KDR-Ig4-6, KDR-Ig5-6 and KDR-Ig5-7 are constructs of cDNA with numbers representing the specific Ig-like domains of KDR. The constructs were cloned by amplifying the cDNA from a Human endothelial cell (aortic, HAAE-1) cDNA library. Primers used for the cloning of each fragment are listed in the Table 4. G129R cDNA was kindly provided by Dr. Chen to be cloned into the fusion clone KDR-Ig4-7-G129R. VEGF portion of the fusion clones KDR-Ig4-7-VEGF and VEGF-KDR-Ig4-7 were amplified by RT-PCR from total RNA isolated from HUVEC. cDNA fragments were ligated separately into TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA) and restriction mapped and sequenced for sequence integrity. Once the cDNA fragments were ligated into pCR2.1 cloning vector, each appropriate clone was
used to generate the final clone consisting of different KDR fragments DNA. To generate clones that express specific proteins, fragments were restriction digested and ligated into NdeI and XhoI restriction sites of pET22b(+) vector (Novagen, Madison, WI) for the expression of KDR-Ig4-7, KDR-Ig4-6, KDR-Ig5-6, KDR-Ig5-7 (Figure 10) and fusion clones KDR-Ig4-7-VEGF, VEGF-KDR-Ig4-7 and KDR-Ig4-7-G129R proteins (Figure 11). For the purpose of generating stable and transient transfected cell lines, all clones were cloned into pUC IG mammalian expression vector (Chen et al., 1990) with metallothionein (MT) promoter. KDR cDNAs were all cloned into NotI and SpeI restriction sites. All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, IA) for use in cloning and RT-PCR. Maps of the plasmid construct are presented in Figure 10.
Table 4. Primer Sequences used in PCR amplification of individual clones to generate KDR proteins.

<table>
<thead>
<tr>
<th>Clones</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KDR-Ig1-3</td>
<td>For 5’-TTTGCGGCCCGCATGGAGAGCAAGGTGCTGCTGGCC-3’</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-CCCACCTAGTTTTATTTTTTGTGGACCCAATATGTGCT-3’</td>
</tr>
<tr>
<td>KDR-Ig4-6</td>
<td>For 5’-CATATGTGTTTGTGCTTTTTGGAAGTGCGCATG-3’</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-CTCGAGTTATAGGACTGTGAGCTGCTGGCTGACCAC-3’</td>
</tr>
<tr>
<td>KDR-Ig4-7</td>
<td>For 5’-CATATGTGTTTGTGCTTTTTGGAAGTGCGCATG-3’</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-CCGCTCGAGTTATAGGACTGTGAGCTGCTGGCTGACCAC-3’</td>
</tr>
<tr>
<td>KDR-Ig5-6</td>
<td>For 5’-CATATGGGTGAGAAATCTCTAATCTCTCTCTCT-3’</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-CTCGAGTTATAGGACTGTGAGCTGCTGGCTGACCAC-3’</td>
</tr>
<tr>
<td>KDR-Ig5-7</td>
<td>For 5’-CATATGGGTGAGAAATCTCTAATCTCTCTCTCT-3’</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-CCGCTCGAGTTATAGGACTGTGAGCTGCTGGCTGACCAC-3’</td>
</tr>
<tr>
<td>VEGF</td>
<td>For 5’-CATATGGGCACCATGGCAGAAGGA-3’</td>
</tr>
<tr>
<td></td>
<td>Rev 5’-CTCGAGTTATAGGACTGTGAGCTGCTGGCTGACCAC-3’</td>
</tr>
</tbody>
</table>
Figure 10. Maps of pET22b(+) vector for the expression of mammalian recombinant KDR monomeric proteins. Individual clones shown above were initially cloned into pCR2.1 T/A cloning vector and subsequently subcloned into pET22b(+) expression vector for production of recombinant proteins. Recombinant plasmid is transformed into E. coli strain and induced by IPTG to produce the target protein.
Figure 11. Maps of pET22b(+) vector for the expression of mammalian recombinant KDR fusion proteins. Individual clones shown above were initially cloned into pCR2.1 T/A cloning vector and subsequently subcloned into pET22b(+) expression vector for production of recombinant proteins. Recombinant plasmid is transformed into E. coli strain and induced by IPTG to produce the target protein.
3.3. Production and Purification of KDR-Ig4-7 and its Variant KDR Proteins

For the production of the proteins, competent \textit{E. coli} cells, BL21 (DE3) Rosetta cells (Invitrogen) were transformed with pET22b(+) vector encoding for KDR-Ig4-7, KDR-Ig4-6, KDR-Ig5-6, and KDR-Ig5-7 and fusion clones KDR-Ig4-7-VEGF, VEGF-KDR-Ig4-7 and KDR-Ig4-7-G129R cDNA. Bacteria were allowed to grow overnight in Luria-Bertani broth (ampicillin 50 μg /ml) at 37°C. The starter culture was then inoculated into a 4 liter LB broth for additional growth for 3hrs and then induced for protein production with 1mM isopropyl thiogalactoside (Alexis Biochemicals, San Diego, CA). Five hours later, cells were pelleted by centrifugation at 6000xg for 5 min and resuspended in Solution I (0.2 M NaPO₄; 10 mM EDTA, pH 8.0; 0.5% Triton X-100) with the addition of lysozyme at (0.1 mg/ml). The cells were incubated for 1 hr at room temperature and then subjected to 5 cycle of 1 minute ultra-sonic dismemberment by 550 Sonic Dismembrator (Fisher Scientific, Pittsburgh, PA) with 1 minute cooling time in between sonication to form inclusion bodies. Inclusion bodies were recovered by centrifugation at 12,000 xg for 20 min at 4°C and resuspended in Sol I and sonicated again for 2-3 cycles of 1 minute sonication. The inclusion bodies were pelleted at 12,000xg for 20 min at 4°C and resuspended in Sol II (0.2 M NaPO₄; 5 mM EDTA, pH 7.0; 0.5% Triton X-100; 1 M urea). The inclusion bodies were recovered by centrifugation and resuspended in Sol III (0.2 M NaPO₄; 8 M urea; 1% v/v β-mercaptoethanol; pH 8.0) and heated at 55°C for 15 min. The proteins were allowed to refold by dialysis in four baths containing 20 mM Tris-HCl, 2 mM EDTA at pH 8.0 with decreasing concentrations of urea (4 M, 2 M, 0 M, 0 M) for 18-24 hrs in each bath.
Refolded proteins were purified using anion-exchange chromatography (5 ml HiTrap Q-Sepharose XL column) from GE Healthcare, Piscataway, NJ. Purified proteins were quantified by Bradford method (Bio-Rad Protein Assay, Bio-Rad, Hercules, CA) and visualized on SDS-PAGE with SYPRO orange protein stain (Bio Rad) to determine the purity. KDR-Ig4-7, KDR-Ig4-6, KDR-Ig5-6 and KDR-Ig5-7 and fusion proteins KDR-Ig4-7-VEGF, VEGF-KDR-Ig4-7 and KDR-Ig4-7-G129R proteins were verified by immunoblotting with anti-VEGFR-2 and prolactin antibody. Purified proteins were sterilized by passing through 0.22 μm Acrodisc syringe filter (Pall, Cornwall U.K.).

3.4. Cross-linking Assay

A cross-linking reagent, [Bis(Sulfosuccinimidyl) suberate] (BS³) (Pierce Biotechnology, Rockford, IL) a water-soluble, homobifunctional N-hydroxysuccinimide ester, was used to cross-link KDR-Ig4-7 to itself or to VEGF. Cross-linking of KDR proteins with itself or VEGF was conducted in conjugation buffer containing 20 mM sodium phosphate, pH 7.5 with 0.15 M NaCl. BS³ was directly added to the sample containing conjugation buffer at a final concentration of 2 mM. When cross-linking KDR-Ig4-7 to VEGF, a molar ratio of 1:1 was applied. The reaction mixture was incubated at room temperature for 30 minutes. The reaction was quenched using 1 M Tris-HCl, pH 7.5 at a final concentration of 20 mM and incubated for 15 minutes at room temperature. The cross-linked reaction mixtures were verified by SDS polyacrylamide gel electrophoresis and stained using SYPRO orange protein stain.
3.5. Stable and Transient Transfection Experiments

Cells were seeded in six-well plates (35mm) at a concentration ranging from 1 to 2.0 x 10^5 cells per well, depending on the type of cell, in order to reach 40-60% confluency for stable transfections and 60-80% confluency for transient transfections. Twenty four hours after seeding the cells they were starved for 2-4 hours with Opti-MEM® Reduced Serum Medium (Invitrogen) before continuing with the transfection. Transient transfections of 1 and 2 μg of each plasmid DNA (pcR3.1 empty vector, pIG KDR-Ig1-3, pIG KDR-Ig4-7, pIG KDR-Ig4-7-VEGF, pIG VEGF-KDR-Ig4-7 or pIG KDR-Ig4-7-G129R) was transfected using Lipofectin Reagent (Invitrogen) according to the manufacturer’s recommendations (Figure 11). The total amount of DNA used for transfections was maintained constant between the samples.

The DNA-lipofectin transfection mixtures were applied to cells for 8 to 12 hours and then fresh growth medium of 2 ml was subsequently added on top of the transfection mixture. Transient transfected cells were harvested after 24 hours for protein lysates and after 48 hours for proliferation assays. T47-D cells (6.0 x 10^3 cells/well) were plated onto 96 well plate and incubated at 37°C for 6 days for the cell proliferation assay. Cell viability was determined by using a MTS-PMS kit (CellTiter 96 Aqueous non-radioactive cell proliferation kit; Promega, Madison, WI). In order to generate stably transfected cells, following transfection (L-cells) the cells were grown to confluency (2 days) and then the cells were trypsinized and transferred to a 100 mm dish with selection media containing 500 μg/ml of G418 sulfate. The cells were allowed to grow at 37°C incubator
for about 2 weeks or until single colonies showed up on the plates. Each individual positive colonies were picked and expanded in a separate plate.

Conditioned media collected from positive colonies were verified of recombinant KDR-Ig4-7-VEGF and VEGF-KDR-Ig4-7 fusion protein expression by Quantikine Human VEGF ELISA (R&D Systems, Minneapolis, MN) and Quantikine Human VEGFR-2 ELISA. The expression of KDR-Ig4-7-G129R and G129R-KDR-Ig4-7 were verified by Coat-A-Count Prolactin IRMA (Diagnostic Products Corp, Los Angeles, CA) kit. Positive conditioned media were also subjected to Western Blotting to verify production of their respective recombinant proteins. The cell lines with high expression levels were expanded and frozen stocks made.
Figure 12. Map of mammalian expression vector constructs for use in transient and stable transfections. Individual recombinant clones were subcloned into the pUC IG mammalian expression plasmid for the generation of stable and transient transfected L-cells.
3.6. Total RNA Isolation from Cells

Confluent cells were lysed with 1.0 ml of cold TRIzol (Sigma) per well (35 mm). Cell lysates were then collected and RNA was extracted with 200 μl of chloroform. Samples were centrifuged at 12,000xg for 15 min at 4°C. The aqueous upper phase (~200 μl) was collected and RNA was precipitated with 500 μl of isopropyl alcohol (Sigma) and centrifuged at 12,000xg for 10 min at 4°C. The RNA pellet was washed with at least 1.0 ml of 75% ethanol and centrifuged at 7,500xg for 5 min at 4°C. The pelleted RNA was left to stand in open air to allow ethanol to evaporate. RNA pellet was dissolved in RNase-free water by incubating in 55°C water bath for 10 min. The RNA concentration was determined spectrophotometrically.

3.7. Reverse Transcription-PCR (RT-PCR)

Reverse Transcription (RT)-PCR technique was used to determine the expression levels of mRNA of various genes in a semi-quantitative manner. Total RNA in the amount of 0.5 - 2 μg was added to the PCR mix included in the Reverse Transcription-PCR kit (Promega, Madison, WI) with 1 μl of appropriate primers (10 μM) and 0.5 μl of reverse-transcriptase enzyme in a 25 μl total reaction volume. The RT-PCR analysis was carried in a Gene Amp PCR System 9700 (Applied Biosystems, Foster City, CA).

Total RNA was first reverse-transcribed for 45 min at 48°C. The cDNAs were initially denatured at 95°C for 1 min and then PCR amplified to 40 cycles using the following standard conditions: denaturation at 94°C for 1 min, annealing for 30 sec (optimal annealing temperatures varied depending on the melting temperature of the
primers), and extension at 72°C for 1-2 min depending upon the size of the final PCR product. A final extension step at 72°C for 6 min was added. RT-PCR samples were stored at -80°C until further use. The presence of mRNA transcripts from positive transfected clones with gene of interest was resolved on a 1% agarose gel-electrophoresis and visualized by ethidium bromide staining and autoradiography. To control for total amount of RNA used, amplification of the G3PDH housekeeping gene was carried simultaneously. To control for the possibility of DNA contamination, RT-PCR was conducted in the absence of reverse transcriptase and separately in the presence of DNase. The photographs of gel electrophoreses were further analyzed with the Kodak 1D Image Analysis software (Eastman Kodak Company Molecular Imaging Systems; Rochester, NY) to compare the mean intensity of the bands.

3.8. Isolation of Cell Lysates

HUVEC and T47-D cells were washed 2 times with ice-cold 1x phosphate buffered saline (PBS) solution and lysed with 100 μl with Lysis Buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 % NP-40; 1 mM EDTA, pH 8.0) containing protease inhibitors (1 μg/ml aprotinin; 1 μg/ml leupeptin; 170 μg/ml PMSF; 180 μg/ml sodium orthovanadate). The cells were then lysed by agitating on a orbital shaker for 15 minutes and collected into a cold 1.5ml tube by scraping the cells off the plates with a cell scraper. The cells were centrifuged at 12,000xg for 15 minutes at 4°C. Cell lysates were transferred to cold sterile tubes and quantified as to its protein concentration. Cell lysates were subjected to Western Blot analysis as described below.
3.9. Western Blot Analysis

Bacterially produced proteins, KDR-Ig4-7, KDR-Ig 4-6, KDR-Ig 5-6 and KDR-Ig5-7 were resolved on a 10 % SDS-PAGE gel for 1 hour at 150V. The proteins were transferred onto a Hybond enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham Biosciences) at 16W for 1 hr. The nitrocellulose membrane was incubated in blocking solution containing TBS with 0.05 % Tween-20 (TBS-T) and 5% milk for 30 min at room temperature while shaking. The membrane was incubated overnight at 4°C with agitation in blocking solution containing the primary antibody. Next day the membrane was washed three times for 5 min each with TBS-T and incubated with secondary antibody for 2 hrs at room temperature while shaking. After 2 hours the membrane was washed two times with TBS-T and one time with purified water. The membrane was treated with ECL Western Blotting Detection Reagents (Amersham Biosciences) for 1 min. Immunoblot analysis of conditioned media from stably transfected cells was treated with ECL Advance Western Blotting Detection Kit (Amersham Biosciences) to detect very low levels of protein expression.

To probe the membrane with additional antibody, membranes were stripped with stripping buffer containing 100mM β-mercaptoethanol, 2%SDS, 62.5 mM Tris-HCl at pH 6.7 in a rotating incubator at 50°C for 30 min. The membrane was re-blocked and re-probed with a primary antibody of choice. To confirm that equivalent amounts of total protein were added to each well, the membranes were stripped and re-probed with anti-β-actin monoclonal antibody when analyzing cell lysates. Membranes were exposed to
Kodak Biomax MR film (Fisher Scientific, Pittsburgh, PA) and developed with Konica SRX-101A processor (Konica Minolta Medical Imagining, Wayne, NJ).

3.10. Cell Proliferation Assay

HUVEC, T47-D, BT-474, SKBR-3, MDA-MB 231, McNeu and HEL and HL-60 cells were grown in their respective growth media. HUVEC were seeded in a gelatin coated 96 well plate (BD Sciences, Palo Alto, CA) at 5,000 cells/well in growth media and grown overnight. After 15 to 18 hrs of incubation, medium was switched to Medium-199 containing 1% FBS from medium containing 10% FBS and depleted overnight. T47-D, BT-474, SKBR-3, MDA-MB 231 and McNeu cells were seeded in a 96-well plate at 15,000 cells/well containing growth media and allowed to grow overnight. The cells were depleted with 0.5% CSS containing cell specific growth media. Varying concentrations of bacterially produced sterile KDR-Ig4-7, KDR-Ig 4-6, KDR-Ig 5-6 and KDR-Ig5-7 were added to the cells in 100 µl volume of 1% FBS containing Medium 199 (HUVEC) or 0.5% CSS containing RPMI 1640 medium or Serum-Free RPMI 1640. VEGF (R&D Systems) when present was at 25 ng/ml concentration. VEGF dependent dosage response curve was conducted at various VEGF concentrations with KDR-Ig4-7 at IC_{50} concentration. The cells were then incubated depending on the cell type an additional 48-96 hrs at 37°C in a humidified 5% CO_{2} incubator. The viability of the cells was determined by MTS-PMS (Promega, Madison, WI) colorimetric assay by measuring absorbance at 490nm using a Benchmark microplate reader (Bio-Rad). All experiments were carried out in triplicate or more.
3.11. Competitive Radioreceptor Binding Assay

A confluent T-75 flask of HUVEC were plated onto a 6-well plate and grown overnight. The cells were depleted with its appropriate serum-free media for 1 hr and then incubated for 2 hrs at room temperature in appropriate serum-free media containing $^{125}\text{I}$-labeled KDR-Ig4-7 (specific activity, 1.7 μCi/μl; Lofstrand Labs, Gaithersburg, MD) with and without various concentrations of unlabeled KDR-Ig4-7. Cells were washed three times with serum-free media and lysed in 0.5 ml of 0.1N NaOH, 1% SDS solution. The bound radioactivity was determined by a scintillation counter. The % specific binding was calculated using the formula: (total binding – non-total binding)/total binding x 100.

3.12. Ligand Tissue Distribution Assay

Tissue distribution of KDR-Ig4-7 was investigated by i.p. injection of $^{125}\text{I}$-labeled KDR-Ig4-7 into FVB mice with and without Her-2 breast tumor xenografts. Approximately, 6.8 μCi of $^{125}\text{I}$-labeled KDR-Ig4-7 was i.p. injected into each mice (n=6). The mice were sacrificed 6 hours after injection and various tissues dissected and weighed. The radioactivity in various tissues was determined using a scintillation counter. The data were expressed as cpm/mg tissue and normalized by reference to serum cpm (% of CPM = cpm in tissue/mg of organ/cpm of 100 μl of serum x 100).
3.13. Tube Formation Assay

Matrigel (BD Biosciences) was allowed to thaw overnight at 4°C. On the day of the experiment, Matrigel was used to coat 48-well plate at 160 μl/well and allowed to polymerize at 37°C incubator for 30 min. HUVECs were then trypsinated and 12,500 cells/well in 150 μl volume of 5 % FBS, 1 x Medium 199 containing EGM-2 singlequot without antibiotics were added onto the solidified matrigel in each well. Each well was then treated with KDR-Ig4-7 at the various concentrations. The cells were incubated for 24 hours at 37°C in a humidified 5 % CO₂ incubator and observed using a CK2 Olympus microscope at 10X magnification. Pictures were taken using a microscope mounted camera at 10X magnification.


T47-D cells were seeded in 12-well plates and grown overnight to allow for 60-80 % confluency and depleted in 0.5% CSS RPMI 1640 medium. After 24 hours, cells were treated with bacterially produced recombinant proteins at varying concentrations and incubated at 37°C in a humidified 5 % CO₂ incubator for 24 hours. Untreated cells and Staurosporine (1μM) were used as negative and positive controls for induction of apoptosis, respectively. Each sample was prepared in triplicates. After 24 hours the non-adherent apoptotic cells in the medium and the viable adherent cells from each individual well were collected and transferred into 1.5 ml tubes and centrifuged at 400 g for 6 min at 4°C. The pellets of adherent and non-adherent cells of the same treatment were combined into one tube and re-suspended in approximately 50-70 μl of growth media and
kept on ice. The re-suspended cells were diluted 1:10 to 1:20 in Guava® ViaCount® Reagent (Guava Technologies, Hayward, CA) which contains two DNA-binding dyes, one of which selectively penetrates apoptotic and dead cells characterized by compromised membrane integrity and one which penetrates all nucleated cells. Following a five minute incubation, the stained cells were analyzed using a Guava Personal Cytometer. Each sample was analyzed using a Guava CytoAnalysis software. The amount of viable/healthy cells, apoptotic cells and dead cells were expressed as a percentage of the total cellular population for each sample. Data were expressed as mean ± SEM and are representative of triplicate experiments.

3.15. HUVEC Migration Assay

HUVEC was seeded on 48-well plate and grown overnight to confluency in regular growth medium. A 1-10 μl micropipette tip was used to scrape a line down the middle of the well to produce a wound/gap. HUVEC with a wound/gap were treated with varying concentrations of bacterially produced KDR-Ig4-7 protein in serum-free growth medium. For an experiment that required VEGF, VEGF was added to the well at 25 ng/ml. HUVEC was incubated at 37°C humidified 5% CO₂ incubator. HUVEC were observed every 4 hours for migration by examining the gap shortening between the HUVEC. The observations were captured under the microscope using a camera.

3.16. Mouse Mammary Gland Tumor 4T1 Lung Metastasis and Xenograft Study

A total of 27 female BALB/c mice that are 3 months old from Jackson Laboratory (Bar Harbor, ME) were used for in vivo 4T1 xenograft model to study the effect of
purified recombinant KDR-Ig4-7 protein on metastasis of 4T1 cells. Nine mice per group were divided into control, high dose and low dose group. Mouse mammary gland tumor 4T1 cells of 100 μl volume containing 1 x 10^5 cells were inoculated into each mouse by tail vein injection. Treatment of mice in each group consisted of 300 μl volume of PBS, 186 μg (high dose) or 20 μg (low dose). The mice were injected intraperitoneally (i.p.) everyday for a period of 2 weeks. After 2 weeks of treatment, mice in each group were sacrificed and lungs removed to determine the level of metastasis of 4T1 tumor cells by determining the number of metastatic pulmonary nodules present on the lobes of the lung. Each lung was stained with Accustain (Bouin’s Solution, Sigma, St. Louis, MO). and viewed under the dissecting microscope in order to count the number of metastatic pulmonary nodules.

For the 4T1 xenograft mice model study, 10 mice of 3 months old female BALB/c were used initially for a control study to determine the correlation of number of 4T1 cells to the body weight, tumor weight and lung weight. The control study had mice divided into 3 groups of 3, 4 and 3 to which 0.5 x 10^6 cells, 0.5 x 10^5 cells and 0.5 x 10^4 cells were respectively inoculated subcutaneously in a 1:1 mixture with Matrigel (BD Biosciences) in volume of 100 μl on both left and right sides of the mammary gland. This resulted in a total of 1.0 x 10^6 cells, 1.0 x 10^5 cells and 1.0 x 10^4 cells per mice. After the control study, six female BALB/c mice of 3 months of age were divided into three groups of 2 mice each for the experiment. A total of 1.0 x 10^6 cells, 1.0 x 10^5 cells and 1.0 x 10^4 cells were inoculated per group and treated with KDR-Ig4-7 recombinant
protein. The treatments were i.p. injected everyday for 2 weeks in a volume of 100 μl containing 72 μg of protein. Control group was injected i.p. with sterile PBS.

3.17. Statistical Analyses

Statistical analyses were performed using GraphPad Prism software. Statistical differences between the groups were determined using one-way and two-way ANOVA and Tukey’s multiple comparison tests unless otherwise mentioned.
4. RESULTS

4.1. Production of various segments of the extracellular domain of KDR

To develop a VEGFR antagonist that could potentially inhibit VEGF-induced KDR activation and ultimately prevent growth and proliferation of cancer cells we produced various extracellular fragments of KDR. To identify the segment or domain that had the ability to bind to the receptor, the entire extracellular component was arbitrarily divided into three segments (KDR-A, KDR-B and KDR-C) as shown in Figure 13. Each segment was PCR amplified using cDNA from a human endothelial cell (aortic, HAAE-1) cDNA library using specific primers and cloned into pCR2.1 vector. Each cDNA was subsequently cloned into a bacterial expression vector pET22b(+) or pET22(+)-G129R as described in the material and methods chapter. VEGF portion of the fusion clones KDR-Ig4-7-VEGF and VEGF-KDR-Ig4-7 were amplified by RT-PCR from total RNA isolated from HUVEC (material and methods chapter). They were then cloned into expression vector pET22(b)+ at either the N-terminal or C-terminal of KDR-Ig4-7, While KDR-B and KDR-C were produced with our established lab protocols, KDR-A production presented great difficulties (data not shown). With the new information from the literature that showed KDR extracellular domains have 7 Ig-like domains, we modified our design and were able to produce most of the KDR for this study other than the fragments containing KDR-Ig1-3 (Figure 13).
Seven extracellular Ig-like domains of KDR

| 1 | 2 | 3 | 4 | 5 | 6 | 7 |

**Initial KDR proteins**
- KDR-A
- KDR-B
- KDR-C

**Modified KDR proteins**
- KDR-Ig1-3
- KDR-Ig4-7
- KDR-Ig4-6
- KDR-Ig5-7
- KDR-Ig5-6

**Figure 13. Schematic representation of initial KDR proteins and modified KDR proteins produced.** The above drawings represent VEGFR proteins and their composition of Ig-like domains. The uppermost drawing shows the extracellular part of KDR and its seven Ig-like domains. Initial VEGFR proteins represent three proteins that were initially made as candidates for VEGFR antagonist with KDR-B being the most potent.
Based on initial studies of KDR-B protein was the one that exhibited the highest KDR antagonistic activity (data not shown). The purified KDR-B protein and G129R-KDR-B fusion protein exhibited a molecular size of 43 kDa and 66 kDa, respectively on SDS-PAGE gel under reducing condition (Figure 14). The production of KDR-B and G129R-KDR-B proteins were verified by Western Blot analysis by using anti-KDR antibody. The gel electrophoresis and Western analysis confirmed the proteins had the correct size and purity for further evaluations of its properties.

After having successfully cloned and produced KDR-B (Figure 14) and KDR-C (data not shown) proteins, further modifications were applied to the overall design to allow for better characterization and identification of the KDR antagonist. We envisioned that this would allow us to produce a protein with enhanced activity and to characterize the Ig-like domain of its significance. The modification was based on the fact that the composition of the extracellular component of the KDR was made up of seven Ig-like domains of which some had been know to have either ligand binding activity or receptor dimerization activity. Therefore, KDR-B was modified to contain precisely four through seven Ig-like domains to produce KDR-Ig4-7 protein. Moreover, KDR variant proteins were designed and produced in order for testing against KDR-Ig4-7 in which each Ig-like domain was either deleted or included to produce different variants of the KDR protein, namely KDR-Ig1-3, KDR-Ig4-6, KDR-Ig5-7 and KDR-Ig5-6.
Figure 14. SDS-PAGE and Western Blot of initial production of KDR-B and its fusion protein, G129R-KDR-B. After purification by chromatography using a Q-sepharose column, the proteins were analyzed by denaturing SDS-PAGE to examine their size and purity. Western blots using anti-KDR was probed to confirm the purified proteins were KDR-B (43 kDa), G129R-KDR-B (66 kDa), arrows indicated.
As was the case for KDR-A, corresponding protein, KDR-Ig1-3 could not be produced into a properly refolded active protein using the bacterial system of production which then led us to use transient and stable cell lines to produce KDR-Ig1-3 for its testing. KDR proteins used in the following studies were cloned and produced as mentioned in the material and methods chapter. As shown in Figure 16 left panel, KDR-Ig4-7, KDR-Ig5-7, KDR-Ig4-6, and KDR-Ig5-6 proteins had molecular weights corresponding to 49kDa, 38kDa, 37kDa, and 28kDa, respectively. These proteins were further verified by Western Blot analysis by probing against anti-KDR antibody (Figure 15 right panel).
Figure 15. SDS-PAGE and Western Blot of KDR antagonist, KDR-Ig4-7 and its variant proteins. After purification by FPLC chromatography using a Q-sepharose column, the proteins were analyzed by denatured SDS-PAGE to examine their size and purity. Western blots were used to confirm the purified proteins with anti-KDR antibody demonstrating the proper molecular sizes (KDR-Ig4-7, 49 kDa, KDR-Ig5-7, 38 kDa, KDR-Ig4-6, 37 kDa, KDR-Ig5-6, 28 kDa).
4.2. KDR-Ig4-7 Binding on Endothelial Cells and Tissue Distribution in Mice

To evaluate the KDR-Ig4-7 as a potential KDR antagonist it was important to examine whether it retained its ability to directly interact with KDR. To address this question, KDR-Ig4-7 protein was subjected to a competitive radio-receptor binding assay using HUVEC (Figure 16), which expresses high levels of KDR on its cell surface (Zeng et al., 2001). $^{125}$I-labeled KDR-Ig4-7 was competitively displaced by increasing amounts of unlabeled KDR-Ig4-7 with an EC$_{50}$ value of approximately 7 μg/ml (143 nM). This assay showed that KDR-Ig4-7 has the ability to bind to HUVEC, which strongly suggested that KDR-Ig4-7 can bind to KDR.
Figure 16. Competitive radioreceptor binding assay of KDR-Ig4-7 using HUVEC. Cells were passed into 6-well plates at $10^6$/well. 100K cpm of $^{125}$I labeled KDR-Ig4-7 was incubated without or with various concentrations of unlabeled KDR-Ig4-7 as indicated for two hours. Cells were then washed with PBS and lysed. The radioactivity was determined by a scintillation counter. The data in this figure is represented as the means ± SD and is from duplicate determinations of seven separate experiments. The EC$_{50}$ value is approximately 7µg/ml.
Tumor angiogenesis is one of the hallmarks of cancer and also the basis for developing a KDR antagonist that can inhibit growth of neovasculature in tumors. Therefore, KDR-Ig4-7, as a potential KDR antagonist, was tested as to its distribution in various organs in mice bearing actively growing tumors of the breast (Figure 17). When $^{125}$I-labeled KDR-Ig4-7 was injected i.p. into the mice and the organs removed after 6 hours, the radioactivity measurements in various organs resulted in high level of $^{125}$I-labeled KDR-Ig4-7 in breast cancer tissue, kidney and lung. The high level measured in kidney and lung can be attributed to the high concentration density of microvessels and capillaries that are lined with endothelial cells that express KDR receptor. The high level of $^{125}$I-labeled KDR-Ig4-7 measured in breast cancer tissue was in agreement with the fact that active tumor angiogenesis was taking place and $^{125}$I-labeled KDR-Ig4-7 was most likely to be targeted to those areas of high tumor angiogenesis.

Furthermore, low level of $^{125}$I-labeled KDR-Ig4-7 measured in mammary gland was in stark contrast to the high level found in breast cancer tissue, which was indicative of $^{125}$I-labeled KDR-Ig4-7 binding to endothelial cells actively proliferating in breast cancer tissue. When one measures the level of $^{125}$I-labeled G129R as opposed to $^{125}$I-labeled KDR-Ig4-7, $^{125}$I-labeled G129R was highest among mammary gland and breast cancer tissue supporting the targeting ability of G129R to bind prolactin receptors (PRLR) found on mammary gland and breast cancer tissues. G129R has been shown to bind to PRLR found on the surface of mammary glands and many breast cancer cell lines (Zhang et al., 2002).
Figure 17. Tissue distribution of $^{125}$I-labeled KDR-Ig4-7 in comparison with G129R. The distribution of KDR-Ig4-7 was assessed by i.p. injection into 6 Balb/c mice bearing human breast cancer xenografts. Approximately, 6.8 μCi of $^{125}$I-labeled KDR-Ig4-7 was i.p. injected into each mouse. Six hours after injection, animals were sacrificed and various tissues were dissected, weighed and the amount of radioactivity was determined by scintillation counter. The data was normalized by reference to the total cpm in 100 μl serum of each animal and expressed (means ± SD) as percent of cpm/mg of tissue. .
Since Ig-like domains four and seven are thought to have receptor dimerization properties (Ruch et al., 2007; Tao et al., 2001), there was a possibility that the fragment would form dimers with itself. If that was the case, the potential of using it as a KDR antagonist was greatly diminished. To examine this possibility, a cross-linking agent, [Bis(Sulfosuccinimidyl) suberate] (BS³) a water-soluble, homobifunctional N-hydroxysuccinimide ester that has a spacer arm length of 11.4 Å was used for protein-protein associations. In Figure 18, when one compared lane 2 to lane 3, incubating KDR-Ig4-7 with cross linking agent BS³ resulted in a smear of bands as shown in lane 3 in contrast to a single band of KDR-Ig4-7 in lane 2. This suggested that it was unlikely for KDR-Ig4-7 to form dimers of itself because one would have expected to find a thick bright band at twice the molecular weight of a single KDR-Ig4-7 instead of a smear of bands. The smear was most likely the result of random interaction of numerous KDR-Ig4-7 with itself and cross-linking agent to form a gradient of multi-complexes. It has been suggested based on deletion studies that Ig-like domains 4-7 in the absence of Ig-like domains 1-3 and VEGF contain structural features that repel receptor-receptor dimerization (Tao et al., 2001).
Figure 18. Cross-linking assay of KDR-Ig4-7 with BS\(^3\) agent. A cross-linking reagent [Bis(Sulfosuccinimidyl) suberate] (BS\(^3\)) a water-soluble, homobifunctional N-hydroxysuccinimide ester was used in the experiment. The reaction mixture was incubated at room temperature for 30 minutes. At the end of the reaction, the reaction mix was subject to 4-12% SDS-PAGE and stained using SYPRO orange protein stain. Lanes are represented by 1, ladder; 2, KDR-Ig4-7; 3, KDR-Ig4-7+BS\(^3\); 4, ladder; 5, KDR-Ig4-7+VEGF; 6, KDR-Ig4-7 +VEGF+BS\(^3\); 7, ladder.
In lane 5 (Figure 18), when KDR-Ig4-7 was incubated with VEGF ligand, one band appeared corresponding to a 49 kDa KDR-Ig4-7 band and the second to an unexplained approximately 66 kDa band. The second band did not fit the total molecular weight of KDR-Ig 4-7 binding with VEGF, since they would be approximately 89 kDa in size. Also Ig-like domains 4 through 7 does not possess ligand binding properties (Davis-Smyth et al., 1996; Shibuya, 2006; Tanaka et al., 1997). When cross-linking agent was added to the KDR-Ig4-7 and VEGF mixture as shown in lane 6, KDR-Ig4-7 monomer band disappeared as shown in lane 3 and also showed a faint but clear second band that was larger than 66 kDa. As with the second band in lane 5, it was unclear as to the nature of this second larger band.

4.3. Functional Studies of KDR-Ig4-7 and its Variants using Endothelial Cells

4.3.1. The Effects of KDR-Ig4-7 and its Variants on HUVEC Proliferation

To determine the effects of KDR-Ig4-7 on endothelial cell growth, VEGF stimulated HUVEC were incubated in the presence of increasing concentrations of KDR-Ig4-7, KDR-Ig4-6 and KDR-Ig5-7 proteins for 48 hrs. As shown in Figure 19, KDR-Ig4-7 was able to inhibit endothelial cell proliferation in a dose-dependent manner with an IC\textsubscript{50} value of approximately 1 µM. In addition, KDR-Ig4-7 showed greater inhibition of endothelial cell proliferation than KDR-Ig4-6 and KDR-Ig5-7 proteins across most of the concentrations tested with a significant inhibition at 1000 nM concentration when compared to KDR-Ig4-6 (p<0.05) and KDR-Ig5-7 (p<0.01).
Figure 19. The effects of KDR-Ig4-7, KDR-Ig5-7 and KDR-Ig4-6 on HUVEC proliferation. HUVEC were cultured in the presence of various concentration of KDR proteins for 48 hr. The viability of the HUVEC was determined by the colorimetric MTS-PMS assay (Promega). Results are presented as the percent of total viable cells compared to control (untreated cells) from at least three separate experiments. KDR-Ig4-7 was significantly different from KDR-Ig5-7 (* p < 0.05) and KDR-Ig4-6 (** p < 0.01).
On the other hand, KDR-Ig4-6 and KDR-Ig5-7 showed similar level of inhibition of endothelial cell proliferation. With the results demonstrating that KDR-Ig4-7 possessed more potent inhibitory effects than that of KDR-Ig4-6 and KDR-Ig5-7 proteins, it is suggested that Ig-like domain 4 and Ig-like domain 7 together might play a role in having stronger affinity towards KDR and therefore resulted in better inhibitory ability on HUVEC proliferation. As negative controls, G129R protein which was produced using the identical production method as KDR-Ig4-7 showed no inhibitory effects on HUVEC (data not shown). Also elution buffer that was used to elute KDR-Ig4-7 from anion-exchange chromatography column was also tested and showed no inhibitory effects (data not shown). This suggested that KDR-Ig4-7 was the sole molecule that exerted the inhibition on HUVEC and not other factors from the production process or the purification process.

4.3.2. VEGF Dependent Inhibitory Effect of KDR-Ig4-7 on Cell Proliferation

VEGF binding to the VEGF receptor is suggested to be a pre-requisite for VEGFR dimerization and activation of VEGF receptor (Ruch et al., 2007; Tao et al., 2001). Therefore it was of interest to determine what effect the presence and absence of VEGF would have on the ability of KDR-Ig4-7 to inhibit HUVEC cell proliferation. As shown on Figure 20, the presence of VEGF significantly enhanced the inhibitory effects of KDR-Ig4-7 on HUVEC proliferation at 1000 nM compared to the absence of VEGF.
Figure 20. The effect of KDR-Ig4-7 on the cell proliferation of HUVEC in the presence and absence of VEGF. HUVEC were cultured with various concentration of KDR proteins in the presence or absence of VEGF for 48 hr. The viability of the HUVEC was determined by the colorimetric MTS-PMS assay (Promega). Results are presented as the percent of total viable cells compare to control (untreated cells) from least three separate experiments. * p < 0.05, ** p < 0.01.
These results suggested additional support for the finding that VEGF binding to the VEGF receptor is a pre-requisite for receptor dimerization and activation and VEGF induced conformational change in the receptor promotes a more favorable condition for receptor-receptor interaction and maybe KDR-Ig4-7-receptor interaction in our study.

4.3.3. The Effects of KDR-Ig4-7 and KDR-Ig5-6 on the Phosphorylation of ERK in HUVEC

With the understanding that the KDR signaling pathway for endothelial cell proliferation and growth utilizes the Raf-MEK-ERK1/2 pathway, we used an ERK phosphorylation assay to test the whether KDR-Ig4-7 can inhibit ERK phosphorylation which may result in the inhibition of cell proliferation seen in Figure 21. HUVECs were treated with various concentrations of KDR-Ig4-7 and KDR-Ig5-6 (10 nM and 1000 nM) either in the presence and absence of VEGF for 2 hours and 24 hours. Cell lysates of HUVEC were isolated and analyzed to determine the level of ERK phosphorylation. As shown in Figure 21, treatment of KDR-Ig-4-7 and KDR-Ig-5-6 resulted in inhibition of ERK phosphorylation with KDR-Ig-4-7 showing more potent inhibition than that of KDR-Ig5-6. This suggested essential roles for Ig-like domain 4 and 7 in the inhibitory actions of KDR-Ig4-7 and was in agreement with the results of the cell proliferation assay (Figure 19). Both KDR proteins showed certain degree of dose dependent inhibition on ERK phosphorylation in HUVEC.
Figure 21. Western blot analysis of KDR-Ig4-7 and KDR-Ig5-6 effect on ERK phosphorylation of HUVEC for 2 hours (A) and 24 hours (B). HUVEC were treated with indicated amounts (nM) of KDR-Ig4-7 and KDR-Ig5-6 in the presence and absence of VEGF. Total cell lysates were isolated and analyzed on a 4-15% gradient SDS-PAGE followed by Western blotting using antiserum against phosphorylated ERK, ERK-1, STAT-5 and β-actin. Panel A represents 2 hours of treatment and panel B represents 24 hours of treatment with indicated proteins.
Interestingly, both KDR proteins seem to affect the level of unphosphorylated ERK-1 level as well. More experiments would be needed to verify this phenomenon. The presence of VEGF seemed to have a greater inhibitory effect by KDR-Ig4-7 on the level of phosphorylation of ERK than in the absence of VEGF, which was also seen with HUVEC cell proliferation assay results (Figure 19). These results further suggested that VEGF interaction with KDR might induce certain conformational change that in turn enhances the binding of KDR fragments resulting in better inhibitory effects. There was no change in STAT-5 level (Figure 21 panel A) which indicated equal loading among samples. However, we also noticed that our original loading control, β-actin level fluctuated with different treatments that could not be readily explained.

4.3.4. The Effect KDR-Ig4-7 on HUVEC Migration and Tube Formation

To further evaluate the anti-angiogenic activity of KDR-Ig4-7, an endothelial cell migration assay and endothelial tube formation assays were conducted. HUVEC cells were used for their known ability to migrate in the presence of VEGF and/or other angiogenic factors during angiogenesis (Goodwin, 2007). A gap or a wound was made down the center of the HUVEC at the beginning of the experiments and cells were incubated to evaluate the ability of HUVECs to migrate and cover the wound as a function of time in the presence or absence of VEGF and KDR-Ig4-7. As shown in Figure 22, VEGF panel, after 16 hours of culture the wound is almost completely filled with VEGF treatment (middle right pictures).
Figure 22. The effects of KDR-Ig4-7 on migration of HUVEC. HUVEC were plated on a 48-well plate and grown to confluency. A wound was made through the middle of the well using a micropipette tip. The cells were treated with designed concentrations of protein and allowed to grow for another 24hrs. The treatments were performed in triplicates. Cells were viewed with an Olympus light microscope and pictures were taken at 10X magnification.
However, treatment with KDR-Ig4-7 (600 nM) prevented the migration of HUVEC since there was a wider gap compared to the control and VEGF treated (bottom right picture). It also appeared that some HUVEC were dead or dying, indicating the induction of apoptosis (Ferrari et al., 2006; Jia et al., 2001).

The formation of tubular structures by HUVEC is reminiscent of blood vessel formation in vivo. Therefore HUVEC tube formation, a standard assay in testing anti-angiogenic drugs was utilized to evaluate KDR-Ig4-7 and KDR-Ig5-6. The assay was conducted in matrigel that provided conditions for endothelial cells to grow and differentiate into tubular structures. HUVEC without any treatment showed the formation of tubular structures (Figure 23). With low concentrations (0.1 µM) of both KDR-Ig4-7 and KDR-Ig5-6 proteins, somewhat normal tubular structures were present but at a reduced level compared to the control. But with high concentrations (3 µM) of treatment there was a difference between the two protein treatments on the formation of tubular structures. While KDR-Ig5-6 treatment still retained some tubular structures, KDR-Ig4-7 treatment completely abolished the tubular structures. As a comparison HUVEC was also treated with bevacizumab, a humanized monoclonal anti-VEGF antibody to show complete absence of tubular structures.
Figure 23. The effects of KDR-Ig4-7 and KDR-Ig5-6 on HUVEC tube formation. HUVEC were plated on Matrigel-coated 48-well plates at 12,500 cells/well in the presence of indicated KDR proteins for 24 hrs at 37°C. bevacizumab was used as positive control. The effects were evaluated for the formation of tubular structures similar to that of blood vessels. Each experiment was conducted in triplicates. Cells were viewed with an Olympus light microscope and pictures taken at 10X magnification.
4.4. Functional Studies of KDR-Ig4-7 and its Variants on Cancer Cells

4.4.1. The Effects of KDR-Ig4-7 and its Variants on T-47D Breast Cancer and Various other Cancer Cells Proliferation

It has been reported that almost all cancer cells express VEGF and many express VEGFRs (Berns et al., 2003; Fujimoto et al., 1998; George et al., 2001; Gorski et al., 2003; Ikeda et al., 1999; Lee et al., 2000; Maeda et al., 1996; Manders et al., 2002; Masood et al., 2001). Hence it is conceivable that anti-angiogenic agents can directly affect tumor cells. Based on this we wanted to determine whether KDR-Ig4-7 protein could inhibit the growth of cancer cells. Cancer cells that were tested in this study included human breast cancer cells T47-D, MDA-MB 231, SKBR-3 and BT-474 and McNeu cells, an epithelial origin isolated from a tumor of MMTV-Neu transgenic mouse and human leukemic cells, HEL and HL-60.

As shown in Figure 24, KDR-Ig4-7 protein was able to inhibit T47-D breast cancer cell proliferation while KDR-Ig5-7 protein showed much less inhibitory activity. The growth of T47-D cells were significantly inhibited at 300 nM (p<0.001) and 600 nM (p<0.01) by KDR-Ig4-7.
Figure 24. The effects of KDR-Ig4-7 and KDR-Ig5-7 on T-47D and MDA-MB 231 human breast cancer cell proliferation. Viability of the T-47D cells was determined by the colorimetric MTS-PMS assay (Promega). Results are percent of viable cells after treatment for 48 hrs and the results are plotted as a percentage of control (cells without treatment) in each experiment. The mean and SD were calculated from at least three experiments. KDR-Ig4-7 was significantly different from KDR-Ig5-7 at 300 nM (** p < 0.01) and 600 nM (*** p < 0.001).
One of the strongest inhibitory activities by KDR-Ig4-7 was seen with SKBR-3 and BT-474 breast cancer cells and McNeu cells treated with KDR-Ig4-7 as shown in Figure 25. A clear inhibition of SKBR-3, BT-474 and McNeu cells started at a low dose and continued up to higher doses in a dose dependent manner showing the highest inhibition of about 90% with SKBR-3 cells. Even though the inhibitory trend was similar there were clear difference in cell type specificity of inhibition by KDR-Ig4-7 between cancer cells of BT-474 and other two cells by a significant margin (100 nM and 300 nM, p<0.01).

The result of human leukemic cells HEL and HL-60 is shown in Figure 26. KDR-Ig4-7 showed a strong but different dose dependent inhibition of HEL and HL-60 cells. KDR-Ig4-7 protein exerted a stronger inhibition with HEL cells compared to HL-60 cells with the maximum inhibition being at approximately 60% with HEL cells compare to 20% with HL-60 cells. MDA-MB 231 cancer cells exhibited the lowest responsiveness to the KDR proteins (Figure 25). A greater level of inhibition by KDR-Ig4-7 protein was seen with T47-D cells in comparison to MDA-MB 231 cells as one could see from the inhibitions of 10 nM through 600 nM concentrations. It would be of great interest to measure the level of VEGF receptors on these cancer cells to determine if expression level and cell type is correlated with level of inhibition. All the cancer cells treated in figures showed a dose dependent inhibition by KDR-Ig4-7.
Figure 25. The effects of KDR-Ig4-7 on the cell proliferation of SKBR-3, BT-474 and McNeu cell lines. Viability of the cells was determined by the colorimetric MTS-PMS assay (Promega). Results are percent of viable cells after treatment for 48 hrs and the results are plotted as a percentage of control (cells without treatment) in each experiment. The mean and SD were calculated from at least three experiments. BT-474 was significantly different from McNeu cells at 100 nM (** p < 0.01) and from both SKBR-3 and McNeu cells at 300 nM (** p < 0.01)
Figure 26. The effects of KDR-Ig4-7, KDR-Ig5-7 and VEGF-KDR-Ig4-7 fusion protein on cell proliferation of human leukemic cell line HEL and HL-60. Viability of the HEL and HL-60 are determined by the colorimetric MTS-PMS assay (Promega). Results are percent of viable cells after treatment for 24 hrs and the results are plotted as a percentage of control (cells without treatment) in each experiment. The mean and SD were calculated from at least three experiments. KDR-Ig4-7-VEGF was significantly different from KDR-Ig4-7 at 300 nM (*p<0.05) in HL-60 cells.
4.4.2. Effects of KDR-Ig4-7 and KDR-Ig5-6 on ERK Phosphorylation in T47-D Breast Cancer Cells

Based on the results of cancer cell proliferation assays, we wanted to determine if the inhibitory effects of KDR-Ig-4-7 on cancer cells was also mediated, at least in part, through the inhibition of ERK phosphorylation as seen in HUVEC. In contrast to the results obtained in HUVEC, at 2 hours of treatment, the level of ERK phosphorylation in T-47D cells was increased with both KDR proteins (Figure 27 panel A). This activation seemed to be transient since at 24 hr treatment, the phosphorylated ERK was inhibited with both KDR proteins (Figure 27 panel B). Again, the level of unphosphorylated ERK was also down regulated with 24 hours (B) but not with 2 hours of incubation. The inhibitory effects of both KDR proteins were not greatly affected by the presence of VEGF, which was also slightly different from what was seen in HUVEC ERK phosphorylation assay. We speculated that it might be related to the difference in VEGF receptor levels between these two types of cells. As in the case of HUVEC, the levels of β-actin were not affected with 2 hours treatment but were affected with 24 hours treatment. However, levels of STAT-5 were not affected in both 2 and 24 hours of treatment.
Figure 27. Western blot analysis of KDR-Ig4-7 and KDR-Ig5-6 effect on ERK phosphorylation of T-47D breast cancer cells for 2 hours (A) and 24 hours (B). T-47D cells were treated with indicated amounts (nM) of KDR-Ig4-7 and KDR-Ig5-6 in the presence and absence of VEGF. Total cell lysates were isolated and analyzed on a 4-15% gradient SDS-PAGE followed by Western blottting using antiserum against phosphorylated ERK, ERK-1, STAT-5 and b-actin. Panel A represents 2 hours of treatment and panel B represents 24 hours of treatment with indicated proteins.
4.4.3. The Effects of KDR-Ig4-7 and KDR-Ig5-6 Proteins on Apoptosis of T47-D breast cancer cells

As cell proliferation assays have shown the inhibitory effects of KDR proteins on different types of normal and cancer cells, we wanted to find out what effects KDR proteins would have on the apoptosis of T47-D breast cancer cells. To determine the state of apoptosis in T47-D breast cancer cells, viable cells were collected, DNA stained with a dye and analyzed using a Guava Cytometer to determine apoptotic, viable and dead cells. In Figure 28, there was a higher percent of apoptotic and dead T47-D cells when treated with KDR-Ig4-7 protein than with cells treated with KDR-Ig5-6 protein. Accordingly there was a lower percent of viable cells when treated with KDR-Ig4-7 protein than with cells treated with KDR-Ig5-6 protein. Again, supporting the finding that Ig-like domains 4 and 7 are important for the anti-angiogenic activity of KDR-Ig4-7 protein. As a positive control, staurosporine, a protein kinase inhibitor, was able to induce apoptosis. The apoptosis assay also suggested that KDR-Ig4-7 inhibits T47-D cell proliferation through the induction of apoptosis. Apoptosis may account for the inhibition of HUVEC cell proliferation as well.
Figure 28. Apoptosis assay of T47-D cells treated with KDR-Ig4-7 and KDR-Ig5-6. T47-D cells were plated on 12-well plates overnight and treated for 24 hrs with Staurosporine (1µM) as positive control, VEGF (25 ng/ml), KDR-Ig5-6 (10, 300, 1000 nM) and KDR-Ig4-7 (10, 300, 1000 nM). Cells were collected analyzed using a Guava Personal Cytometer with Guava CytoAnalysis software. Each sample was tested in triplicates and is representative of three independent experiments. The data in this figure is represented as the means ± SD. EB1 and EB2 represent negative controls consisting of elution buffer. Significant difference was in comparison to the Elution buffer controls (**p<0.01).
4.5. Effects of KDR Fusion Proteins on HUVEC and Leukemic cells

In an effort to improve the biological activity of KDR-Ig-4-7 after initial testing, novel fusion proteins were designed and produced. G129R-KDR-Ig4-7, KDR-Ig4-7-VEGF and VEGF-KDR-Ig4-7 were successfully produced and verified by SDS-PAGE and Western blot analysis (Figure 29). Fusion proteins that were produced exhibited enough purity to be used for evaluating its efficacy in inhibiting VEGF-induced KDR activation in HUVEC and T47-D breast cancer cells.

So far we have shown that KDR-Ig4-7 protein can inhibit cell proliferation in human endothelial cells, human breast cancer cells, mouse derived epithelial cells and leukemic cells. We also suggest that Ig-like domains 4 and 7 might play an even greater role in KDR-Ig4-7’s anti-angiogenic properties. Furthermore, the presence of VEGF exhibited an enhancement of the inhibitory activity of KDR-Ig4-7 protein. However, KDR-Ig4-7 protein’s binding affinity for KDR seem to be less than expected ($EC_{50}=7 \mu g/ml$) which motivated us to make modifications to improve on the binding affinity of KDR-Ig4-7 protein. KDR ligand, VEGF binds to the Ig-like domains 1 to 3 of the KDR, with strong affinity. Therefore we postulated that attaching VEGF to KDR-Ig4-7 protein could enhance the binding ability of the KDR protein. KDR fusion proteins were produced with VEGF attached to either ends of the KDR protein, C-terminal or N-terminal end. This could potentially enhance the KDR fusion proteins by the binding of VEGF moiety to the ligand binding site of KDR receptor and further promoting KDR-Ig4-7 moiety to bind to the receptor dimerization domains of the KDR.
Figure 29. SDS-PAGE and Western Blot analysis of KDR fusion proteins. After purification by chromatography using a Q-sepharose column, the proteins were analyzed by denaturing SDS-PAGE to examine their size and purity. Western blots using anti-KDR was performed to confirm the purified proteins. G129R-KDR represents G129R-KDR-Ig4-7 (72 kDa), KDR-VEGF represents KDR-Ig4-7-VEGF (68 kDa), and VEGF-KDR represents VEGF-KDR-Ig4-7 (68 kDa).
The reason for attaching VEGF to different sites was to determine which end-terminal attachment would provide the enhanced inhibitory activity we had hoped for since different orientations showed varying effects on the efficacy of other inhibitors.

To determine the effects of the KDR fusion proteins on cell proliferation they were tested on endothelial cells and leukemic cells. VEGF-stimulated HUVEC, HEL and HL-60 cells were incubated in the presence of increasing concentrations of KDR-Ig4-7-VEGF and VEGF-KDR-Ig4-7 proteins. As shown in Figure 30 and 31, the fusion proteins exerted inhibition with all three cells but with varying degrees of inhibition based on cell type, concentration and attachment site of VEGF. Overall, KDR-Ig4-7-VEGF fusion protein exerted higher inhibition on the proliferation of HUVEC and HL-60 cells than VEGF-KDR-Ig4-7 fusion protein with significant inhibition observed at 300 nM treatment. Based on the cell proliferation assay of HUVEC it is difficult to judge whether the KDR fusion protein has enhanced binding affinity over the KDR-Ig4-7 protein.
Figure 30. The effects of VEGF-KDR-Ig4-7 and KDR-Ig4-7-VEGF fusion proteins on cell proliferation of HUVEC. Viability of the HUVEC is determined by the colorimetric MTS-PMS assay (Promega). Results are percent of viable cells after treatment for 48 hrs and the results are plotted as a percentage of control (cells without treatment) in each experiment. The mean and SD were calculated from at least three experiments. KDR-Ig4-7-VEGF was significantly different from VEGF-KDR-Ig4-7 (*** p < 0.001).
Figure 31. The effects of KDR-Ig4-7, KDR-Ig5-7 and VEGF-KDR-Ig4-7 fusion protein on cell proliferation of human leukemic cell line HEL and HL-60. Viability of the HEL and HL-60 are determined by the colorimetric MTS-PMS assay (Promega). Results are percent of viable cells after treatment for 24 hrs and the results are plotted as a percentage of control (cells without treatment) in each experiment. The mean and SD were calculated from at least three experiments. KDR-Ig4-7-VEGF was significantly different from KDR-Ig4-7 at 300 nM (*p<0.05) in HL-60 cells.
4.6. Transient and Stable production of KDR proteins and its effect on HUVEC and T47-D Breast Cancer Cells

Having used a bacterial system to produce KDR proteins to evaluate its activity, we wanted to see if production of KDR proteins in a mammalian system can exert the full potential as a KDR antagonist. *E. coli* does not possess post-translational modification that is found in eukaryotic system. To address this we generated transient and stable cell lines to secrete the various KDR proteins by including the native signal peptide to each clone that were produced. They were evaluated using cell proliferation assay with HUVEC and T47-D breast cancer cells. As shown in Figure 32 various KDR proteins were evaluated as to its effect on cancer cell growth by transiently transfecting T47-D breast cancer cells. Surprisingly, we did not see any significant growth inhibition by KDR-Ig4-7 or any other KDR proteins expressed transiently compared to the empty vector (negative control) on T47-D breast cancer cells. The expression of KDR mRNA transcripts was confirmed by RT-PCR (data not shown). There could be many reasons for the results but one could be that KDR proteins did not get produced in high enough level to overcome the proliferative nature of the cells. More will be pondered in the discussion section.

With transiently expressed KDR proteins showing no significant inhibitory effect, we turned to stably transfected L-cells. We were successful in producing KDR-Ig1-3 protein and other KDR proteins by stably transfecting L-cells in contrast to the bacterial production method which did not produce KDR-Ig1-3. Total RNAs were isolated from each stably transfected positive L-cells and the presence of mRNA transcripts from L-cell clones were verified by RT-PCR (Figure 33). The results show that stably transfected L-
Figure 32. The effects of transient transfection of various genes on the cell proliferation of T-47D breast cancer cell line. Cells were seeded in six-well plates (35mm) at a concentration ranging from 1 to 2.0 x10^5 cells per well. Transient transfections of 1 and 2 μg of DNA plasmids (pcR3.1 empty vector, pIG KDR-Ig1-3, pIG KDR-Ig4-7, pIG KDR-Ig4-7-VEGF, pIG VEGF-KDR-Ig4-7 or pIG-KDR-Ig4-7-G129R) alone were performed using Lipofectin Reagent (Invitrogen). Transiently transfected cells were harvested after 24 hours for protein lysates. Cells in the number of 6.0 x 10^3 cells/well were plated onto 96 well plate and incubated at 37°C for 6 days and level of proliferation was measured using MTS-PMS (Promega). Results were plotted as a percentage of control (cells without treatment) in each experiment. The mean and SD were calculated from at least three experiments.
Figure 33. Verification of the expression of recombinant KDR mRNA transcripts by stably transfected L-cells. Total RNA were isolated from each stably transfected L-cells using Trizol reagent (Sigma). RT-PCR was conducted using specific primers to confirm the expression of mRNA transcripts of KDR-Ig 1-3, 4-7, and fusion clones KDR-VEGF, and KDR-G129R.

*Lanes, 2, 6, 10: negative controls; Lanes 3, 7, 11, 13: + positive controls; Lane 4: KDR-Ig1-3; Lane 8: KDR-Ig4-7; Lane 12: KDR-Ig4-7-VEGF; Lane 14: KDR-Ig4-7-G129R; and Lane 15: G3PDH (loading control).
cells with plasmid DNAs of KDR-Ig1-3, KDR-Ig4-7, KDR-Ig4-7-VEGF and KDR-Ig4-7-G129R were expressing the correct size mRNA transcript. For further verification of KDR protein expression, VEGF and VEGFR-2 ELISA were used to confirm the presence of KDR proteins (data not shown). The presence of KDR-Ig4-7 was not detected by ELISA which suggested that the KDR antibody was not recognizing KDR-Ig4-7. KDR-Ig4-7-G129R fusion protein was also verified of its presence by prolactin IRMA (Immunoradiometric Assay) that detects the G1219R moiety (data not shown).

Conditioned medium was collected from each stably transfected L-cells that were transfected with specific KDR plasmid DNA. As shown in Figure 34, KDR-Ig1-3 protein with Ig-like domains 1 to 3 responsible for ligand binding, exerted little or no inhibition on T47-D cell and HUVEC proliferation compared to the negative control (NC). KDR-Ig4-7 protein also exerted very little inhibition on both cell lines. However, fusion proteins of KDR-Ig4-7-VEGF, VEGF-KDR-Ig4-7, and KDR-Ig4-7-G129R exerted significant inhibition on both cells. The two KDR fusion proteins showed inhibition in the 40% range and KDR-Ig4-7-G129R showed slightly better inhibition of about 50%. The KDR fusion proteins seemed overall to have similar levels of inhibitory activity on both cell lines indicating VEGF attachment on either ends confer enhanced activity to the KDR fusion proteins. This KDR-Ig4-7-G129R fusion protein takes advantage of G129R moiety, a prolactin receptor antagonist that has a single amino acid substitution from a glycine to an arginine at position 129 of prolactin (Chen et al., 1999). G129R moiety is able to bind and inhibit prolactin receptor activation (Chen et al., 1999).
Figure 34. The effects of conditioned media from stably transfected L-cell on cell proliferation of HUVEC and T-47D breast cancer cell line. Cells were seeded in six-well plates (35mm) at a concentration ranging from 1 to 2.0 x10^5 cells per well. Stable transfections of 2 μg of DNA plasmids (pIG KDR-Ig1-3, pIG KDR-Ig4-7, pIG KDR-Ig4-7-VEGF, pIG VEGF-KDR-Ig4-7 or pIG-KDR-Ig4-7-G129R) alone were performed using Lipofectin Reagent (Invitrogen). Positive clones were selected using media containing 500 μg/ml of G418 sulfate. Results were plotted as a percentage of control (cells without treatment) in each experiment. The mean value and SD of the mean value of least three experiments was reported. All three fusion proteins were significantly different from the negative control (*** p < 0.001).
4.7 *In vivo* Study of the Effects of KDR-Ig4-7 on Lung Metastasis and Mammary Gland Tumor 4T1 Xenograft Model

The next logical test in assessing the anti-angiogenic activity of KDR-Ig4-7 protein was to conduct an *in vivo* study. To begin our study, 4T1 mouse mammary gland tumor cells, an aggressive cell line, was established in 6 Balb/c mice and divided into 3 groups of 2 mice each. Purified KDR-Ig4-7 protein (72 µg) was injected (i.p.) everyday for a period of two weeks. The control group was treated with identical volume of PBS. Contrary to the inhibitory effects observed with *in vitro* assays, tumor xenograft model displayed tumor weights that were significantly increased by the treatment of KDR-Ig4-7 protein in mice with $1.0 \times 10^5$ 4T1 cells (p<0.01) and $1.0 \times 10^4$ 4T1 cells (p<0.05) (Figure 35). Mice with $1.0 \times 10^6$ 4T1 cells showed almost no difference in tumor weight between the treated and untreated. It is difficult to explain the stimulatory effect KDR-Ig4-7 had on the 4T1 cancer cells but we can speculate that KDR-Ig4-7 protein, which is based on human DNA, could not recognize mouse KDR receptors on the mouse endothelial cells. Other possibilities are discussed in the discussion section.
Figure 3.5. The effect of KDR-Ig4-7 treatment on 4T1 mouse mammary gland tumor cells xenografts in Balb/c mice. Mice were divided into 3 groups of 3, 4 and 3 to which $0.5 \times 10^6$ cells, $0.5 \times 10^5$ cells and $0.5 \times 10^4$ cells were respectively inoculated subcutaneously in a 1:1 mixture with Matrigel (BD Biosciences) in volume of 100 μl on both left and right sides of the mammary gland to give a total of $1.0 \times 10^6$ cells, $1.0 \times 10^5$ cells and $1.0 \times 10^4$ cells per mice. After the control study, six female BALB/c mice of 3 months of age were divided into three groups of 2 mice each for the experiment. A total of $1.0 \times 10^6$ cells, $1.0 \times 10^5$ cells and $1.0 \times 10^4$ cells were inoculated per group and treated with KDR-Ig4-7 recombinant protein. The KDR-Ig4-7 was i.p. injected everyday for 2 weeks in a volume of 100 μl containing 72 μg of protein. Control group was injected i.p. with sterile PBS. The mean and SD were calculated from at least three experiments.

* $p < 0.05$. ** $p < 0.01$
Metastasis is one of the major characteristics of tumor angiogenesis and it is the leading cause of death among majority of the cancer patients. Anti-angiogenic drugs have been found to have properties that are able to limit or inhibit metastasis of tumor cells. We wanted to determine whether KDR-Ig4-7 protein had any inhibitory effect on metastasis of tumor cells, specifically 4T1 mouse mammary gland tumor which are known for their aggressive phenotype. 4T1 tumor cells were injected into tail vein of 27 Balb/c mice and randomly divided into 3 groups of 9 mice. The 3 groups were either treated with high (186 µg) or low (20 µg) dose of purified KDR-Ig4-7 protein or PBS (control). After 2 weeks of treatment, the results showed increased mouse pulmonary nodules when compared to the control group. As shown in Figure 36 the low dose treatment resulted in a significant (p<0.01) increase in the number of metastasized 4T1 cells found on the lung compared to the PBS treated control. The group treated with the high dose did not show any significant increase or decrease in 4T1 metastasis to the lung compared to the control. It is difficult to explain the reasons for this observation but attempting to treat mouse endothelial cells with human KDR proteins could be the reason for showing no inhibitory effects. But it still does not explain the stimulatory effects seen. However, one still notices a decrease in number of 4T1 metastasis to the lung with the high dose treated mice when compared to the low dose treated mice, even though they are not significant.
Figure 36. The effect of KDR-Ig4-7 treatment on lung metastasis of 4T1 mouse mammary gland tumor cells in Balb/c mice. 27 female BALB/c mice that were 3 months old were used in vivo 4T1 xenograft model to study the effect of purified recombinant KDR-Ig4-7 protein on metastasis of 4T1 cells. 9 mice per group were divided into control, high dose and low dose group. 4T1 cells of 100 μl volume containing 1 x 10^5 cells were inoculated into each mouse by tail vein injection. Treatment of mice in each group consisted of 300 μl volume of PBS, 186 μg (high dose) or 20 μg (low dose) everyday for a period of 2 weeks. After 2 weeks of treatment, mice in each group were sacrificed and lungs removed to determine the level of metastasis of 4T1 tumor cells. Each lung was stained with Accustain (Bouin’s Solution, Sigma) and viewed under the dissecting microscope in order to count the number of metastatic lung nodules. The mean and SD were calculated from at least three experiments. ** p < 0.01.
5. DISCUSSION

5.1. Current Strategies of Anti-angiogenesis in Cancer Therapy

VEGFR-2 is the main receptor that binds VEGF in both physiological and pathological vascular development. This makes VEGF/VEGFR-2 pathway a good target for anti-angiogenic therapies. Many of the current anti-angiogenic therapies have centered on either targeting the VEGF ligand to prevent binding to its receptor by the use of proteins (monoclonal antibody and soluble aptamers) or VEGFR-2 to prevent its activation by the use of proteins and small RTKI (Blagosklonny, 2004). Currently there are two FDA approved drugs solely for anti-angiogenesis treatment. They are bevacizumab (Avastin) and pegaptinib (Macugen) and are in clinical use to treat metastatic colorectal cancer and age-related macular degeneration, respectively (Sato, 2006). We have designed, produced and characterized the anti-angiogenic activity of a novel protein, KDR-Ig4-7 and its KDR variants.

Current strategies applied to inhibit VEGF signaling in anti-angiogenic therapy can be divided into two major strategies. The first strategy employs the use of neutralizing monoclonal antibody or soluble VEGF receptor or aptamers against VEGF/VEGFR to inhibit angiogenesis (Eskens and Verweij, 2006; Ferrara and Kerbel, 2005). This strategy has resulted in the development of drugs such as bevacizumab (Genentech), VEGF-Trap (Regeneron), vertsporfin (Novartis) and pegaptinib (OSI) (Ferrara and Kerbel, 2005; Folkman, 2007; Kowanetz and Ferrara, 2006). These agents either bind VEGF and prevent VEGF from binding to its native receptor or, bind to the
receptor and prevent activation by VEGF, ultimately inhibiting receptor activation and signal transduction. The second strategy employs the use of small molecule VEGFR tyrosine kinase inhibitors, or RTKIs, that act on the intracellular domain of the receptor to prevent receptor phosphorylation following ligand binding. These inhibitors include sunitinib (Pfizer), and sorafenib (Onyx) and vatalanib (Novartis). (Ferrara and Kerbel, 2005; Folkman, 2007; Kowanetz and Ferrara, 2006).

Bevacizumab, VEGF-Trap and aptamers have been designed to specifically neutralize the VEGF ligand from activating the receptor thereby inhibiting angiogenesis. Small molecule RTKIs, that have been developed to inhibit tyrosine kinase activity, not only inhibit VEGFRs but also PDGFRs, raf kinase, c-Kit receptors and fms-related tyrosine kinase 3 (Kowanetz and Ferrara, 2006). Both strategies have advantages and disadvantages in their attempts at blocking angiogenesis. The first strategy of using anti-angiogenic agents like monoclonal antibody to neutralize VEGF or VEGFR has an advantage of being highly specific and having a long half-life. But it also has disadvantages like high cost of production, high cost in drugs, long developmental phase and difficulties in producing high specificity. The second strategy of using RTKIs has its own advantages in that it has a shorter developmental phase, a lower cost of production, and a lower cost in drugs, but on the other hand, these inhibitors have downsides that are prone to drug resistance, low specificity, and short half-life.

5.2. The Rationale of Developing a Protein Based Novel Anti-Angiogenic Agents

In our study, we have come up with a novel design that could potentially be used as an anti-angiogenic agent. We proposed to produce a novel soluble protein that can bind
to the VEGF receptor and block the receptor from forming a dimer, thereby preventing receptor activation (Figure 9). This idea was based on several reports that showed specific Ig-like domains of the extracellular portion of the receptor were involved in the process of receptor dimerization. As all receptor tyrosine kinases form a receptor dimer for activation, Blechman et al. (1995) initially reported that their study of stem cell factor (SCF) receptors revealed the fourth Ig-like domain was essential for receptor dimerization and signal transduction. This suggested other receptor tyrosine kinases’ fourth Ig-like domain to have a similar function. Another finding by Barleon et al. (1997) showed that VEGFR-1 extracellular domains with varying deletions of the Ig-like domains demonstrated that Ig-like domains 1 through 3 was involved in VEGF binding. Also Ig-like domain four was shown to be essential in stabilizing receptor dimerization in association with VEGF. In that same year, Tanaka et al. (1997) reported virtually the same results for VEGFR-1, showing Ig-like domains 1 through 3 to have a strong ligand binding activity, and domains 4 through 7 to be involved in receptor dimerization. They also suggest that Ig-like domains 5 through 7 might potentially be involved in dimer formation as well. In 2001 it was reported that, based on mutational studies, Ig-like domains 4 through 7 of VEGFR-2 had structural features that allowed for receptor dimerization (Tao et al., 2001). This was the first report based on VEGFR-2. These findings suggested that a novel anti-angiogenic protein could be produced with specific Ig-like domains that possessed the receptor dimerization activity.

Our design of KDR proteins are novel in that we utilize a part of the receptor itself to act as an inhibitor to target VEGF receptor for inhibition. Currently available anti-
angiogenic drugs that target VEGFR are either monoclonal antibodies or tyrosine kinase inhibitors. As yet there has not been any report in literature of utilizing a part of the receptor to target VEGFR.

5.3. Pros and Cons of Using Bacterial or Mammalian Production System

In order for us to test our design, we produced the KDR proteins using an established bacterial (E. coli) system (Chen et al., 1999). This method of production allowed us to produce ample quantities with relative ease of production and at moderate cost. As shown in Figure 13 Initial VEGFR proteins, based on our initial design three KDR proteins, KDR-A, KDR-B and KDR-C were produced. We succeeded in producing KDR-B (Figure 13), KDR-C (not shown) and KDR fusion proteins (Figure 13). However, KDR-A protein presented a problem that prevented us from producing the protein. The problem was KDR-A protein did not remain dissolved in refolding buffer, but instead precipitated out during refolding process possibly due to its low PI value. Different bacterial production methods and refolding conditions were unsuccessful in resolving the issue and allowing the production of KDR-A protein. With the production of KDR-B and KDR-C, initial cell proliferation assays were conducted to test their anti-angiogenic activities.

Our results indicated that KDR-B had the highest anti-angiogenic activity in comparison to KDR-C (data not shown). But with the findings of Blechman et al (1995), Tanaka et al (1997), Barleon et al. (1997) and Tao et al. (2001), we decided that some modification to the protein could enhance the binding ability which affected the anti-angiogenic activity. We made modifications to KDR-B that allowed it to possess specific
combinations of the Ig-like domains of the extracellular KDR domains. The new design of the various KDR proteins is shown in Figure 13 Modified VEGFR Proteins: which include KDR-Ig1-3, KDR-Ig4-7, KDr-Ig4-6, KDR-Ig5-7 and KDR-Ig5-6. Of these proteins, KDR-Ig1-3 (equivalent form of KDR-A), was the only one produced using a different production strategy. Instead of using the bacterial system, we utilized the mammalian system of the transient and stable transfection method to produce the protein because of refolding problems. Therefore a different strategy was employed to produce all of the KDR proteins.

This raised the question of whether KDR proteins might exhibit different levels of activity depending on a bacterial or mammalian system of production. Before we discuss the difference in activity of the KDR proteins, there are advantages and disadvantages to the two systems of production. The use of the bacterial system of production has been the most widely and classically used method for the production of recombinant proteins. It has many advantages in having a relatively simple and straightforward procedure for production, well-studied physiology, simple genetics and availability of advanced genetic tools, rapid growth, high-level protein production, ease of handling in a standard molecular biology laboratory, low cost and the ability to combine both expression screening and protein production (Baneyx, 1999; Knaust and Nordlund, 2001; Lesley, 2001; Swartz, 2001). In contrast, the disadvantages of the bacterial system are found particularly in the production of eukaryotic proteins such as the lack of eukaryotic chaperones, specialized post-translational modifications, the ability to be targeted to subcellular locations or to form complexes with stabilizing binding partners that can result in
protein mis-folding and aggregation (Baneyx, 1999; Swartz, 2001). On the other hand, using a eukaryotic system has its advantages that compensate the bacterial method’s disadvantages, allowing correct folding or genuine post-translational modifications to the recombinant eukaryotic protein of interest. There are few disadvantages of using the mammalian method including the fact that it is more complex, time consuming, laborious and results in a relatively low quantity production of recombinant proteins.

Our evaluations of the anti-angiogenic activity of the recombinant KDR proteins were carried out mainly using the bacterially produced proteins (Figure 14, 15 and 29), with the exception of the cell proliferation assays of the transiently and stably transfected cells in Figure 32 and 34, respectively. Most of the bacterially produced KDR proteins were inhibitory in various cell-based assays, suggesting that KDR-Ig4-7 had KDR antagonistic properties, but the mammalian-based KDR proteins had mixed results. As shown in Figure 32, transiently produced KDR monomeric and fusion proteins had very little or no inhibitory effect on T47-D breast cancer cells over the empty vector control. However in Figure 34, the fusion proteins of KDR-Ig4-7-VEGF, VEGF-KDR-Ig4-7 and KDR-Ig4-7-G129R showed significant inhibition in both HUVEC and T47-D breast cancer cells. To explain the mixed results seen with the mammalian-based KDR monomeric and fusion proteins’ effect on the cell proliferation, the expression level of the transiently produced KDR proteins might not have been high enough to effectively inhibit the proliferation of T47-D breast cancer cells (Figure 32).

Of the results shown in Figure 32, very little inhibition shown by KDR-Ig1-3 protein is attributed to its ability to bind VEGF and not KDR. The reasons for that are
first, KDR-Ig1-3 is composed of Ig-like domains 1 through 3 (VEGF ligand binding properties); therefore, it could not directly block VEGFR-2 receptor activation on the cell surface to inhibit proliferation (Barleon et al., 1997; Tanaka et al., 1997). Second, even though KDR-Ig1-3 might have VEGF neutralizing properties similar to the actions of bevacizumab, it would not affect the proliferation of T47-D breast cancer cells because KDR-Ig1-3 does not directly interact with the cancer cell. However, the very low inhibition shown by KDR-Ig4-7 and other KDR fusion proteins was not as expected. We speculate that the low level of expression by transiently transfected T47-D cells could be the reason for the results.

5.4. The Binding Characteristics and the Pharmacodynamic Distribution of KDR-Ig 4-7 in Cell Culture and in Mice

With the production of modified KDR proteins, we asked whether the KDR-Ig4-7 protein had the ability to bind to its target KDR to inhibit its activation. As shown in Figure 16, 125I labeled KDR-Ig 4-7, when incubated with HUVEC cells, was competitively displaced by the increasing amounts of unlabeled KDR-Ig4-7. Knowing HUVECs express high level of KDR receptors on its surface, it is most likely that 125I labeled KDR-Ig 4-7 was binding to KDR and being displaced when unlabelled KDR-Ig4-7 was present in high amounts to compete for its binding. This result suggested that KDR-Ig4-7 was binding to it target receptor on HUVEC. We measured the binding affinity of KDR-Ig4-7 to be 7µg/ml or 143 nM. When this is compared to other VEGFR-2 binding molecules, it shows it has a relatively weak binding affinity to KDR. The binding affinity of VEGF-A to VEGFR-2 has been measured to be around 75-125 pM,
and the binding to VEGFR-1 to be 10-fold higher than that of VEGFR-2 (Fuh et al., 1998; Shinkai et al., 1998). Another example is an anti-angiogenic drug pegaptanib, an anti-VEGF aptamer having a binding affinity of 140 pM and IMC 1121B, a human anti-VEGFR-2 monoclonal antibody being developed by Imclone showing about 50 pM binding affinity to VEGFR-2 (Ng et al., 2006; Youssoufian et al., 2007). These data show the binding affinity of KDR-Ig4-7 is more than a 1000 fold lower in comparison. Even though KDR-Ig4-7 might have a low binding affinity, this does not preclude it from exerting anti-angiogenic activity.

Since tumor angiogenesis is required for tumor progression, KDR-Ig4-7, if active, should be concentrated in areas of high tumor vasculature. As shown in Figure 17, KDR-Ig4-7 was detected mostly in breast cancer, kidney and lung tissues in mice bearing Her2/neu breast tumor. This is in agreement with KDR-Ig4-7’s affinity for KDR receptor on endothelial cells, as kidney and lung tissues have high a concentration of microvessels and capillaries lined with endothelial cells. Endothelial cells are known for the high expression of KDR on its surface, and that KDR functions as targets for KDR-Ig4-7 proteins (Nakatsu et al., 2003). Also breast cancer tissue would provide an optimum environment for attracting KDR-Ig4-7 proteins because of the tumor angiogenesis driven highly developed tumor vasculature of the breast cancer tissue. In comparison, the G129R, a PRL receptor antagonist, was mostly detected in mammary tissue and breast cancer tissue, indicating its antagonistic properties by binding to highly expressed PRL receptors on those tissues (Chen et al., 1999). The difference shown by the two proteins concentrating in different tissues suggested their differing properties to be attracted to
specific sites. This data gave further support for the notion that KDR-Ig4-7 has the ability to bind to KDR receptors.

Since KDR-Ig4-7 is a soluble fragment containing Ig-like domains 4 to 7, we examined whether it would form homodimers. If KDR-Ig4-7 is found to form dimers, it would defeat the purpose of using KDR-Ig4-7 as an anti-angiogenic agent. With the use of cross-linking agent, [Bis(Sulfosuccinimidyl) suberate] (BS³), we showed in Figure 18 lane 3 and lane 6 that KDR-Ig4-7 did not form dimers of itself but remained in a monomeric form (lane 2). A smearing of bands (lane 3 and 6) suggested that a gradient of multi-complexes of KDR-Ig4-7 was formed from the random collision of KDR-Ig4-7 with the cross-linking agent. If dimers had actually formed, they would have formed a band with a size of about 100 kDa (two KDR-Ig4-7). When KDR-Ig4-7 was incubated with VEGF in the presence of the cross-linker, a band that measured 70-75 kDa appeared in lane 6. This band could be the result of cross-linking between KDR-Ig4-7 (49 kDa) and VEGF monomer (~23 kDa) under reducing conditions of SDS-PAGE gel. Native VEGF is a homodimer (46 kDa) linked by disulfide bonds (Holmes et al., 2007). A band that is difficult to explain was shown in lane 5 with a size of about 66 kDa in the absence of the cross-linking agent. KDR-Ig4-7, which does not possess VEGF binding domain, could not bind VEGF to form a complex in the absence of the cross-linking agent. In summary, we concluded that KDR-Ig4-7 does not form dimers but stays in a monomeric form as seen lane 2 of Figure 18.
5.5 The Anti-Angiogenic Effects of KDR-Ig4-7 and its Variants in HUVEC

With the KDR-Ig4-7 showing binding affinity to KDR, we examined the inhibitory activity of KDR-Ig4-7 and other KDR variant proteins. As HUVEC is utilized in many assays as the standard cell type for testing anti-angiogenic activity, we used it in cell-based assays. As shown in Figure 19, it is clear that KDR-Ig4-7 was able to inhibit VEGF-stimulated HUVEC cell proliferation. The results also showed that when tested in comparison to the other KDR proteins, KDR-Ig4-6 and KDR-Ig5-7, KDR-Ig4-7 exhibited significantly more potent inhibitory activity (50%) than that of the other 2 KDR proteins (30%) at 1000 nM concentrations. We could see from the results that KDR-Ig4-7’s inhibitory activity was dose dependent. Furthermore, increased inhibitory activity shown by KDR-Ig4-7, when compared to KDR-Ig4-6 and KDR-Ig5-7, suggested that Ig-like domains 4 through 7 and in particular Ig-like domains 4 and 7 might play a greater role than Ig-like domains 4 to 6 or 5 to 7 in receptor dimerization.

Experiments have shown that Ig-like domain 4 and 7 are involved in receptor dimerization of KDR, VEGFR-1 and PDGFR (Barleon et al., 1997; Blechman et al., 1995; Herren et al., 1993; Tanaka et al., 1997; Tao et al., 2001). More recently Ruch et al. (2007) have reported that in the presence of VEGF, Ig-like domain 4 functions to stabilize receptor dimerization and lock the receptor-ligand complex. In addition, Ig-like domain 7 is suggested to be responsible for bringing the transmembrane and cytoplasmic domains of the two VEGFR-2 into close proximity for receptor activation. Therefore we suggest, based on our findings of KDR-Ig4-7’s ability to exhibit a higher inhibitory activity in the presence of VEGF than other KDR proteins that are missing either Ig-like
domains 4 or 7, Ig-like domains 4 and 7 have greater roles in receptor binding leading to inhibitory activity of KDR-Ig4-7. This does not rule out the roles of Ig-like domains 5 and 6 in receptor dimerization and with inhibitory activity but that they seem to have reduced roles in the process compared to Ig-like domains 4 and 7.

As VEGFR-2 activation leads to endothelial cell proliferation through the Raf-MEK-ERK1/2 pathway, KDR-Ig4-7 was tested for its ability to inhibit ERK phosphorylation in HUVEC (Cebe-Suarez et al., 2006; Otrock et al., 2007; Rousseau et al., 1997; Shibuya, 2006). A dose dependent inhibition of ERK phosphorylation was seen with KDR-Ig4-7 at both low and high dose treatments at 2 and 24 hour incubations (Figure 21). This indicated that the inhibition of cell proliferation seen in Figure 19 was due to in part by the down regulation of ERK phosphorylation by KDR-Ig4-7. This inhibition of cell proliferation of HUVEC was also shown by Belloni et al. (2007). When VEGF induced ERK phosphorylation was blocked by a protein called vasostatin I, it resulted in the inhibition of HUVEC cell proliferation. In another study utilizing a cysteine-rich and basic domain fragment of a Tat protein, a polypeptide encoded by HIV-1 virus, it was able to inhibit VEGF receptor binding, cell proliferation and ERK phosphorylation in endothelial cells (Jia et al., 2001). In addition, our data demonstrate that the level of inhibition by KDR-Ig5-6 was less potent than that of KDR-Ig4-7 further supporting the involvement of Ig-like domains 4 to 7 in blocking KDR activation through receptor binding than the domains of 5 and 6 alone. Surprisingly the levels of loading controls of ERK-1, β-actin and STAT-5 were down-regulated in both 2 and 24 hours of incubation at varying degrees. We cautiously speculate that KDR-Ig4-7 and KDR-Ig5-6
proteins might be reducing the translation of ERK-1, β-actin and STAT-5 or increasing the degradation of these proteins.

Endothelial cell migration, proliferation and differentiation have been shown to occur through the pathway of VEGF induced VEGFR-2 activation (Ferrara and Davis-Smyth, 1997; Holmes et al., 2007; Olsson et al., 2006; Shibuya, 2006). Endothelial cell migration is one of the critical responses to VEGF that enables cells to move through the protease degraded basement membrane in the direction of high VEGF gradient in the process of angiogenesis (Holmes et al., 2007). When we examined the effects of KDR-Ig4-7 on HUVEC migration, KDR-Ig4-7 was able to prevent HUVEC migration (Figure 22). Treatment of HUVEC with KDR-Ig4-7 at 600 nM for 16 hours resulted in the inhibition of HUVEC migration compared with the control and VEGF treated HUVEC. This inhibition of migration by HUVEC at 600 nM was not due to cell death because the morphology of the HUVEC when compared to the control or VEGF treated indicated they looked almost identical and healthy and was still attached to the plate. When HUVECs were treated with diallyl trisulfide (DATS), a cancer-chemopreventive constituent of garlic, it was able to downregulate KDR expression and inhibit HUVEC migration (Xiao et al., 2006). This provides further support to the notion that KDR-Ig4-7 is affecting HUVEC via the KDR receptor to inhibit VEGFR-2 activation leading to the inhibition of cell migration.

The formation of tubes on Matrigel represents the ability of HUVEC to proliferate, migrate and differentiate in a highly regulated process to form a capillary-like tubular structures (Murray, 2001). In Figure 22 HUVEC tube formation assay, KDR-Ig4-
7 was able to inhibit tube formation in a dose dependent manner and exhibit much greater inhibitory effect on HUVEC tube formation than KDR-Ig5-6. Reduced inhibitory effects of KDR-Ig5-6 when compared to KDR-Ig4-7 on tube formation assay, is in agreement with the results of the cell proliferation (Figure 19) and ERK phosphorylation (Figure 21) suggesting Ig-like domains 4 and 7 play a greater role in receptor dimerization and inhibitory activity of HUVEC tube formation. It was shown that DATS, a cancer-chemopreventive constituent of garlic, was also able to inhibit HUVEC tube formation in part by inhibiting ERK1/2 (Xiao et al., 2006). This suggests that KDR-Ig4-7’s inhibition of ERK phosphorylation could also in part explain the inhibition of HUVEC tube formation as well. A receptor tyrosine kinase inhibitor, SU11248, that inhibits VEGFR-2, was shown to inhibit endothelial migration and tubule formation of endothelial cells in culture and blood vessel formation in vivo (Osusky et al., 2004). As would be expected bevacizumab was also able to inhibit tube formation (Figure 22). The results of the tube formation assay in addition to other \textit{in vitro} results demonstrated that KDR-Ig4-7 in HUVEC has potent anti-angiogenic properties that may function as a VEGFR-2 antagonist.

5.6 The Anti-angiogenic Effects of KDR-Ig4-7 is Enhanced in the Presence of VEGF

VEGF receptor activation requires two events, binding of VEGF to its binding site and receptors dimerization (Barleon et al., 1997; Tanaka et al., 1997; Tao et al., 2001). It was further shown that VEGF binding is a pre-requisite for receptor dimerization followed by receptor activation (Barleon et al., 1997; Ruch et al., 2007; Tao et al., 2001).
We noticed a difference in the inhibitory effect of KDR-Ig4-7 on HUVEC cell proliferation when VEGF was present as to when it was absent (Figure 20). In the presence of VEGF, KDR-Ig4-7 caused significantly higher inhibitory effects (100 nM and 1000 nM) on HUVEC cell proliferation than in the absence of VEGF. This was also observed in ERK phosphorylation assay (Figure 21) where VEGF presence resulted in a greater reduction of ERK phosphorylation level by KDR-Ig4-7 and KDR-Ig5-6 proteins at 10 nM and 1000 nM at both 2 hr and 24 hr treatment when compared to VEGF absence. Our data suggest that the presence of VEGF augments the inhibitory effects by KDR-Ig4-7 and to a lesser degree also the effects by KDR-Ig5-6. These findings suggest that VEGF binding to the VEGFR-2 receptor causes a conformational change in the receptor that might promote better interaction between KDR-Ig4-7 and VEGFR-2 that results in receptor inactivation.

Tao et al. (2001) showed VEGF binding to Ig-like domains 1 to 3 relieved the structural impediments imposed by Ig-like domains 4 through 7 that prevented receptor dimerization in the absence of the ligand. Our results showing KDR-Ig4-7 in the presence of VEGF has a higher inhibitory activity than in the absence of VEGF give further support for the findings of Tao et al. (2001) and Shibuya et al. (2006). In papers by Blechman et al. (1995), Barleon et al. (1997), and Ruch et al. (2007), they further discuss the likelihood of Ig-like domain 4 as the site for ligand dependent dimerization and that Ig-like domain 4 is involved in the stabilization of the VEGF bound VEGFR receptor dimer.
5.7 The Inhibitory Effects of KDR-Ig4-7 in Multiple Cancer Cells

Almost all cancer cells have been shown to express VEGF and VEGF receptors on its surface (Berns et al., 2003; Ferrara et al., 2003; Fujimoto et al., 1998; George et al., 2001; Gorski et al., 2003; Ikeda et al., 1999; Lee et al., 2000; Maeda et al., 1996; Manders et al., 2002; Masood et al., 2001). These cancer cells have been implicated in expressing VEGF and KDR for autocrine and paracrine mediated effects involved in tumor angiogenesis (Papetti and Herman, 2002). Therefore, anti-angiogenic therapies could be used for inhibiting cancer cell growth and proliferation directly by targeting the cancer cells or indirectly by targeting the tumor vasculature such as the endothelial cells.

When the effects of KDR-Ig4-7 and KDR variants on various cancer cells including human breast cancer cells, T47-D, MDA-MB 231 (Figure 24), SKBR-3, BT-474 and McNeu cells (Figure 25) and leukemic cells, HEL and HL-60 (Figure 26) were tested, we observed the inhibitory activity of KDR-Ig4-7 with varying degrees depending on the cell lines. The level of response to KDR-Ig4-7 from most sensitive to the lease sensitive was seen in the order of SKBR-3, BT-474 and McNeu cells showing the best response, followed by T47-D cells and the least response by MDA-MB 231 cells. We further demonstrated a dose dependent inhibition in these cells by KDR proteins and showed KDR-Ig4-7 is more potent than KDR-Ig5-7 (Figure 24). These results were similar to that of the results obtained with HUVEC. We believe that the different level of inhibition observed with different cancer cell lines is mostly likely due to the different KDR receptor levels on the cell surface. An interesting observation was noted with T47-D cells where 2-3 hours of treatment with high concentration of KDR-Ig4-7 caused
increasing amounts of cells to detach from the plate and stay rounded in the medium (data not shown). The cells that detached from the plate when reseeded in fresh growth medium in the absence of KDR-Ig4-7 were able to reattach and grow normally. This phenomenon was not seen with other cancer cell lines.

Similar to the results in HUVEC (Figure 21), the inhibitory effect of KDR-Ig4-7 on T47-D cell proliferation was also in part mediated through the inhibition of ERK phosphorylation (Figure 27). Despite of a transient elevation of ERK phosphorylation by KDR-Ig4-7 and KDR-Ig5-6 at 2 hours (Figure 27 panel A), the phosphorylated ERK level was inhibited in a dose dependent manner by both KDR proteins (Figure 27, panel B). The level of ERK, β-actin and STAT-5 was unaffected at 2 hours time but was reduced at 24 hours with the high dose treatment. KDR-Ig4-7 seemed to have no effect on level of the ERK, β-actin and STAT-5 when the level of phosphorylated ERK was elevated (2 hrs) but when phosphorylated ERK level was reduced (24 hrs), the level of ERK, β-actin and STAT-5 were reduced as well. The difference in the level of inhibition of phosphorylated ERK by KDR-Ig4-7 on T47-D when compared to HUVEC could be in part due to the difference in the level of KDR expression between HUVEC and T47-D cells. It has been shown that HUVEC expresses high level of KDR while T47-D cell does not express as much KDR on its surface (Stewart et al., 2003; Weigand et al., 2005). The overall results showed similarities between HUVEC and T47-D in that inhibition of T47-D cell proliferation was also mediated in part through the inhibition of ERK phosphorylation.
The cellular process in part responsible for the inhibition of cell proliferation was revealed with the apoptosis assay. T47-D cells treated with increasing doses of KDR-Ig4-7 caused cells to become apoptotic, have reduce viability and increase the number of dead cells whereas KDR-Ig5-6 had much less of an effect (Figure 27). Others have reported that angiogenesis inhibitors can induce apoptosis. For example, a cysteine-rich and basic domain of HIV-1 Tat peptide was shown to inhibit angiogenesis through the induction of endothelial cell apoptosis (Jia et al., 2001). Also in a study of pulmonary emphysema using rat as a model, VEGFR blocker SU5416 was shown to induce alveolar cell apoptosis (Kasahara et al., 2000). SU5416 was also able to inhibit growth of colon cancer and liver metastasis by inducing tumor and endothelial cell apoptosis (Shaheen et al., 1999). Based on our apoptosis assay results, we believe that the inhibitory effects of KDR-Ig4-7 in cell proliferation, is partly due to the induction of apoptosis.

5.8 KDR-Ig4-7 is not Effective in Inhibiting 4T1 Xenograft Growth and Metastasis in Mice

Based on the positive data from our in vitro assays, we further tested KDR-Ig4-7’s anti-tumor activity in vivo. Balb/c mice carrying 4T1 mouse mammary gland tumor was injected i.p. daily with KDR-Ig4-7 for a period of 2 weeks. The data seen in Figure 35 show the tumor weights of mice injected with 10^4 and 10^5 number of 4T1 cells and treated with KDR-Ig4-7 had significantly higher tumor weights than the untreated control group. But in mice with 10^6 number of 4T1 cells, there was no difference between the treated group versus the untreated group. The in vivo effect of KDR-Ig-4-7 was further evaluated by measuring the metastasis lesion of 4T1 cells to the lung. Again, no
inhibitory effect was observed. As a matter of a fact (Figure 36), there was an increase in total number of metastasized lung nodules in group treated with KDR-Ig-4-7 as compare to that of PBS.

One could speculate on different reasons for no inhibition of tumor growth by treatment with KDR-Ig4-7. One explanation could be that the there are many pro-angiogenic factors in addition to VEGF such as bFGF, IL-8, P1GF and PDGF that can regulate angiogenesis. Therefore the inhibitory effect of KDR-Ig4-7 could have been circumvented by other angiogenic pathways. Second, the negative in vivo result could be due to the relatively short serum half-life of KDR-Ig4-7 protein, which is based on the size of the molecule. KDR-Ig4-7 would have been cleared from circulation after few hours post injection therefore unable to maintain the effective concentration in serum. Third, the aggressive nature of 4T1 cells, make it insensitive to anti-angiogenic treatment. Fourth, KDR-Ig4-7 is a human version protein that may not recognize mouse VEGFR-2 on the endothelial cells hence rendering the KDR-Ig4-7 unable to bind to its intended receptor to exert its inhibitory activity. In short, further studies would be required to reach any conclusion.

5.9 Attempts to Improve the Potency of KDR-Ig4-7

We have shown the anti-angiogenic activity of KDR-Ig4-7 and the KDR variants produced in E. coli using various in vitro assays. We wanted to investigate the possibility of enhancing the anti-angiogenic properties of KDR-Ig4-7 and the KDR variants by utilizing a eukaryotic system of production. To meet our objectives of improving KDR-
Ig4-7, we decided to produce fusion proteins by fusing either VEGF or G129R to KDR-Ig4-7 through molecular cloning to enhance the KDR binding affinity or breast cancer specificity, respectively.

VEGF is a disulfide-linked homodimeric glycoprotein with a molecular weight of 40,000-46,000 Da, and it has a high affinity for VEGFR-1 ($K_d \sim 7.5-12.5$ pM) and VEGFR-2 ($K_d \sim 75-125$ pM) leading to angiogenesis (Ferrara and Davis-Smyth, 1997; Ferrara et al., 2003; Neufeld et al., 1999). Our logic behind the fusion of VEGF to KDR was that the fusion protein will have a high affinity through VEGF binding to Ig-like domains 1 through 3, but would not form a receptor dimer due to the KDR-Ig4-7 part of the fusion protein. Two fusion protein constructs were produced with the VEGF moiety attached to either end of the KDR-Ig4-7 protein. In addition, G129R fusion proteins were produced utilizing the G129R moiety as the targeting moiety to recognize the cancer cells with high level of PRL receptors. The G129R moiety is virtually a PRL with a single amino acid substitution at position 129 from a glycine to an arginine to generate a PRL receptor antagonist (Chen et al., 1999). This PRL antagonist has shown its inhibitory effects in many breast cancer cells as a single agent and also as a targeting moiety on a fusion protein with other effector molecules such as endostatin and IL-2 (Beck et al., 2003; Ramamoorthy et al., 2001; Zhang et al., 2002).

Cell proliferation assays tested with fusion proteins, KDR-Ig4-7-VEGF and VEGF-KDR-Ig4-7 made from *E. coli* were conducted with HUVEC and leukemic cell lines HEL and HL-60, shown in Figure 30 and 31. The fusion proteins exerted a dose dependent inhibition of cell proliferation in HUVEC and leukemic cells. HUVEC cell
proliferation assay (Figure 30) did not show a noticeable inhibition by both fusion proteins until the concentration reached 300 nM. KDR-Ig4-7-VEGF fusion protein (~50%) exhibited a significantly higher inhibition than VEGF-KDR-Ig4-7 fusion protein (0%). The maximum inhibition of 90% was reached with 600 nM treatment with both fusion proteins but we believe it is due to non-specific inhibition (Figure 30). When the fusion proteins were tested in HEL and HL-60 leukemic cells, it did not show a clear difference in efficacy between the two fusion proteins and also between the fusion proteins and KDR-Ig4-7. However, KDR-Ig4-7-VEGF did show a significant inhibition at 300 nM in HL-60 cells (Figure 31). Despite the potential for increasing the binding affinity and efficacy of the KDR proteins, the *E. coli* produced KDR fusion proteins did not exhibit a greater level of inhibition of cell proliferation in HUVEC and leukemic cells beyond that of KDR-Ig4-7. The two KDR fusion proteins could have exhibited less than expected inhibitory activity because of their production using a bacterial system that lack post-translational modifications.

The two KDR fusion proteins (KDR-Ig4-7-VEGF and VEGF-KDR-Ig4-7) and KDR-Ig4-7-G129R fusion protein were also produced in a mammalian system since mammalian production provides the necessary post-translational modification to the protein. KDR is known to require post-translational modification, i.e. glycosylation, to form a mature 230 kDa receptor on the surface of endothelial cells (Boldicke et al., 2005; Takahashi and Shibuya, 1997). T47-D breast cancer cells were transiently transfected with each fusion plasmid DNA using lipofectin to deliver the fusion protein gene. As shown in Figure 32, after 72 hours of incubation, T47-D cells that were transiently
transfected did not show any significant inhibition of cell proliferation compared to the empty vector control. We suspect that even though mRNA transcripts were detected (data not shown), there may not have been enough KDR proteins to inhibit cell proliferation.

L-cells stably transfected with KDR-Ig4-7-VEGF, VEGF-KDR-Ig4-7 and KDR-Ig4-7-G129R were generated. The presence of mRNA transcripts of the three fusion proteins was verified by RT-PCR from positive L-cells (Figure 33) and the presence of the three fusion proteins in the conditioned media were detected using VEGF ELISA kit and PRL IRMA kit at around 1-10 ng/ml (data not shown). The conditioned media containing fusion proteins were collected and their effects on HUVEC and T47-D cells were tested. We found that each conditioned media containing KDR-Ig4-7-VEGF, VEGF-KDR-Ig4-7 and KDR-Ig4-7-G129R individually, were able to inhibit cell proliferation (Figure 34). The fusion of VEGF to either C- or N-terminus of KDR-Ig4-7 resulted in two fusion proteins with similar potency in the level of inhibition. These results suggest that eukaryotic proteins produced in a mammalian system might have better activity than eukaryotic proteins produced in a bacterial system lacking a post-translational modification process. We would need to further evaluate the bacterially and eukaryotically produced proteins to determine which system would produce KDR proteins with enhanced activity.
6. CONCLUSION

Since the first report of angiogenesis as one of the hallmarks that supports the growth of tumors, disruption of tumor blood supply has become a hotly pursued strategy in developing new cancer therapy. In this study we have designed, produced and tested a potential KDR antagonist, KDR-Ig4-7.

Based on in vitro assay results, we demonstrated that KDR-Ig4-7 was able to bind specifically to KDR expressing HUVEC. Furthermore, KDR-Ig4-7 was shown to be able to accumulate in blood vessel rich tissue, especially in the breast tumors, its intended target in mice, suggesting its affinity to endothelial cells in the tumor vasculature. KDR-Ig4-7 was the most potent among all of the KDR variants tested in inhibiting proliferation of HUVEC and multiple cancer cells, which suggested that the Ig-like domains 4 and 7 play a greater role in KDR dimerization. The inhibitory effects of KDR-Ig4-7 were, in part due, to inhibition of ERK phosphorylation, which leads to apoptosis. The anti-angiogenic activity of KDR-Ig4-7 was further supported with the evidence from HUVEC tube formation and migration assays. Interestingly, the inhibitory effect of KDR-Ig4-7 was enhanced when VEGF was present, which suggested that the binding of VEGF to KDR induced a conformational change, which favors the interaction between KDR-Ig4-7 and KDR. To our disappointment, however, KDR-Ig4-7 treatment in 4T1 xenograft model was not effective in inhibiting tumor growth and metastasis. In an effort to improve the efficacy of KDR-Ig-4-7, we designed and tested a few novel fusion proteins, KDR-Ig4-7-VEGF, VEGF-KDR-Ig4-7 and KDR-Ig4-7-G129R. The preliminary results
indicated that these fusion proteins exhibited better anti-angiogenic activity in HUVEC and T47-D breast cancer cells. We believe that the KDR fusion proteins warrant further investigation.
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