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IDENTIFICATION OF A NOVEL PEPTIDE, SP5.2, THAT INHIBITS VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) MEDIATED ANGIOGENESIS BY BINDING TO ITS FMS-LIKE TYROSINE KINASE-1 (FLT-1) RECEPTOR: AN *IN VITRO* STUDY USING A PHAGE DISPLAY LIBRARY

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IDENTIFICATION PAR BANQUE PHAGIQUE D'UN PEPTIDE, SP5.2, SPECIFIQUE AU RECEPTEUR Flt-1 AYANT UN POTENTIEL ANTI-ANGIOGENIQUE

Par

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"O my Lord! advance me in knowledge."
Quran 20 :114

To my dear parents, to my dear husband
to my dear brothers and sisters,
to my family.

Résumé

Le cancer est une maladie génétique étant donné qu'elle peut être retracée jusqu'à des altérations au niveau de gènes spécifiques sans pour autant, dans la plupart des cas, être d'origine héréditaire. Les altérations génétiques conduisant à la plupart des cancers apparaissent dans l'ADN des cellules somatiques au cours de la vie de l'individu affecté. Suite à ces changements génétiques, les cellules cancéreuses prolifèrent de façon incontrôlée, pouvant produire des tumeurs malignes qui envahissent le tissue environnant. Les cellules des tumeurs malignes peuvent métastaser et se propager dans le corps en entrant dans la circulation lymphatique et vasculaire, et former des tumeurs secondaires. En se développant, la tumeur stimule la formation de nouveaux vaisseaux sanguins; ce processus est appelé angiogenèse. La cellule tumorale stimule l'angiogenèse en sécrétant des facteurs de croissance qui agissent au niveau des cellules endothéliales des vaisseaux sanguins environnants, les stimulant ainsi à proliférer et à développer de nouveaux vaisseaux. Parmi ces facteurs de croissance, le VEGF (*Vascular Endothelial Growth Factor*) est retrouvé dans plusieurs types de tumeurs. Son activité angiogénique a été démontrée *in vitro* et *in vivo*, ce qui en fait une cible de choix pour le développement d'une thérapie anti-tumorale.

Le VEGF et ses récepteurs sont la cible d'un intérêt important par leur rôle dans la formation des vaisseaux sanguins (angiogenèse et vasculogenèse) dans une variété de processus physiologiques vitaux incluant l'embryogenèse et la guérison des liaisons tissulaires, mais également dans des processus patho-physiologiques, tels que la croissance tumorale, la réthinopatie proliférative et certaines maladies inflammatoires chroniques (Folkman 1995, Ferrara 1996). Parmi les facteurs de croissance, le VEGF joue aussi le rôle exclusif de mitogène spécifique des cellules endothéliales via son action sur la prolifération et le mouvement des cellules endothéliales, le remodelage de la matrice extracellulaire, la formation des tubules capillaires et la perméabilité vasculaire (Wang et *al.*, 2000).

L'activité du VEGF est médiée par l'attachement à deux récepteurs de haute affinité pour le VEGF, le *Human Kinase Domain Receptor* (KDR) et le *Fms-like Tyrosine Kinase receptor* (Flt-1) (Shibuya et *al.*, 1990), qui sont exprimés sélectivement par les cellules endothéliales au cours de l'embryogenèse et lors des pathologies reliées au VEGF (Millauer et *al.*, 1993). Ces deux récepteurs sont des tyrosine kinases de type III (Vaisman

et *al.*, 1990, Kaipainen et *al.*, 1993) qui subissent une dimérisation induite par le ligand, déclenchant ainsi un signal de transduction. Les études chez les souris ont démontré que l'expression de KDR atteint son plus haut niveau au cours de l'angiogenèse et de la vasculogenèse embryonnaires (Millauer et *al.*, 1993). De plus, un faible niveau d'ARNm de Flt-1 est détecté pendant la croissance du fœtus, un niveau moyen au cours de l'organogenèse, et un haut niveau chez la souris nouveau-née (Peters et *al.*, 1993). Il a été démontré qu'un troisième récepteur tyrosine kinase semblable au fms, le Flt-4, et dont l'expression est limitée à la vasculature lymphatique, jouerait un rôle dans l'angiogenèse lymphatique (Dumont et *al.*, 1998). De plus, il se lierait au VEGF-C et au VEGF-D mais pas au VEGF (Achen et *al.*, 1998). Les expériences chez des souris déficientes en Flt-1 ou en KDR ont révélé que le KDR est essentiel dans le développement des cellules endothéliales, alors que Flt-1 serait nécessaire pour l'organisation de la vascularisation embryonnaire (Fong et *al.*, 1995; Shalaby et *al.*, 1995). Finalement, le système VEGF-Flt-1 jouerait un rôle important dans la stimulation de l'angiogenèse tumorale, faisant de Flt-1 une cible intéressante dans le développement d'agents anti-angiogéniques (Shibuya 1995).

Le récepteur humain Flt-1 est composé de 7 domaines immunoglobulaires extracellulaires contenant la région de liaison du ligand, une petite séquence trans-membranaire simple, et une région intracellulaire contenant le domaine tyrosine kinase. Les séquences en acides aminés de Flt-1 et KDR possèdent une homologie d'environ 45%. Par contre, Flt-1 possède une plus haute affinité pour VEGF (K_D=10-20pM) comparée à KDR (K_D=75-125pM). L'activation du récepteur Flt-1 par VEGF régule l'interaction des cellules endothéliales entre elles ou avec la membrane basale sur laquelle elles résident (Quinn 1993, Waltenberg 1994). L'ARNm du récepteur Flt-1 peut subir un épissage alternatif codant pour le récepteur Flt-1 complet ou pour une forme soluble, Flt-1s. Flt-1s retient sa haute affinité de liaison au VEGF et inhibe complètement, par un mécanisme de dominant négatif, la mitogenèse des cellules endothéliales stimulée par le VEGF (He et *al.*, 1999). De plus, il a été suggéré que Flt-1s peut former un complexe hétérodimérique avec KDR pouvant avoir un effet négatif sur la transduction de signal de KDR (Kendall *et al.*, 1996).

Étant donné que les récepteurs de VEGF sont associés à plusieurs pathologies, l'interférence de l'action du VEGF par des antagonistes représente une stratégie attrayante cliniquement. En effet, des anticorps neutralisants humanisés qui interagissent avec le

VEGF près du site de liaison Flt-1 et de KDR (Kim et *al.*, 1993; Muller et *al.*, 1997; Muller et *al.*, 1998), et des SELEX *(Systemic Evolution of Ligands by Exponential Enrichment)* dérivés molécules d'ARN (Jellinek et *al.*, 1994) ont démontré une capacité à bloquer la croissance tumorale dépendante de la vascularisation des tissus normaux adjacents (Plate et *al.*, 1994). De plus, il a été démontré qu'un anticorps monoclonal anti-KDR peut inhiber l'activité du VEGF et posséderait une activité anti-tumorale (Witte et *al.*, 1998). Des études plus récentes portant sur le récepteur Flt-1 soluble, sur des fragments de VEGF, et sur des petites molécules inhibitrices de l'activité tyrosine kinase des récepteurs de VEGF, comme le PTK787/ZK222584 (Drevs et *al.*, 2000) et le ZD4190 (Wedge et *al.*, 2000) ont démontré leur capacité à inhiber l'angiogenèse *in vivo* (Gehlbach et *al.*, 2003). Finalement, un oligonucléotide antisens anti-VEGF conçu pour inhiber l'expression de VEGF a bloqué la néovascularisation et la croissance de tumeurs implantées (Melinyk et *al.*, 1996; Benjamin et *al.*, 1997; Cheng et *al.*, 2002). Cependant, il n'y a aucun antagoniste sélectif du récepteur Flt-1 disponible à ce jour.

Dans la présente étude, le SP5.2 (NGYEIEWYSWVTHGMY), un nouveau peptide identifié en utilisant un criblage sur une librairie phagique, a été montré capable de se lier spécifiquement au Flt-1 tout en inhibant un large spectre d'évènements médiés par le VEGF. Aucune interaction croisée n'a été observée entre le SP5.2 et plusieurs récepteurs endothéliaux humains incluant le KDR et le récepteur à l'ICAM-1. Des analyses par cytofluorimétrie ont démontré que le SP5.2 marqué à la fluorescéine était capable de se lier spécifiquement aux cellules endothéliales cérébrales primaires humaines (HCEC) stimulées au VEGF, alors qu'aucune interaction n'a été observée sur des HCEC non-stimulées ou des cellules de neuroblastôme humain (ShyY). De plus, le SP5.2 empêche la prolifération des cellules endothéliales ombilicales humaines primaires (HUVEC) induite par le $VEGF_{165}$ recombinant humain à un IC_{50} de 5μM. Il a aussi été démontré que le SP5.2 peut être un antagoniste de la prolifération induite par le VEGF ou le PIGF (Placenta Growth Factor) des HCEC, sans avoir d'effet sur la prolifération induite par le bFGF (basic Fibroblast Growth Factor). En utilisant un test de formation de capillaires *in vitro*, il a été démontré que l'activité angiogénique induite par le VEGF sur des HCEC en milieu Matrigel™ a complètement été inhibée par la présence de 10 μM de SP5.2. Le peptide a aussi inhibé la formation de capillaires de HCEC exposées à un milieu préparé par des astrocytes

hypoxiques ou par des cellules de glioblastome humain qui sont reconnues pour sécréter une grande quantité de VEGF. D'autres études ont démontré que le SP5.2 inhibe complètement la perméabilité à travers une monocouche de HCEC. Finalement, le SP5.2 est aussi capable d'inhiber la chémotaxie induite par le VEGF sur des HCEC, mais pas la migration de celles-ci si elle est induite par d'autres chemoattractants tels que IL-8. Des peptides à séquence aléatoire n'ont eu aucun effet.

Pour explorer la possibilité d'utiliser le SP5.2 comme élément de ciblage, des conjugués recombinants et chimiques du SP5.2 avec des protéines rapportrices (Peroxidase ou β-galactosidase) ont été préparés. Ces protéines de fusion ont augmenté de façon significative le potentiel de liaison (200 fois pour le SP5.2-β-gal, et 400 fois pour le SP5.2-Peroxidase) au Flt-1 recombinant par rapport au peptide synthétique, ce qui suggère que des agents thérapeutiques ayant un activité de l'ordre du nanomolaire peuvent être développés et basés sur l'activité de SP5.2.

Ce mémoire de maîtrise est divisé en quatre chapitres. Le premier chapitre présente une revue de littérature concise portant sur le facteur VEGF, ses récepteurs, ainsi que l'application des librairies phagiques. Le deuxième chapitre présente le matériel et les méthodes utilisées pour la sélection et l'analyse du peptide sélectionné. Le troisième chapitre décrit les résultats obtenus. Finalement, le quatrième chapitre présente une discussion et une conclusion reflétant l'analyse des principaux résultats obtenus au cours de ce projet de recherche.

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ABTS	2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)
Anti-hIgGFc	anti-human IgG (Fc specific)
bFGF	basic Fibroblast Growth Factor
BSA	Bovise Serum Albumin
DMEM	Dulbecco's Modification of Eagle's Medium
EGM	Endothelial Growth Media
ELISA	Enzyme Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
Flt-1	Fms-like tyrosine kinase receptor
Flt-1-MP	Flt-1 coated Magnetic Particles
HCEC	Human Cerebral Endothelial Cells
HGF/SF	Hepatocyte Growth Factor/Scatter Factor
HPLC	High Performance Liquid Chromatography
HRP	Horse Radish Peroxidase
HUVEC	Human Umbilical Vein Endothelial Cells
ICAM-1	Intracellular Adhesion Molecule 1
IgG	Immunoglobulin G
IL-8	Interleukin 8
KDR	Kinase Domain Receptor
NRP	Neuropilin
PBS	Phosphate Buffered Saline
PDGF	Platelet Derived Growth Factor
PECAM-1	Platelet Endothelial Cell Adhesion Molecule 1
PIGF	Placenta Growth Factor
RF	Replicative Form
rhFlt-1	Recombinant human Flt-1 receptor
SDS	Sodium Dodecyl Sulfate
SELEX	Systemic Evolution of Ligands by Exponential Enrichment
sFlt-1	Soluble Flt-1 receptor
ShyY	Neuroblastoma cells

TNF-α	Tumor Necrosis Factor α
VEGF	Vascular Endothelial Growth Factor
VEGFR-I	Vascular Endothelial Growth Factor Receptor I (Flt-1)
VEGFR-II	Vascular Endothelial Growth Factor Receptor II (KDR)

Summary

Vascular endothelial growth factor (VEGF) is known to play a predominant role in tumor angiogenesis and metastasis formation that is mediated by its interactions with two tyrosine receptors, VEGFRI or Fms-like Tyrosine Kinase Receptor-1 (Flt-1) and VEGFRII or Kinase Domain Receptor (KDR). Inhibition of VEGF-dependant events in tumor tissues is known to enhance apoptosis and to suppress tumor growth. In the present study, a new peptide, SP5.2, that selectively binds with Flt-1 and inhibits a broad range of VEGF mediated events, was identified using a phage-display library screening. SP5.2 did not demonstrate cross-interactions with two other human endothelial receptors, KDR and ICAM-1 (Intracellular Adhesion Molecule). A cytofluorometry assay revealed that fluorescein labeled SP5.2 was capable of specific binding to VEGF-stimulated primary human cerebral endothelial cells (HCEC), while non-stimulated HCEC, as well as human neuroblastoma cells (ShyY) did not show any interaction with the peptide. The peptide also prevented proliferation of cultured primary human umbilical vein endothelial cells (HUVEC) induced by recombinant human $VEGF_{165}$ at an IC_{50} of 5μM. Furthermore, SP5.2 was also shown to antagonize VEGF- and PIGF (Placenta Growth Factor) -induced proliferation of HCEC but did not affect bFGF (basic Fibroblast Growth Factor) -mediated proliferation. The *in vitro* analysis of antiangiogenic activity of SP5.2, using a capillary-like tube formation assay, showed that VEGF-induced angiogenesis of HCEC grown on Matrigel™ was completely inhibited in the presence of 10 μM SP5.2. The peptide also inhibited capillary-like tube formation by HCEC exposed to media conditioned by hypoxic astrocytes or human glioblastoma cells, known to produce high levels of VEGF. Further studies demonstrated that SP5.2 completely inhibited VEGF-induced increase in permeability of HCEC monolayers. SP5.2 was also found to selectively inhibit VEGF-stimulated chemotaxis of HCEC, but not cell migration induced by other chemoattractants such as IL-8, while a control scrambled peptide did not affect either of the processes.

These *in vitro* results clearly demonstrate that SP5.2 is an effective antagonist of VEGF-Flt-1 binding, and suggests that this peptide may serve to be a potent inhibitor of tumor angiogenesis and metastasis. Furthermore, to explore a possibility of using SP5.2 as a targeting vector for functionalized delivery in combination with other therapeutic diagnostic particles, chemical and recombinant conjugates of SP5.2 with two reporter

proteins, peroxidase and β-galactosidase, were produced. The resulted products not only exhibited binding with recombinant Flt-1, but showed a significant increase in the binding potency (200-fold for SP5.2-β-galactosidase and 400-fold for SP5.2-peroxidase) compared to the original synthetic SP5.2, suggesting that highly potent, in nanomolar range, therapeutic or diagnostic agents may potentially be developed based on the structure of this novel peptide.

Introduction

Cancer is the second leading cause of death in USA. One in four fatalities is caused by cancer, costing 180 billion in direct medical costs (Reichert et Paquette, 2002). It is a genetic disease, because it can be traced to alterations within specific genes, but in most cases it is not inherited disease. The genetic alterations that lead to most cancers arise in the DNA of a somatic cell during the lifetime of the affected individual. Because of these genetic changes, cancer cells proliferate uncontrollably, producing malignant tumors that invade surrounding healthy tissue. Malignant tumors tend to metastasize, enter the lymphatic or vascular circulation and spread to distant sites in the body where they may establish lethal secondary tumors. As a tumor grows in size, it stimulates the formation of new blood vessels, a process termed angiogenesis. Cancer cells promote angiogenesis by secreting growth factors that act on the endothelial cells of surrounding blood vessels, stimulating them to proliferate and develop into new vessels. Among those growth factors Vascular Endothelial Growth Factor (VEGF) was found in several types of tumors. It has shown a tumoral angiogenic activity *in vitro* and *in vivo*, and thus is a privileged target for the control of angiogenesis in an anti-tumoral goal, a very promising anti-cancer strategy. The present work was an attempt in this direction undertaken to search for new antiangiogenic ligand for cancer therapy.

VEGF and its receptors are the focus of intense interest because of their role in blood vessel formation (angiogenesis and vasculogenesis) in a variety of a vital physiological and patho-physiological processes, including embryogenesis, wound healing, tumor growth, proliferative retinopathies and chronic inflammatory diseases such as rheumatoid arthritis (Folkman 1995; Ferrara 1996). VEGF plays an important role in the development of the fetal cardiovascular system (Folkman 1995; Ferrara 1996, Zachary 1998). Among the growth factors, it plays also unique role, being an endothelial cell-specific mitogen that promotes the proliferation and movement of endothelial cells, remodeling of the extracellular matrix, formation of capillary tubules and vascular leakage (Wang et *al.*, 2000).

VEGF activities are mediated through binding to two high affinity VEGF receptors, Kinase Domain Receptor (KDR) and Fms-like tyrosine kinase receptor (Flt-1) (Shibuya et *al.*, 1990), that are selectively expressed on endothelial cells during embryogenesis and

VEGF related pathologies (Millauer et *al.*, 1993). Both of these receptors are class III tyrosine kinases (Vaisman et *al.*, 1990; Kaipainen et *al.*, 1993) that undergo ligand-induced dimerization that triggers signal transduction. Studies in mice have shown that the expression of KDR reaches the highest levels during embryonic vasculogenesis and angiogenesis (Millauer et *al.*, 1993). In contrast, only low levels of mRNA for Flt-1 were found during fetal growth and moderate levels during organogenesis, but high levels in newborn mice (Peters et *al.*, 1993). A third fms-like tyrosine kinase receptor, Flt-4, that is largely confined to the lymphatic vasculature, has been shown to play a consistent role in lymph angiogenesis (Dumont et *al.*, 1998). Furthermore it binds to VEGF-C and –D but not to VEGF (Achen et *al.*, 1998). Experiments with knockout mice deficient in Flt-1 or KDR receptor revealed that KDR is essential for the development of endothelial cells, whereas Flt-1 is necessary for the organization of embryonic vasculature (Fong et *al.*, 1995; Shalaby et *al.*, 1995). Also VEGF-Flt-1 receptor system plays an important role in the simulation of tumor angiogenesis, which makes Flt-1 an interesting target for anti-angiogenic drugs (Shibuya 1995).

Human Flt-1 receptor is composed of 7 extracellular Ig-like domains containing the ligand-binding region, a single short membrane-spanning sequence, and an intracellular region containing tyrosine kinase domain. The amino acid sequences of Flt-1 and KDR are identical to each other by approximately 45%, however, Flt-1 has the higher affinity for VEGF (K_D =10-20pM) compared to 75-125pM for KDR. Activation of Flt-1 receptor by VEGF regulates interactions of endothelial cells with each other or with the basement membrane on which they reside (Quinn 1993, Waltenberger 1994). The Flt-1 receptor mRNA can be spliced to generate forms encoding either the full-length membrane-spanning receptor or a soluble form, denoted sFlt-1. Isolated sFlt-1 retains specific high affinity binding for VEGF and fully inhibits VEGF-stimulated endothelial cell mitogenesis by dominant negative mechanism (He et *al.*, 1999). Furthermore, it was suggested that sFlt-1 might form heterodimeric complexes with KDR which could potentially exert a negative effect on KDR signal transduction (Kendall et *al.*, 1996).

Because VEGF receptors are associated with several pathologies, interference of VEGF action by VEGF antagonists seems clinically attractive. Humanized neutralizing antibodies that interact with VEGF near the KDR and Flt-1 binding sites (Kim et *al.*, 1993;

Muller et *al.*, 1997; Muller et *al.*, 1998), and SELEX (Systemic Evolution of Ligands by Exponential Enrichment) derived RNA molecules (Jellinek et *al.*, 1994) has been shown to induce blockade of tumor growth that is dependent on vascularization of adjacent normal tissue (Plate et *al.*, 1994). Also anti KDR monoclonal antibodies have been shown to inhibit VEGF induced signaling and demonstrate a high anti-tumor activity (Witte et *al.*, 1998). Most recently soluble Flt receptor, fragments of VEGF have been shown to inhibit angiogenesis *in vivo* (Gehlbach et *al.*, 2003), as well as small molecule inhibitors of the tyrosine kinase activity of the VEGF receptors, such as PTK787/ZK222584 (Drevs et *al.*, 2000) and ZD4190 (Wedge et *al.*, 2000). Anti-VEGF antisense oligonucleotide was designed to inhibit VEGF expression, VEGF induced neovascularization of tumor implantation and growth (Melinyk et *al.*, 1996; Benjamin et *al.*, 1997; Cheng et *al.*, 2002). However, there is no selective antagonist of Flt-1 receptor available yet.

In the present study we have identified a new peptide motif that inhibits VEGF binding to Flt-1 by using a phage-displayed peptide library. A random 16-mer peptide library displayed on the surface of the filamentous phage M13 was screened *in vitro* against the extracellular domain of Flt-1. The screening resulted in the identification of a peptide (SP5.2) that competed with VEGF binding to Flt-1 and inhibited a broad range of VEGF induced events in cultured endothelial cells. In addition, the potential of using SP5.2 as a targeting agent, in combination with other therapeutic or diagnostic particles for targeted or functionalized delivery, was also explored by both synthetic and genetically constructed recombinant conjugates of the peptide in the two reporter proteins.

The thesis is divided into 4 chapters. The first chapter of this master's project presents a concise literature review concerning VEGF factor, its receptors and phage display library application. The second chapter presents materials and different techniques used for the selection and the analysis of the selected peptide. The third chapter includes obtained results. The fourth chapter presents the discussion and a conclusion, reflecting the analysis of the major results obtained during the course of this research project. Finally, the thesis ends with Annex 1 which includes the article "A VEGF high affinity receptor 1 specific peptide with antiangiogenic activity identified using a phage display peptide library" which is accepted by Journal of Biological Chemistry.

CHAPTER 1: Literature Review

1.1 Vascular network, neovascularization, vasculogenesis and angiogenesis.

The vascular network in the body is important for the development and maintenance of a variety of normal tissues in vertebrates. Vascular formation, or neovascularization, includes vasculogenesis and angiogenesis. Vasculogenesis is blood vessel formation from endothelial precursor cells in the mesenchyme during the early embryogenesis and angiogenesis is the branching out of new vessels from pre existing small blood vessels. Angiogenesis is required for development and differentiation of organs during embryogenesis as well as for wound healing and reproductive functions in the adult (Folkman 1991) such as the formation of corpus luteum (Shibuya 1995). Angiogenesis is also implicated in the pathogenesis of a variety of disorders such as proliferative retinopathies, age-related macular degeneration, rheumatoid arthritis, psoriasis, Kaposi's sarcoma, ocular neovascularization, atherosclerosis, hemangioma, tumor growth and metastasis.

Activator and inhibitor molecules regulate angiogenesis, normally the inhibitors predominates and block growth. Whenever a need for new blood vessels arise, angiogenesis activators increase in number and inhibitors decrease. This prompts the growth and division of vascular endothelial cells and, ultimately, the formation of new blood vessels (Iruela-Arispe and Dvorak, 1997).

The principal growth factors stimulating angiogenesis are VEGF, bFGF and hepatocyte growth factor/scatter factor (HGF/SF) (Schmidt et *al.,* 1999). After they are synthesized, they are secreted into the surrounding tissues. They bind to specific receptors expressed on the endothelial cells which causes the activation of series of proteins. Those relay proteins transmits a signal into the nucleus and prompts a group of genes to produce several endothelial growth factors (Figure 1.1). Other positive regulators of angiogenesis are angiopoietin-1, angiotropin, angiogenin, epidermal growth factor, granulocyte colony stimulating factor, interleukin-1 (IL-1), IL-6, IL-8, platelet derived growth factor (PDGF), and tumor necrosis factor-alfa (TNF-α) (Ferrara 2000). Angiogenesis is negatively regulated by one or more of the known endogeneous inhibitors including angiopoietin-2 , angiostatin , endostatin , interferon-α, platelet factor-4, prolactin, thrombospondin,

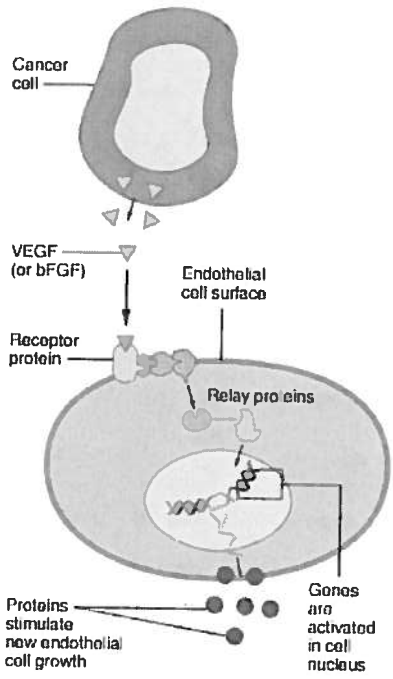


Figure 1.1 The angiogenesis signalling cascade.
VEGF and bFGF are first synthesized inside cells and then secreted into the surrounding tissue. When they encounter endothelial cells, they bind to specific proteins, called receptors, sitting on the outer surface of the cells. The binding of either VEGF or bFGF to its appropriate receptor activates a series of relay proteins that transmits a signal into the nucleus of the endothelial cells. The nuclear signal ultimately prompts a group of genes to make products needed for new endothelial cell growth.

tissue inhibitors of metalloproteinase (TIMP-1, TIMP-2 and TIMP-3), and troponin I.

In recent years, with the pace of discovery increasing, the search for an effective, non-toxic, anti-angiogenesis therapy appears to be within the grasp of oncology. Research in anti-angiogenesis therapy currently is a robust with numerous promising avenues. The major targets of pharmacologic therapies are VEGF and bFGF.

1.1.1 Role of Vascular Endothelial Growth Factor (VEGF) in health: angiogenesis

The VEGF gene has been isolated from a variety of animals: human, mouse, rat, bovine and guinea pig (Breier et *al.*, 1992). The human VEGF gene has been assigned to chromosome 6p21.3 (Vincenti 1996) that is composed of 8 polypeptide-coding exons separated by 7 introns, and its coding region spans approximately 14 kb (Tischer et *al.* 1991). The analysis of the cDNA sequence of several human VEGF clones showed that VEGF might exist in one of four different isoforms having 121, 165, 189 or 206 amino acids (Thomas 1996), which is determined by alternative exon splicing of a single VEGF gene. $VEGF_{165}$ lacks the region encoded by exon 6, and compared to $VEGF_{165}$, $VEGF_{121}$ lacks 44 amino acids which is the region encoded by both exon 6 and 7; $VEGF_{189}$ has an insertion of a highly basic stretch of 24 amino acids while $VEGF_{206}$ has an additional stretch of 17 amino acids. The major subtype of VEGF produced by both normal and tumor tissues is $VEGF_{165}$, although $VEGF_{121}$ and $VEGF_{189}$ transcripts are detected in most cells and tissues expressing VEGF gene (Tisher et *al.*, 1991; Houck et *al.*, 1991); $VEGF_{206}$ is a rare form and has been detected only in human fetal liver cDNA library (Houck et *al.*, 1991). Murine VEGF gene is similar to human gene in that it comprises 8 exons interrupted by 7 introns, also the coding region spans approximately 14 kb (Shima et *al.*, 1996). However, the analysis of murine gene exons suggested the generation of 3 isoforms of 120, 164 and 188 amino acids which are one amino acid shorter than the human gene. A murine isoform comparable to human rare $VEGF_{206}$ was not detected (Levy et *al.*, 1995).

The originally characterized form of VEGF is a homodimeric glycoprotein of 34-46 kDa. $VEGF_{165}$ is the major isoform and it is referred as VEGF; it is basic and binds to heparin and to heparan proteoglycans. It can be found as a freely soluble protein or bound to the cell surface and extracellular matrix. $VEGF_{121}$ is a weakly acidic polypeptide but fails to bind to heparin (Houk et *al.*, 1992) and is considered as a freely soluble protein.

$VEGF_{189}$ and $VEGF_{206}$ are more basic and bind to heparin with higher affinity to $VEGF_{165}$ and they are found completely sequestered in the extracellular matrix (Park et *al.*, 1993).

1.1.2 Physiological role of VEGF

VEGF mRNA is found to play important role in a variety of physiological processes such as embryonic development, normal growth and differentiation, wound healing and is suggested to be a mediator of the cyclical growth of blood vessels that occurs in the female reproductive tract (Phillips et *al.*, 1990; Ravindranath et *al.*, 1992; Cullinan-Bove et *al.*, 1993; Shweiki et *al.*, 1993). During embryonic development, VEGF expression is detected during the first few days after implantation in the giant cells of the trophoblast, which shows the participation of this factor in the induction of vascular growth in the deciduas, placenta and vascular membranes. Two studies from 1996 have generated direct evidence for the important role of VEGF in embryonic vasculogenesis, by showing that a targeted inactivation of a single VEGF allele in mice resulted defective angiogenesis and embryonic lethality between day 11 and 12 (Carmeliet et *al.*, 1996; Ferrara et *al.*, 1996).

VEGF mRNA is expressed in different organs at later developmental stages in mouse or rat embryos, including heart, vertebral column, kidney, and along the surface of the spinal cord and the brain (Jakeman et *al.*, 1993; Breier et *al.*, 1992). In the human fetus of 16-22 weeks, VEGF mRNA is expressed in all tissues but most abundant in lung, kidney, and spleen. However, VEGF protein is mostly expressed in epithelial cells and monocytes (Shifren et *al.*, 1994) and around microvessels in areas where endothelial cells are normally quiescent, such as kidney glomerulus, pituitary, heart, lung, and brain (Ferrara et *al.*, 1992; Monacci et *al.*, 1993; Brown et *al.*, 1992). Those studies show the importance of VEGF for the maintenance of the differentiated state of blood vessels (Ferrara et *al.*, 1992). The role of VEGF in endochondral bone formation, which is a fundamental mechanism for longitudinal bone growth during vertebrate development, was examined (Gerber et *al.*, 1999; Carlevaro et *al.*, 2000). In those recent studies, it was suggested that VEGF mRNA is expressed by hypertrophic chondrocytes in the epiphyseal growth plate; and blocking the VEGF activity resulted in almost complete suppression of blood vessel invasion, concomitant with impaired trabecular bone formation.

Follicular growth and development and endocrine function of the ovarian corpus luteum (CL) are all dependant on the proliferation of new capillary vessels (Bassett 1943).

Some studies have suggested the involvement of angiogenic inhibitors and inducers during the course of the ovarian cycle (Goede et *al.*, 1998; Maisonpierre et *al.*, 1997). Earlier studies have shown that VEGF mRNA is temporally and spatially related to blood vessels proliferation in rat, mouse and primate ovary and rat uterus (Phillips et *al.*, 1990; Ravindranath et *al.*, 1992; Shweiki et *al.*, 1993; Cullinan-bove et *al.*, 1993). Other studies have demonstrated the essential role of VEGF to mediate angiogenesis for luteal function in different species such as rats (Ferrara et *al.*, 1998), Cynomologous monkeys (Ryan et *al.*, 1998) and marmoset (Fraser et *al.*, 2000).

It has been suggested that VEGF is involved in the wound healing process, which is an important pathophysiological process. Keratinocytes in a healing wound express VEGF mRNA (Frank et *al.*, 1995; Detmar et *al.*, 1994; Detmar et *al.*, 1995). Frank et al has shown that an altered VEGF regulation leads to defective angiogenesis and impaired wound healing in genetically diabetic db/db mice (Frank et *al.*, 1995).

1.1.3 VEGF Related molecules

Over the last decade, a few close structural homologues to VEGF genes have been identified from mammalian sources such as PIGF, VEGF-B and VEGF-C also two sequences in the genome of the parapoxvirus *orf* virus. VEGF shares ~ 20% amino acid sequence identity with PDGF. PDGF and VEGF members contain 8 conserved Cysteine residues.

The first VEGF related molecule identified is PIGF which shares 53% amino acid sequence identity with the PDGF-like region of human VEGF. It exists in three isoforms PIGF-1 ($PIGF_{131}$), PIGF-2 ($PIGF_{152}$), PIGF-3 ($PIGF_{183}$) (Maglione et *al.*, 1993). PIGF expression is restricted to placenta, and it is similar to VEGF in that they are dimeric glycoproteins. The only signaling receptor identified to PIGF is Flt-1 and it binds with high affinity (K_D ~250 pM) (Park et *al.*, 1994). It has been found recently that mice lacking PIGF exhibit deficiencies in certain models of adult vascular remodeling (Carmeliet 2000). VEGF-B consists of 188 amino acids. It is expressed as a membrane-bound protein that can be released in a soluble form after the addition of heparin. It exists in two isoforms VEGF-B_{167} and VEGF-B_{186} which can both bind and activate Flt-1 receptor (Olofsson et *al.*, 1996). VEGF and both VEGF-B isoforms are able to form heterodimers when coexpressed (Olofsson et *al.*, 1996, Olofsson et *al.*, 1996). Mice lacking VEGF-B are overtly normal

and fertile but their heart is reduced in size suggesting that VEGF-B may have an important role in coronary vascularization and growth (Bellomo 2000).

VEGF-C or VRP is a secreted protein with 399 amino acid residues and has 32% homology to VEGF. It binds and activates the receptor Flt-4 and KDR. They are synthesized as precursor proteins, but require C- and N-termini processing to release the VEGF homology domain (Joukov 1996). The incompletely processed VEGF-C may cause lower or no affinity to the previously mentioned receptor. VEGF-C seems to play an important role in lymphatic development basing on its ability to bind to Flt-4. Transgenic overexpression of VEGF-C may cause lymphatic hyperplasia (Olofsson et *al.*, 1999).

1.1.4 Biological activities of VEGF

VEGF is the most selective mitogen known (ED_{50} 2-10pM) for micro- and macrovascular endothelial cells derived from arteries, veins and lymphatics (Ferrara et *al.*, 1989; Conn et *al.*, 1990). Other studies have reported mitogenic effects of VEGF on other non-endothelial cell types, for example retinal pigment epithelial cells (Guerrin et *al.*, 1995), pancreatic duct cells (Oberg-Welsh et *al.*, 1997) and schwann cells (Sondell et *al.*, 1999). VEGF is a key survival factor for endothelial cells and promotes angiogenesis in a variety of *in vitro* and *in vivo* models such as chick chorioallantoic membrane (Leung et *al.*, 1989), the rabbit cornea (Philipps et *al.*, 1995), the primate iris (tolentino et *al.*, 1996) and the rabbit bone (Connolly et *al.*, 1989).

VEGF also elicits non-mitogenic responses by vascular endothelial cells such as chemotaxis and the expression of plasminogen activators and collagenases, which permits the penetration of growing capillaries into tissues and sprouting of endothelial cells. VEGF induces both the expression of metalloproteinase interstitial collagenase in human umbilical vein endothelial cells (HUVEC) (Unemori et *al.*, 1992), and the expression of serine proteases urokinase type and tissue type plasminogen activators (PA) in cultured bovine microvascular endothelial cells (Pepper et *al.*, 1991). VEGF was reported to promote monocyte chemotaxis (Clauss et *al.*, 1990) and colony formation by mature subsets of granulocyte-macrophage progenitor cells (Broxmeyer et *al.*, 1995). VEGF was found to have a negative effect on the maturation of host professional antigen presenting cells, such as dendritic cells, without affecting the function of mature cells, which enhances tumor growth by avoiding the activation of the immune response (Gabrilovich et *al.*, 1996).

VEGF is also known as a vascular permeability factor based on its ability to induce vascular leakage that has been found in guinea pig skin (Senger et *al.*, 1983; Dvorak et *al.*, 1995). Dvorak proposed that an increase in microvascular permeability to proteins is a crucial step in angiogenesis of tumors and wound healing (Dvorak 1986). Therefore, mitogenic growth factors such as VEGF would induce plasma protein leakage and then enhancing protein extravasation. Recent study has shown that Src or Yes specifically mediates the vascular permeability enhancing activity of VEGF, because mice lacking *src* and *yes* display a normal angiogenic response to VEGF, develop normally but do not show any overt defect in the vasculature (Eliceiri et *al.*, 1999). This has led to the conclusion that enhanced vascular permeability is not a requirement for VEGF-dependant angiogenesis.

1.1.5 Regulation of VEGF gene expression

1.1.5.1 Oxygen tension

VEGF is a potent and critical vascular regulator, and its dosage must be exquisitely regulated in spatial, temporal and quantitative manner to avoid vascular disaster. Different chemicals and growth factors, with the participation of several mechanisms, regulate the expression of VEGF gene. Among those is hypoxia or oxygen tension. Hypoxia is known to induce angiogenesis by providing an alternative way by which tissues can increase oxygenation. Therefore, decreased level of O_2 is one of the most transcriptional reversible inducers for VEGF (Shweiki et *al.*, 1992) and its receptors (Tuder et *al.*, 1995) in normal and transformed cells. Induction of VEGF is seen at reduced pO_2 in ischemic pig and rat myocardium caused by occlusion of the left anterior descending coronary artery (Banai et *al.*, 1994). HIF-1 has been purified and cloned as a mediator of transcriptional responses to hypoxia and is a basic, heterodimeric, helix-loop-helix protein (Wang et *al.*, 1995; Wang et *al.*, 1995). More direct evidence was presented by Forsythe et *al.* (Forsythe et *al.*, 1996) that HIF-1 is implicated in the activation of the VEGF gene transcription during hypoxia. However, the mechanism by which hypoxia increases expression of VEGF is partially understood.

1.1.5.2 Hormones and cytokines

A number of serum-derived and paracrine growth factors and cytokines promote the up-regulation of VEGF mRNA expression in several cell types. Keratinocyte growth factor

(fibroblast growth factor-7), epidermal growth factor, tumor necrosis factor-α (Frank et *al.*, 1995), transforming growth factor β1 (Brogi et *al.*, 1994; Frank et *al.*, 1995), interleukin 1α (Ben-Av et *al.*, 1995), 1β (Li et *al.*, 1995) and 6 (Cohen et *al.*, 1996) were all shown to induce expression of VEGF by 3- to 20-fold in a variety of cultured cells. In addition to protein growth factors, several small mediators have been shown to modulate VEGF expression such as phorbol esters and prostaglandin E2. Phorbol esters could increase VEGF protein levels to more than 5 folds in human keratinocytes. Prostaglandin E2 increases expression of VEGF in cultured synovial fibroblasts, suggesting the participation of this inductive mechanism in inflammatory angiogenesis (Ben-Av et *al.*, 1995).

1.1.6 VEGF receptors

VEGF binding to different receptors appears on proliferating and quiescent endothelial cells (Jakeman et *al.*, 1993; Jakeman et *al.*, 1992). Various members of VEGF family may have overlapping abilities to bind to cell-surface receptors (Figure 1.2). The most important receptors that seem to be involved in initiating a signal transduction cascade in response to VEGF interaction are VEGFR-1 (Flt-1), VEGFR-2 (KDR) and VEGFR-3 (Flt-3 or Flt-4). They are closely related receptor tyrosine kinases and they form the *flt* family. They are characterized by seven extracellular immunoglobulin-like domains followed by a membrane spanning region and a conserved intracellular tyrosine kinase domain interrupted by a kinase insert sequence (Shibuya et *al.*, 1990; Matthews et *al.*, 1991). Other receptors, such as neuropillins, are involved in modulating the binding to the main receptors (Soker et *al.*, 1998).

1.1.6.1 VEGFR-1

VEGFR-1 is a 180-kDa glycoprotein, which is also known as fms-like tyrosine kinase receptor (Flt-1). It is the highest affinity VEGF receptor (Kd 10-30 pM) (de Vries et *al.*, 1992; Quinn et *al.*, 1993) and is the first to be identified among the flt family. It is found in the adult mice endothelium, in embryonic tissues and in the neovasculature of healing skin wounds (Peters et *al.*, 1993), which shows a continued role in endothelial maintenance due to its presence in the quiescent and proliferating endothelial cells. It was shown that VEGFR-$1^{-/-}$ mice die at embryonic day 8.5 (Fong et *al.*, 1995). Few years after it was found that in these mice, endothelial cells form normally at embryonic and extra-

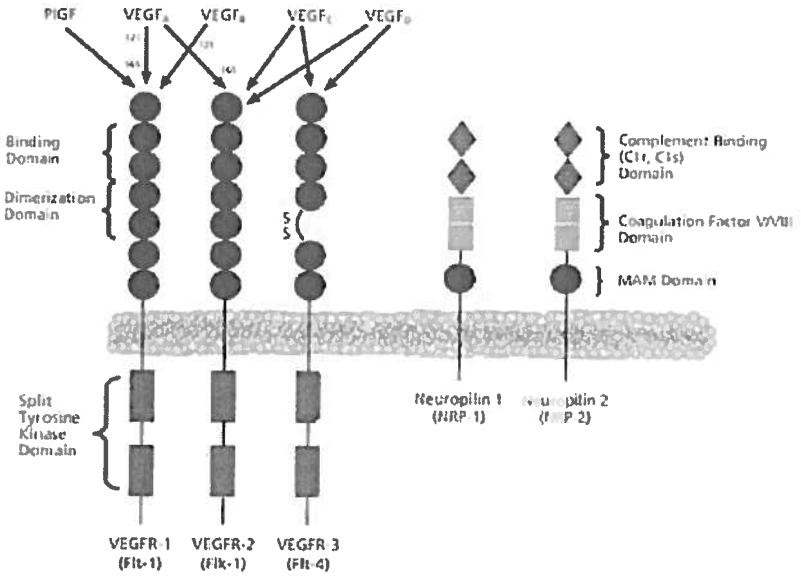


Figure 1.2 VEGF receptors and ligands.
The receptors for vascular endothelial growth factor (VEGF) and related ligands include VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (Flt-4), neuropillin-1 (NRP-1) and neuropillin-2. The interaction of VEGFR with either Neuropillin-1 or heparan sulfate proteoglycan may facilitate the presentation and binding of VEGF to its receptor. These receptors have multiple immunoglobulin G-like extracellular domains and intracellular tyrosine kinase activity. There are several splice variants of VEGF, including VEGF-121, 165, 189, and 206, with VEGF 165 being the predominant form. Other members of the VEGF family have been cloned including VEGF-B, -C, and –D and PIGF.

embryonic sites but fail to assemble correctly into organized blood vessels (Fong et *al.*, 1999). Those studies suggested that VEGFR-1 is essential during vasculogenesis. VEGFR-1 is involved in upregulated endothelial expression of tissue factor, urokinase-type plasminogen activator and plasminogen activator inhibitor 1 (Clauss et *al.*, 1996; Landgren et *al.*, 1998; Olofsson et *al.*, 1998). VEGFR-1 plays different roles in other cell types such as tissue factor induction and chemotaxis in monocytes and enhancing matrix metalloproteinase expression by vascular smooth muscle cells (Barleon et *al.*, 1996; Clauss et *al.*, 1996; Wang and Keiser, 1998). Tyrosine phosphorylation of VEGFR-1 is hardly detected after VEGF stimulation and no direct proliferative, migratory or cytoskeletal effects mediated by this receptor is shown clearly (Park et *al.*, 1994, Waltenberger et *al.*, 1994; Seetharamgotoh et *al.*, 1995). Other related growth factors can bind to VEGFR-1 such as PIGF and VEGF-B. VEGFR-1 can exist in a soluble truncated form (sFlt-1) that includes only the first six Ig-like domains. It is found in high levels in the placenta, where it might control VEGF activity at particular stages of pregnancy (Clark et *al.*, 1998; He et *al.*, 1999). It can bind VEGF as strong as the full length VEGFR-1 but inhibits VEGF activity by sequestering it from signaling receptors and by forming non-signalling heterodimers with VEGFR-2.

1.1.6.2 VEGFR-2

VEGFR-2, also known as kinase insert domain containing receptor (KDR), is a 200-230-kDa high affinity receptor for VEGF with a K_D of 75-760pM (Terman et *al.*, 1992; Quinn et *al.*, 1993; Waltenberger et *al.*, 1994). It seems to mediate the major growth and permeability actions of VEGF. It shares 85% identity with its murine homolog: mouse fetal liver kinase 1 (Flk-1) (Matthews et *al.*, 1991; Quinn et *al.*, 1993) and 45% identity to VEGFR-1 (Thomas 1996). This receptor was identified in humans after screening endothelial cDNA for tyrosine kinase receptors (Terman et *al.*, 1991). It was shown that VEGFR-$2^{-/-}$ mice die by embryonic day 9.5 after they exhibit defects in the development of hematopoietic precursors and fail to develop a vasculature and have very few endothelial cells (Shalaby et *al.*, 1995). In quiescent adult vasculature, VEGFR-2 mRNA appears to be downregulated (Millauer 1993; Quinn 1993, Eichmann 1997) but it is normally expressed in hematopoietic precursors, endothelial cells, nascent haematopoietic stem cells and the umbilical cord stroma. Upon binding to its ligand VEGF, VEGFR-2 can be efficiently

tyrosine phosphorylated and in endothelial cells, it leads to chemotaxis, mitogenesis and cell morphology (Quinn 1993; Waltenberger 1994). Other related growth factors can bind to VEGFR-2 with high affinity such as VEGF-C and VEGF-D. A truncated form of VEGFR-2 lacking the C-terminal, half of the kinase domain is expressed at lower levels than the full-length in normal rat retina (Wen et *al.*, 1998); but it seems to be activated by VEGF as efficiently.

1.1.6.3 VEGFR-3

VEGFR-3 may be important during blood vessel development but is most unique and critical based on its expression on lymphatic vessels of adult tissues. It is thought to control lymphangiogenesis. It does not bind VEGF but VEGF-C and VEGF-D (Pajusola et *al.*, 1992; Finnerty et *al.*, 1993; Kaipainen et *al.*, 1995; Taipale et *al.*, 1999).

1.1.6.4 Receptor expression

Several growth factors can affect VEGF receptors expression. VEGF stimulation of VEGFR-2 upregulates expression of *VEGFR-2* gene and increase cellular VEGFR-2 levels (Shen et *al.*, 1998). bFGF is known to synergize with VEGF and upregulating VEGFR-2 in endothelial cells, thus inducing angiogenesis (Pepper et *al.*, 1998). Platelet endothelial cell adhesion molecule 1 (PECAM-1) can influence the level of expression of both VEGFR-1 and VEGFR-2 (Sheibani et *al.*, 1998).

Similarly to VEFG, hypoxia is also a potent stimulator of VEGFR-1 and VEGFR-2 expression *in vivo* (Tuder et *al.*, 1995; Li et *al.*, 1996). *VEGF and VEGFR-1* genes have the HIF-1 consensus in its promoter region (Gerber et *al.*, 1997) but not *VEGFR-2* gene, which may cause a slight downregulation (Thieme et *al.*, 1995).

1.1.6.5 Signal transduction

VEGF has separate but overlapping binding sites for VEGFR-1 and VEGFR-2 at each pole at the dimer interface. The interaction of receptor monomers at these sites of VEGF induces receptor dimerisation that triggers signal transduction through transphosphorylation (Wiesman et *al.*, 1997; Fuh et *al.*, 1998). VEGFR-1 and VEGFR-2 can form homodimers but can also heterodimerise: as previously mentioned, sFlt-1 and VEGFR-2 that can form non-signaling complexes. VEGFR-2 seems to mediate almost all observed endothelial responses to VEGF. However, VEGFR-1 promotes monocyte

migration (Barleon et *al.*, 1996), but endothelial cell migration in response to VEGFR-1 has not been shown clearly. Mice embryos expressing VEGFR-1 lacking the tyrosine kinase domain, affects VEGF-mediated macrophage migration but not embryo development and angiogenesis (Hiratsuka et *al.*, 1998).

1.1.6.6 Neuropilin 1 and 2

Neuropilin 1 (NRP1) is a receptor that recognizes the exon-7-encoded domain of VEGF but do not correspond to the existing VEGFRs. It is a 120-130 kDa glycoprotein and is a cell-surface receptor for $VEGF_{165}$ (Soker et *al.*, 1996) and class 3 semaphorins that is expressed by neurons and endothelial cells (He et *al.*, 1997; Kolodkin et *al.*, 1997). NRP-1 is involved in the control of axon growth and guidance on embryonic neurons (Kawakami et *al.*, 1996; Takagi et *al.*, 1995). NRP1 overexpression causes death at day 17.5 with an excessively high density dilated blood vessels prone to hemorrhaging (Kitsukawa et *al.*, 1995). *NRP1*$^{-/-}$ mice die at embryonic day 10.5-12.5 after cardiovascular anomalies (Kitsukawa et *al.*, 1997). Studies on mice suggested that NRP1 is required for normal developmental angiogenesis, vasculogenesis and the development of the nervous system. Other VEGF-related factors can interact with the NRP1 receptor such as PIGF-2 and VEGF-B. They bind through regions homologous to the exon-7-encoded domain of VEGF. The biological function of binding of NRP1 to VEGF-B and PIGF-2 is still unclear. NRP1 can form homomultimers or heteromultimers with NRP2, but the significant role of those complexes is still not clear (Giger et *al.*, 1998). NRP2 represents a separate cell-surface $VEGF_{165}$ receptor. It was identified during the expression cloning of NRP1 from breast carcinoma cells (Soker 1998).

1.1.7 Role of VEGF in disease: pathological angiogenesis

VEGF is found to play a major role in angiogenic processes critical for the pathogenesis of cancer, diabetic retinopathy and other vascularizing diseases (Folkman 1995; Ferrara et *al.*, 1992).

It was demonstrated in several studies that VEGF mRNA is clearly up-regulated in the majority of human tumors. These include: thyroid (Viglietto et *al.*, 1995), lung (Mattern et *al.*, 1996), breast (Brown et *al.*, 1995; Yoshiji et *al.*, 1996), intracranial (Phillips et *al.*, 1993; Wizigmann-voss et *al.*, 1994), gastrointestinal tract (Brown et *al.*, 1993), urinary tract (Brown et *al.*, 1993), female reproductive tract (Olson et *al.*, 1994) and germ cells

(Viglietto et *al.*, 1996). And in all specimens studied, VEGF mRNA was expressed in tumor cells and not in endothelial cells unlike mRNA of KDR and Flt-1, they are up-regulated in the endothelial cells associated with the tumor (Brown et *al.*, 1993; Plate et *al.*, 1992).

Intraocular neovascularization is considered the major cause of vitreous hemorrhage, retinal detachment, nonvascular glaucoma and eventual blindness (Garner 1994). The new blood vessels are associated with diabetes mellitus, occlusion of central retinal vein or prematurity with subsequent exposure to oxygen (Patz 1980). Also, it can lead to age-related macular degeneration (AMD), which is the overall cause of blindness (Garner A: vascular diseases, edn 2. New york 1994). It was proposed by Michaelson that all these pathogenesis are associated to the release by the ischemic retina into the vitreous of a diffusible angiogenic factors responsible for retinal and iris neovascularization (Michaelson 1948).

Other studies have demonstrated that VEGF is associated to other pathological conditions. VEGF was highly detected in the synovial fluid of rheumatoid arthritis patients and not in patients with other type of arthritis (Koch et *al.*, 1994; Fava et *al.*, 1994). VEGF was also shown to be expressed increasingly in the psoriatic skin (Detamar et *al.*, 1994) and VEGF mRNA was up-regulated in the epidermis over blisters and in areas adjacent to dermal inflammatory infiltrates (Brown et *al.*, 1995). VEGF was shown to be implicated in the pathogenesis of Graves' disease (Sato et *al.*, 1995). Other study has shown elevation of VEGF level in the peritoneal fluid of patients with endemetriosis (Mclaren et *al.*, 1996; Shifren et *al.*, 1996), which is a condition characterized by ectopic endometrium implants in the peritoneal cavity (Buttram et *al.*, 1979).

1.2 Phage Display : a tool to screen for peptides targeted for specific sites of ligands

One very effective way for producing large numbers (up to 10^{10}) of diverse peptides and proteins with different characteristics is "Phage Display", first introduced in 1985 by G. Smith (Smith 1985). This technique is also used effectively for the study of protein-ligand interactions (Cesarini 1992), for recognition of receptor and antibody –binding sites (Griffiths 1993; Winter et *al.*, 1994), manipulation, enhancement and improvement of the affinity of proteins for their binding partners (Burton 1995; Neri et *al.*, 1995). It involves the fusion , from DNA sequence, of proteins or peptides to M13 bacteriophage coat proteins that permits them to be expressed or "displayed" on the surface of the filamentous phage (Smith 1985). In this way a physical linkage could also be established between phenotype and genotype: antigenic peptides were displayed on the surface of phage particles that also contained the coding DNA. This permits the selection and amplification of specific clones of phage representing desired binding specific sequences from pools of billions of phage clones from non-binding variants. The other section will describe this filamentous bacteriophage.

1.2.1 Filamentous bacteriophages

Bacteriophages, or simply phages, are viruses infecting a variety of Gram-negative bacteria using bacterial pili as receptors. They have a single-stranded, covalently closed DNA genome, which is encased in a long cylinder approximately 7 nm in width and 900 to 2000 nm in length. The best characterized of these phages are M13, fl, and fd. The genomes of these three phages have been completely sequenced and are 98% homologous (Van Wezenbeek et *al.*, 1980; Beck and Zink 1981; Hill and Peterson 1982) consisting of 10-11 genes (Figure 1.3A). A viable phage expresses about 2700 copies of gene 8 protein (pVIII), the major capsid protein, and 3 to 5 copies of gene 3 (pIII) encoded adsorption protein, one of the minor coat proteins on the tip of the filamentous phage. Filamentous phages do not produce a lytic infection in *E.coli*, but induce a state in which the infected bacteria produce and secrete phage particles without undergoing lysis. The infection is initiated by the specific interaction of the phage pIII with the tip of the f pilus of a male *E.coli*. The circular phage ssDNA enters the bacterial cytoplasm where it is converted by the host DNA replication machinery into the replicative form (RF), which is like a double-

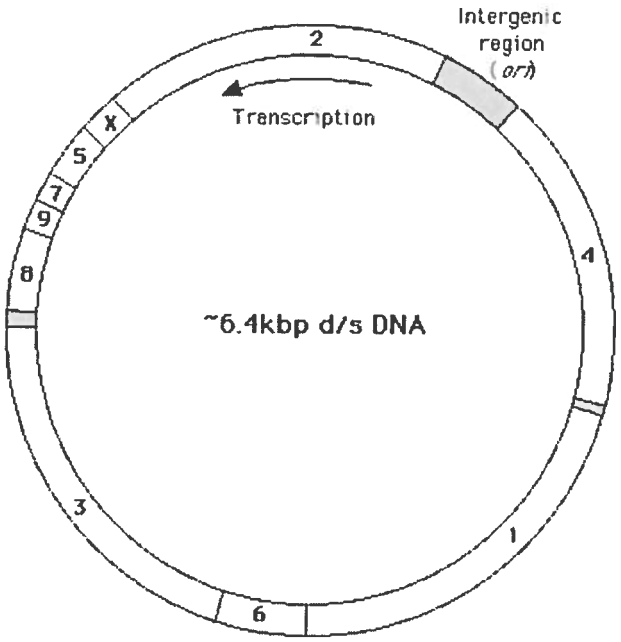


Figure 1.3A M13 genome.
The M13 genome is used as a cloning vector. The viral genes are shown as boxes on the map. The single-stranded DNA contains 10 genes. It has 6407 nucleotides, which are numbered from the unique HindII site located in gene 2. The phage genome encodes 10 proteins of which 5 are virion structural proteins, 3 are required for phage DNA synthesis, and two serve assembly functions. In addition there is an intergenic region which contains signals for the initiation of + and – strand synthesis, the initiation of capsid formation and the termination of RNA synthesis.

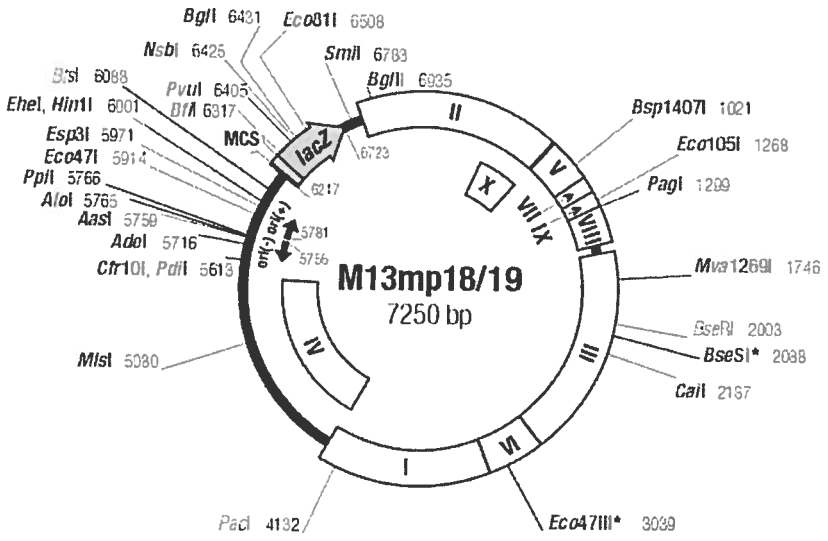


Figure 1.3B Map of M13mp18/19 genome.
M13mp vectors are derived from M13, contain the lacZ gene and differ with each other by the cloning sites embedded within it. Unique restriction sites are shown on the circular map. The 10 viral genes are shown as boxes on the map; they are transcribed in a clockwise fashion. The origin of replication can be in either orientation to direct the replication and packaging of either the plus or minus strand.

stranded plasmid. The RF undergoes rolling circle replication to make ssDNA and serves as a template for expression of the phage protein pIII and pVIII. The phage components are assembled by packaging of ssDNA into protein coats and extruded through the bacterial membrane into the medium. There are two forms of the phage DNA with the M13 phage, ssDNA templates that are isolated from the media for sequencing, and dsDNA RF that is extracted from the infected bacterial host and used for cloning of a specific fragment.

A more popular vector for phage display, called phagemids, are hybrids of phage and plasmid vectors and are designed to contain the origins of replication of M13 and *E.coli*, a copy of gene III, a multiple cloning sites and an antibiotic-resistance gene. A common phagemid used is M13mp18/19 (Mead 1988) (Figure 1.3B).

1.2.2 Phage display of synthetic oligonucleotides and biopanning

Synthetic oligonucleotides are used to prepare a bank of phages expressing a variety of randomly selected peptides of a constant length with unspecified codons and randomized through site-directed mutagenesis using degenerate oligodeoxynucleotides. They are cloned as fusions to phage coat genes III or VIII of M13 phage where they are expressed as peptide-capsid fusion proteins. Consequently, each phage displays a different peptide as a fusion protein on its surface. A phage displayed random peptide library is constructed by first synthesizing oligonucleotides that encode the random peptides. Because the synthetic chemistry for each base is essentially identical, one can create oligonucleotides encoding completely random or biased amino acid sequences by mixing different combinations of nucleotide triphosphates at different molar ratios. For example, a common strategy to generate random amino acids is to synthesize oligonucleotides with $(NNK)_n$, where N represents an equal mixture of all four nucleotides, and K is a 1:1 mixture of Guanine (G) and Thymine (T). Incorporation of these oligonucleotides in frame with gIII or gVIII will produce a stretch of n residues of random amino acids as part of the coat proteins. Sequences coding for peptides ranging in size from 6 to 38 amino acids have been successfully fused to pIII (Scott and Smith 1990; Kay et *al.*, 1993), however, fusion to pVIII has been usually restricted to relatively short peptides of 6 to 8 amino acids (Kishchenko et *al.*, 1994).

The display of random peptides on filamentous bacteriophage as fusion to either gene III or gene VIII coat proteins (Greenwood et *al.*, 1991; Sternberg et *al.*, 1995) allows

and is exploited for the identification of peptides that specifically bind to a variety of targets including antibodies (Scott et *al.*, 1990), streptavidin (Devlin et *al.*, 1990), ribonuclease S (Smith et *al.*, 1993), concavalin A (Scott et *al.*, 1992) and receptors (Binetruy-Tournaire et *al.*, 2000; Hetian et *al.*, 2002) using a form of affinity selection known as "biopanning" (Parmely and Smith 1988).

Biopanning is a technique to screen and enrich antibody libraries for antigen-specific clones. The library is incubated first with a target molecule followed by the capture of the target molecule with a bound phage. Unbound phages are eliminated by washing, and then phages that bind the antigen are eluted. Phages recovered in this manner are then amplified and again selected for binding to the target molecule, thus enriching for those phages that bind specifically to the antigen. Because binding of non-specific phage limits the enrichment that can be achieved per cycle , several , usually three to four rounds of selection are necessary to recover phages with specific binding. The primary structure of the specifically binding peptides is deduced by nucleotide sequencing of individual clones.

1.2.3 *In vitro* enrichment of phages expressing specific functions by affinity selection

In vitro selection can be achieved by using free or immobilized antigens. Immobilized antigens can be performed against antigen adsorbed onto plastic surfaces or enzyme-linked immunosorbant assay (ELISA) plates (Marks et *al.*, 1991; Kang et *al.*, 1991). It is recommended to employ indirect antigen coating, such as antigen-specific capture antibodies (Sanna et *al.*, 1995), because some phage antibodies selected against an adsorbed antigen may not be able to recognize the native form because of conformational integrity of the immobilized antigen. Biotinylated antigens can also be immobilized on streptavidin-coated beads. Phages are added to the immobilized antigen, followed by several washings, the bound phages are eluted from their specific antigens with acidic solutions such as HCL or glycine buffer (Kang et *al.*, 1991; Roberts et *al.*, 1992), with basic solutions such as triethylamine (Marks et *al.*, 1991), or by displacement by competition with excess antigen (Clackson et *al.*, 1991) (Figure 1.4). Selection using antigen in solution allows overcoming the problems with conformational changes that are encountered upon coating antigens on solid surfaces. Furthermore, the use of labelled biotinylated soluble antigens allows a more accurate quantification of the antigen used during selection (Hawkins et *al.*, 1992), thus enhances the possibility of using a lower

concentration of the antigen to favor selection of high-affinity phage antibody. Phage-antibodies are incubated. Phages bound to the labelled antigen are recovered with avidin or streptavidin-coated paramagnetic beads. The magnetic beads are pulled out of solution by a magnet and washed. The phages are recovered by different ways of elutions as described previously and then characterized.

Selection on cells using markers on cell surfaces has been carried out efficiently on either monolayers of adherent cells or on cells in suspension. Phages are incubated with the cells and unbound phage is washed away by rinsing tissue culture flasks in the case of monolayers or by centrifugation in the case of cell suspension. A simultaneous positive and negative selection maybe applied to minimize the irrelevant binding (de Kruif et *al.*, 1995; Binetruy-tournaire et *al.*, 2000). For this purpose, a competition is set up between a small number of antigen-positive target cells and an excess of antigen-negative cells which can serve to minimize the non-specific binding. FACS (Fluorescence Activated Cell sorting) assay is used to isolate the target cells binding the specific phage using a fluorescent labelled antibody against the irrelevant antigen. Using a similar technique, three rounds of positive selection on human melanoma cells and negative selection on human peripheral blood mononuclear cells using scFv library and two clones that recognize melanoma cells have been isolated (Kupsch et *al.*, 1999).

1.2.4 *In vivo* selection for phages targeted for specific organs

This technique was first used by Pasqualini and Ruoslati to isolate phage-displayed peptides homing to the brain and kidney. In this method, phage libraries are injected intravenously in animals. Tissues are collected and washed, phages are recovered and amplified *in vitro* and then re-injected to obtain sufficient enrichment. This approach permits the isolation of phage-displayed peptides homing to intact targets and also eliminates phage-displayed peptides that recognize plasma and cell surface proteins. Other organs, such as liver and lung capture too many phages to be used as target organs for selection (Pasqualini and Ruoslati 1996).

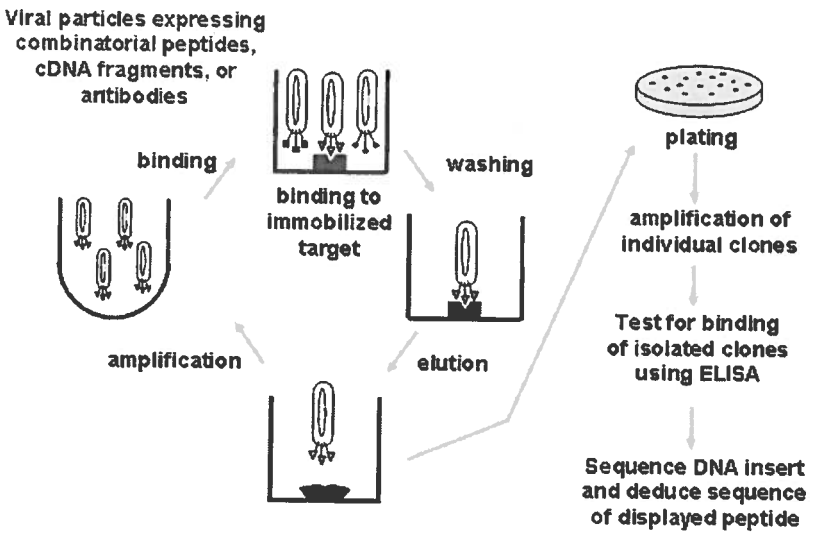


Figure 1.4 Phage display selection using immobilized antigen.
A library of phage is incubated with an immobilized target. Unbound phages are washed with washing buffer and bound phages are eluted by acidic buffer or by displacing factor. Recovered phages are amplified and analyzed by sequencing.

1.3 Objectives of the study.

The screening of phage-displayed libraries is a powerful technique for identifying peptides with desirable biological or physiological properties, particularly when it is combined with iterative cycles of phage selection and amplification. New agonists and antagonists for cell membrane receptors, such as KDR, have been successfully identified using this process (Hetian et *al.*, 2002). However, to the best of our knowledge, no truly antagonistic compounds that would selectively discriminate between Flt-1 and KDR receptors are available yet.

The objective of the present study was to select, using a phage-displayed peptide library, a new peptide motif that inhibits VEGF binding to Flt-1 by antagonizing Flt-1 receptor. A random 16-mer peptide library displayed on the surface of the filamentous phage M13 was screened *in vitro* against the extracellular domain of Flt-1. The screening resulted in a peptide (SP5.2) that competed with VEGF for the Flt-1 binding and inhibited a broad range of VEGF induced events in cultured endothelial cells. Potential use of SP5.2 as a targeting agent was evaluated using both synthetic and genetically constructed conjugates of the peptide and reporter proteins.

CHAPTER 2: Materials and Methods

2.1 General materials used

2.1.1 Proteins and peptides

A recombinant Flt-1 receptor chimera (rhFlt-1) consisting of the six NH_2-terminal extracellular domains of human Flt receptor and Fc fragment of human IgG was obtained from R&D Systems (Minneapolis, USA). The goat polyclonal anti-hFlt-1 antibody, $rhVEGF_{165}$, hKDR/Fc chimera, hsICAM-1, mFlt-1/Fc chimera and mFlk-1/Fc chimera were obtained from R&D Systems. Biotin labelled anti-human IgG (Fc specific) (anti-hIgGFc) rabbit polyclonal antibody was purchased from ICN (Costa Mesa, CA). The mouse monoclonal anti-β-galactosidase clone Gal-13 antibody and anti-mouse IgG peroxidase conjugate antibody developed in goat and absorbed with rat serum proteins were obtained from Sigma (Oakville, ON). The anti-Histidine HRP (Horse Radish Peroxidase) conjugate antibody was purchased from Invitrogen (Burlington, Ontario, Canada). Non-relevant scrambled control peptide, NGSAIAASSAVTHGMS, was synthesized at Supratek Pharma Inc. (Laval, Quebec).

2.1.2 Cell cultures

Human umbilical vein endothelial cells (HUVEC) were purchased from BioWhittaker Inc. (Walkersville, MD). The cells were cultured in the endothelial growth medium, Clonetics media EGM & EGM BulletKit (BioWhittaler Inc.). The cells were used for the experiments at their 3^{rd} or 4^{th} growth passages. Human cerebral endothelial cells (HCEC) were isolated from human temporal cortex using previously described protocols (Gerhard et *al.*, 1988; Stanimirovic et *al.*, 1996). Purity of HCEC cultures generated by these procedures was routinely assessed by the immunocytochemical staining for Factor VIII-related antigen and the lack of staining for smooth muscle β-actin, and was estimated to be >95%. The morphological, phenotypic, biochemical and functional characteristics of these HCEC cultures have been described in detail previously (Stanimirovic et *al.*, 1996; Muruganandam et *al.*, 1997).

2.1.3 Bacterial strains, bacteriophages and plasmid vectors

E.coli strain, K91Kan cells, obtained from Smith G. (University of Missouri, Columbia, MO) were grown on LB agar (Sigma) supplemented with kanamycin (100μg/ml), and K91Kan *E.coli* cells were made competent using the protocol described

previously by Smith and Scott (1993). M15 was purchased from Qiagen (Mississauga, ON), and cells made competent following the protocol from Qiagen. Vector pQE16 was purchased also from Qiagen (Mississauga, ON) and vector pCMVb from Clontech (Palo Alto, CA).

The 16-mer phage-displayed library used in this study was constructed at Supratek Pharma Inc., using fUSE5 as the phage vector essentially by a protocol described by Popkov et *al.* (1998). This linear library consisted of 10^9 independent recombinant phages recovered as tetracycline-resistant colonies. The majority of these phages (>90%) contained inserts as was indicated by the sequencing of randomly selected clones.

2.2 Screening the phage display library with Flt-1 coated magnetic particles

The receptor, rhFlt-1 chimera, (10μg in 50 μl of 0.1%BSA/PBS), was immobilized on streptavidin-coated magnetic particles (Roche Diagnostics, Laval, Canada) using biotinylated anti-hIgG(Fc) antibody. The Flt-1-coated magnetic particles (Flt-1-MP) were blocked with 3%BSA/PBS for 2 hours at room temperature. For selection, phage ($1x10^{11}$cfu) from the linear 16 a.a. random peptide phage display library diluted in 0.1%BSA/PBS were added to Flt-1-MP and incubated overnight at 4°C. After extensive washing with 0.1%BSA/PBS, the bound phage were either eluted with a low pH buffer (0.2 M Glycine-HCl, pH2.2) or displaced from Flt-1-MP with 10 μg/ml $rhVEGF_{165}$ for 1 hour at 22°C. Recovered phages were amplified using competent K91Kan *E. coli* cells and then subjected to four subsequent rounds of selection on Flt-1-MP. Phage binding was quantified by counting the phage titer in eluted aliquots from Flt-1-MP as described earlier (Smith and Scott, 1993).

2.3 Sequencing phage clones

Phages from selected clones were sequenced at Sheldon Biotechnology sequencing centre (McGill, Montreal).

2.4 ELISA of synthesized positive phages binding to Flt-1 receptor

The rhFlt-1 receptor chimera were immobilized on Streptavidin-coated microtiter plates (total binding capacity for biotin-labeled antibody = 1.5 μg/well, Roche Diagnostics). First, Biotin-labeled antibody anti-hIgGFc at a concentration of 10μg/ml in 0.1%BSA (Bovine Serum Albumin)/PBS (Phosphate Buffered Saline) was added into each well of the plate. The plate with assay mixtures were incubated at 4°C for 8 hours in a

humidified container, and then washed four times with 0.1%BSA/PBS. Recombinant human Flt-1/Fc chimera (1 μg/well in 0.1%BSA/PBS, R&D Systems, Minneapolis) was added into each well, and the plates were incubated overnight at 4°C in a sealed container to let receptor attach to ligand. Unbound receptor was washed away with 2 ml of 0.1%BSA/PBS. Each well was then filled with 250 μl of blocking buffer (2% nonfat dry milk in PBS) and incubated at room temperature for 2-3 hours. As a negative control, 3 of the wells were blocked with the blocking buffer without any antibody added. Phage particles ($5x10^9$-$1x10^{10}$ cfu/well) were added to each well, including the negative control ones, and incubated for 2 hours at room temperature. In competition experiments, phage suspension was premixed with various concentrations of competing agent (synthetic SP5.2 peptide, $rhVEGF_{165}$) and then added to immobilized receptor. Wells were washed with 0.1%BSA/PBS and amount of bound phages was detected with peroxidase-conjugated anti-M13 antibody (Pharmacia Biotech, Illinois). After the addition of the substrate, ABTS (2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic acid)) and H_2O_2, antibody reaction was analyzed at 405 nm using a Microplate Fluorescence Reader FL600 (BioTek, Vermont).

2.5 Production of mutant variants of the positive phage V5.2

Several single point mutations in Flt-1 binding peptide coding oligonucleotides insert in V5.2 phage were produced using protocols described previously (Hoess et *al.*, 1993.). Briefly, series of peptide-coding oligonucleotides, with a particular amino acid coding triplet replaced with GCT (alanine coding triplet), was synthesized by Invitrogen (Invitrogen, ON). The mutant oligonucleotides were cloned into fUSE5 phage vector as described previously (Popkov et *al.*, 1998). All mutant phages were purified and verified by DNA sequencing.

2.6 Synthesis of the selected Flt-1 receptor binding peptide, SP5.2

Peptide amides were synthesized manually on solid Rink amide resin (Nova Biochem, CA) using standard Fmoc protocol and PyBOP activation. The fluorescein residue was introduced into the peptide by standard coupling of N-terminal deprotected peptide with fluorescein-5-carboxylic acid. Peptide amides were cleaved from the resin with trifluoroacetic acid : water : etanedithiol : triisopropylsilane (95:5:2.5:2.5 [vol/vol/vol/vol]) and were recovered by precipitation with ice-cold diethyl ether. Crude products were purified by high performance liquid chromatography (HPLC) on Vydac C18

column using a linear gradient of 30% to 70% acetonitrile/water (0.1% trifluoroacetic acid), for 60 minutes at 5 ml/minutes flow rate. The identity of peptides was verified by electrospray mass spectrometry (PE Sciex API III Biomolecular Mass Analyzer, Applied Biosystems, CA). Fluorescein labelled SP5.2 peptide was synthesized using the method described above.

2.7 Competition assay for the binding of the synthetic peptide to Flt-1 receptor

Fluorescein-labeled peptide in 0.1%BSA/PBS buffer, pH 8.5 was added at various concentrations (0 μM, 0.1 μM, 1 μM, 10 μM, 50 μM and 100 μM) into wells of ELISA plate coated with immobilized rhFlt-1 receptor. In competition experiments, the various concentrations of phage or $VEGF_{165}$ were mixed and added into wells. After 2 hours incubation, microtiter plates were washed 10 times with 0.1%BSA/PBS buffer, pH 8.5 and the bound peptide was measured using Microplate Fluorescence Reader FL600 (BioTek, Vermont) (λ_{ex} =485 nm; λ_{em} = 530 nm).

2.8 Endothelial cell proliferation assay

HUVE cells were placed in 96-well plates (Costar) at 10^4 cells per well in 200 μl of EGM-2 medium (Clonetics) supplemented with 0.5% heat-inactivated fetal bovine serum. Cells were incubated to grow for 24 hours at 37 °C in 5% CO_2. HCEC cells were placed in 12 well plates and allowed to grow for 3 days until sub-confluent. Cells were then washed once by PBS and incubated in a serum- and glucose-free DMEM (Dulbecco's Modification of Eagle's Medium) (Invitrogen) for 24 hours to suppress cell growth. Both HCEC and HUVEC cells were then exposed to various treatments, (i.e., 10% FBS, $VEGF_{165}$ (20ng/ml), PIGF (100ng/ml), bFGF (10ng/ml) in the absence or presence of SP5.2 peptide antagonist). Scrambled control peptide NGSAIAASSAVTHGMS at the same concentration was used as a negative control. After 48 hours of incubation, 1μCi of [methyl-^{3}H]-thymidine (20 Ci/mmol; ICN) was added per well and plates were incubated for an additional 24 hours. The cells were then placed on ice, washed twice with EGM-2 medium containing 10% FBS (Fetal Bovine Serum) (Invitrogen) and fixed for 10 minutes by adding 200 μl of ice-cold 10% trichloroacetic acid per well. After washing with ice-cold water, cells were lysed and DNA was solubilized in 50 μl of 2% SDS (Sodium Dodecyl Sulfate) (Invitrogen). [^{3}H]-Thymidine incorporation was determined by scintillation counting.

2.9 Angiogenesis assay using capillary tube formation in Matrigel™

HCEC were seeded ($8x10^4$ cells) on a semi-solid basement membrane matrix, Matrigel™ (Collaborative Biomedical Products, Bedford, MA)-coated 24-well plates and exposed to serum-free media containing various additives. Cells were treated with $VEGF_{165}$ or glioblastoma cell (U-87MG, ATCC, Rockville, MD)-conditioned media (serum-free DME conditioned for 48-72 hours) in the absence or presence of synthetic SP5.2 or non-relevant scrambled control peptide for 24 hours. HCEC were then labelled with a vital dye, calcein-AM (2 μM) for 30 minutes. Cells were then washed in PBS and capillary-like tube formation, a measure of *in vitro* angiogenesis, was observed using a Zeiss LSM 410 inverted laser scanning microscope (Thornwood, NY) with an Argon/Krypton ion laser and a Zeiss LD achroplan 5X, 0.6 NA objective. Confocal apertures for each recorded wavelength were adjusted to a full width half maximum of 20 μm. The emitted light was collected through a 515-540 nm band-pass filter using an excitation of 488 nm. Eight to 32 individual optical sections were collected and the average was calculated. Standard image processing was performed to enhance brightness and contrast using ImageSpace software (Molecular Dynamics Inc., Sunnyvale, CA).

2.10 Chemotaxis set up

Chemotaxis of calcein-AM labeled HCEC induced by various stimuli (including $VEGF_{165}$ and IL-8) was tested in the absence or presence of synthetic SP5.2 or scrambled control peptide using a ChemoTx # 101-5 assembly (Neuro Probe, Inc., Gaithersburg, MD) consisting of a 96-well plate and a polycarbonate filter membrane, as described by Junger et *al.* (1993). Briefly, the wells of the plates were loaded with buffer containing various additives or phenol red- and serum-free conditioned media. The framed filter membrane was positioned on top and 50,000 calcein-AM-labeled HCEC suspension in 20μl of matching non-conditioned media was applied on the top of each membrane/well. The assembly was incubated for 120 minutes at 37°C, and the numbers of endothelial cells transmigrated into wells of the 96-well plate were quantified by measuring the intensity of fluorescence (excitation/emission: 485/530 nm) in a CytoFluor 2350 reader (Millipore, Bedford).

2.11 Immunocytochemical microscopy

HCEC were grown on glass cover slips in a 24 well dish for 3 days until sub-confluent. Media was aspirated out; cells were washed 2 times in PBS, and fixed with 4% paraformaldehyde for 10 minutes at room temperature. Cells were subsequently washed 3 times in PBS, incubated in blocking solution 5% goat serum in PBS for 1 hour at room temperature, and exposed to the primary antibody (5μg/ml goat anti-human Flt-1 in blocking solution; R&D Systems Inc) for 1 hour at room temperature. After washing 3 times in blocking solution, cells were exposed to the secondary antibody (1:20 dilution of donkey anti-goat IgG-immunogold (R & D Systems) in blocking solution; Accurate Chemical and Scientifif Corp.) for 30 minutes at room temperature. Cells were then extensively washed (3 times with blocking solution and 3 times with distilled water) and incubated in silver enhancing solution for 30 minutes (1:1 dilution of reagent A and B). After washing 2 times for 5 minutes each in distilled water, cells were viewed under microscope.

2.12 Binding of synthetic peptide SP5.2 to HCEC cells

Fluorescein-labelled SP5.2 peptide was added to HCEC cells at different concentrations, at 1 μM, 10 μM and 50 μM and incubated for 30 minutes. Neuroblastoma ShyY (NRC, Ottawa) cell line was used as a negative control with 50 μM Fluorescein-labelled synthetic SP5.2 peptide. Media were aspirated and replaced with clear M199 (Earle's salts, 25 mM Hepes, 4.35g/l sodium bicarbonate and 3 mM L-glutamine). Cells were observed under fluorescent microscope.

2.13 Construction and purification of fusion protein with β-galactosidase

The prokaryotic expression vector, pQE-16 purchased from Qiagen (Mississauga, ON) was used to construct the Flt-1 binding fusion protein with β-gal, containing SP5.2 peptide on N-terminus and $(His)_6$ on C-terminus of β-galactosidase. For this the vector pCMV-β from Clontech (Palo Alto, CA) was digested with *BamH1* (NEB), the resulting β-galactosidase gene was purified from agarose gel and modified by PCR as so to introduce *Bgl II* (NEB) at the 3'end for cloning into pQE116 instead of DHFR, that has been removed by digestion of plasmid with *BamH1* and *Bgl II.* SP5.2 insert containing *EcoR1* site at 5' end and *BamH1* site at 3' end was prepared by annealing of two single stranded oligonucleotides synthesized by INRS-IAF, Laval, QC and inserted in the pQE16-β-gal

vector. pQE16-SP5.2-β-gal was used to transform competent *E.coli* M15 cells. The resulting clones were sequenced to verify the fusion gene.

For further purification of the fusion protein, an overnight culture of a single colony was grown in 400 ml LB medium containing 100 μg/ml Ampicillin and 25 μg/ml Kanamycin to an O.D. of ~0.6 at 600 nm. The culture was induced with 1 mM IPTG (Invitrogen) for 4 hours at 37°C, centrifuged and the pellet was stored at –80°C. The expression of β-galactosidase and the presence of His-tag were verified by Western-blot using anti-β-galactosidase and anti-His-HRP antibody. The pellet was thawed and resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysozyme was added to a concentration of 1 mg/ml. After incubation for 30 minutes at 4°C, 400 μl of 50% Ni-NTA resin (Qiagen, ON) was added to the supernatant and incubated 1 hour shaking at 4°C. The lysate-Ni-NTA mixture was loaded into a column (Qiagen, ON), washed with washing buffer (50 mM NaH_2PO_4, 10 mM NaCl, 20 mM imidazole, pH8.0) and the protein SP5.2-β-gal was eluted with the elution buffer (50 mM NaH_2PO_4, 10 mM NaCl, 250 mM imidazole, pH8.0). The eluted protein was washed by PBS to decrease imidazole concentration and concentrated by Centricons (Fisher) to get a final concentration of approximately 1μg/ml.

2.14 Assay for the binding of the β-galactosidase fusion protein to Flt-1 receptor

Streptavidin-coated microtiter plates (total binding capacity for biotin-labeled AB = 1.5 μg/well, Roche Diagnostics) were used to immobilize the rhFlt-1 receptor chimera. Biotin-labeled anti-hIgGFc antibody 10 μg/ml in 0.1%BSA/PBS were added into each well of streptavidin-coated plate. The microtiter plates were incubated at 4°C for 16 hours in a humidified container and then washed for 4 times with 0.1%BSA/PBS. Recombinant human Flt-1/Fc chimera (1 μg/well in 0.1%BSA/PBS) was added into each well. Plates were incubated at 4°C 8 hours in sealed container to let receptor to immobilize. Unbound receptor was washed away with 2 ml of 0.1%BSA/PBS. Then each well was filled with 350 μl of Blocking Buffer (2% nonfat dry milk in PBS) and blocked for 16 hours at 4°C. As a negative control, 3 wells with immobilized anti-hIgGFc antibody were blocked with 2% nonfat milk in PBS. SP5.2-β-gal fusion protein and recombinant β-gal from Sigma (Oakville, Ontario) (used as control) were added to each well and incubated for 2 hours at room temperature. Wells were washed with 0.1%BSA/PBS and amount of bound phages

expressing fusion protein was detected with anti-β-gal and IgG anti-mouse HRP conjugate. After substrate addition (ABTS containing H_2O_2), wells were analyzed in microtiter plate reader at 405 nm.

2.15 Construction and purification of the fusion protein with Peroxidase

Three mg Peroxidase (ICN, CA) was dissolved in 1ml of phosphate buffer (0.1 M Na_2HPO_4, 0.1 M NaCl, 1 mM EDTA, pH8.5). N-succinimidy 1-3-(2-pyridylthio) propionate (SPDP; Sigma, ON) was dissolved in dimethylformamide (DMFA) in a proportion of 0.234 mg SPDP/39 µl DMFA. The solution of SPDP was added to the solution of peroxidase and stirred 30 minutes at room temperature. The solution of activated peroxidase was applied to Sephadex G-25 column (Fisher) and eluted with 50 ml phosphate buffer, fractions of 1 ml were collected and detected at 280nm at sensitivity level of 50, lamp intensity 0.005, and recorder prints at 5mm/minutes. The fractions containing modified peroxidase were selected and combined. One mg of synthetic SP5.2 peptide (Supratek) was dissolved in phosphate buffer, added to active peroxidase and incubated stirring for 24 hours at room temperature. The reaction was controlled by UV detection at 343 nm. The conjugate was applied on G-25 Sephadex column for purification and the conjugate fractions were collected and combined. Activity of conjugate was determined by checking peroxidase activity itself using ABTS substrate containing H_2O_2. Cysteine conjugated to peroxidase used as a negative control was synthesized at Supratek (Supratek, Laval).

2.16 Assay for the binding of the Peroxidase fusion protein to Flt-1 receptor

Streptavidin-coated microtiter plates (total binding capacity for biotin-labeled AB = 1.5 µg/well, Roche Diagnostics) were used to immobilize the rhFlt-1 receptor chimera. Biotin-labelled anti-human FC antibody (ICN) 10 µg/ml in 0.1%BSA/PBS were added into each well of streptavidin-coated plate. The microtiter plates were incubated at 4°C for 16 hours in a humidified container and then washed for 4 times with 0.1%BSA/PBS. Recombinant human Flt-1/Fc chimera (1 µg/well in 0.1%BSA/PBS, R&D Systems, Mineapolis) was added into each well. Plates were incubated at 4°C 8 hours in sealed container to let receptor to immobilize. Unbound receptor was washed away with 2 ml of 0.1%BSA/PBS. Then each well was filled with 350 µl of Blocking Buffer (2% nonfat dry milk in PBS) and blocked for 16 hours at 4°C. As a negative control, 3 wells with

immobilized anti Fc-antibody were blocked with 2% nonfat milk in PBS. SP5.2-peroxidase conjugate and Cys-peroxidase used as control, were added to each well and incubated for 2 hours at room temperature. Wells were washed with 0.1%BSA/PBS and amount of bound conjugate was detected with peroxidase activity. After substrate addition (ABTS containing H_2O_2), wells were analyzed in microtiter plate reader at 405nm.

CHAPTER 3: Results

Le travail présenté dans ce mémoire a grandement bénéficié de la collaboration de beaucoup de chercheurs qui ont généreusement partagé leur expertise et leurs équipements et laboratoires de recherches afin d'exécuter certaines expériences dans l'étude que j'avais mené à bonne fin sous le conseil de mes directeurs de recherches. La liste suivante décrit leurs contributions :

1. Les analyses de la prolifération des cellules endothéliales (liaison de SP5.2 au récepteur Flt-1, présenté dans la figure 3.7), analyses d'angiogenèse (inhibition de la prolifération médiée par VEGF des cellules endothéliales et cervicales (HCEC); formation de tubes capillaires; perméabilité, résultats présentés dans les figures 3.8 3.9, 3.10 et 3.11 respectivement) ont été effectuées dans les laboratoires du Dr. Stanimirovic et du Dr. Moreno de CNRC, Ottawa.
2. Le criblage des bactériophages et la synthèse du peptide (conjugé de peroxydase SP5.2) ont été respectivement exécutés en collaboration avec le Dr. Tchistiakova et le Dr. Pietrzynski de Supratek Pharma Inc., Laval.
3. La protéine de fusion SP5.2-ß-gal a été construit en collaboration avec le Dr. Yurchenko à Supratek.

3.1 Screening and enrichment (biopanning) of a 16-mer phage display library for clones displaying peptides binding specifically to human Flt-1 receptor

The phage display technique was used to identify the clones expressing the peptide sequences that bind to a receptor of VEGF Flt-1, and thus compete for the receptor inhibiting VEGF interaction with the Flt-1 receptor. A random 16mer peptide phage display library composed of approximately $1x10^9$ independent peptide sequences was obtained from Supratek Pharma and screened for binding to immobilized human Flt-1 receptor fused with Fc fragment of human IgG antibody (rhFlt-1/Fc chimera) using a two step elution protocols. The Flt-1 bound phages were recovered by elution with acidic buffer (0.2M Glycine-HCl, pH2.2). In an alternate elution set up, the positive Flt-1 binding clones were further analyzed for their interaction with the VEGF binding site of Flt-1, by measuring the displacement of the bound phages with $rhVEGF_{165}$. In both assays, a pronounced enrichment in the phage binding to Flt-1 (relative to bovine serum albumin (BSA) used as a negative control) was detected. The results, presented in Figure 3.1, show highest level of enrichment after 3rd round of panning (2,500-fold in the case of acidic elution and 400-fold in the case of elution with VEGF). At the end of the selection, 20 positive clones were isolated and analyzed for the DNA sequence.

3.2 DNA sequencing of positive phage clones and multiple alignment and analysis of the deduced amino acid sequences

DNA sequencing and the deduced amino acid (a. a.) sequence analysis of the positive clones showed that 11 of the clones selected with acid elution protocol code for two sequences A1 and A2, and four of the clones isolated with VEGF displacement method code for four different peptide sequences (V5.2, V5.10, V40 and V5.8). The clones A2 and V5.2 had identical sequences (Table 3.1).

3.3 Binding of the isolated positive phage clones to hFlt-1

The binding of each of the selected phage clones (A1, V5.2, V5.8, V5.10, V40) to Flt-1 receptor was tested by ELISA on immobilized rhFlt-1/Fc chimera. The results presented in Figure 3.2, show that the phage clone V5.2 exhibited the strongest binding

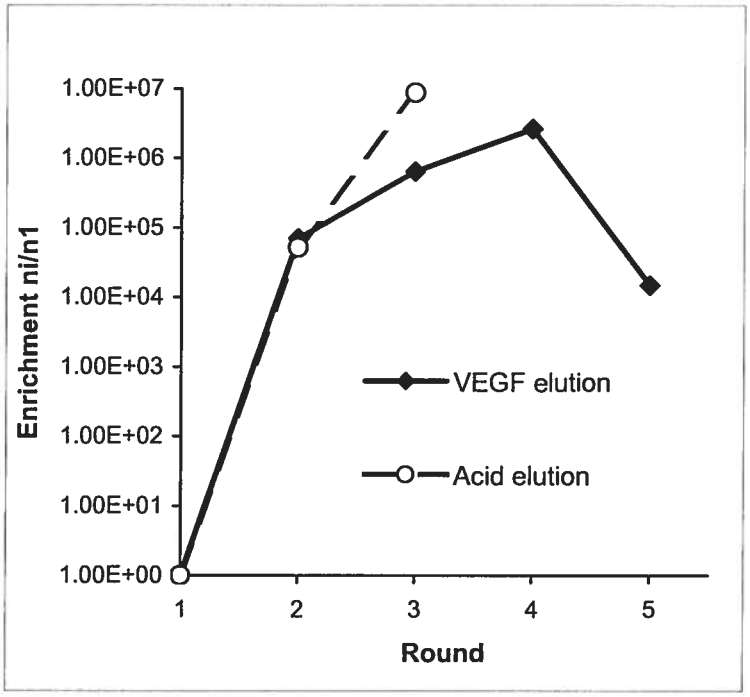


Figure 3.1 Phage library enrichment during selection.
A random 16mer peptide phage display library was screened for binding to immobilized Flt-1 receptor. Phages were recovered either by acid elution or by VEGF displacement. A pronounced enrichment in the phage binding reaching the maximum after the 3rd round of the panning.

Table 3.1 Sequences of the phage clones binding to the receptor Flt-1 and deduced amino acid sequences of displayed peptides.
20 clones were isolated and analyzed by DNA sequencing after 5 rounds of panning. A1 and A2 were selected with acidic elution; V5.2, V5.10, V40, V5.8 were isolated with VEGF displacement. A2 and V5.2 sequences were identical.

Positive phage clones	Insert peptide sequence	Elution at/with	Frequency
A1	WFLLTM	Acidic pH	8
A2	NGYEIEWYSWVTHGMY	Acidic pH	3
V5.8	RIKYHVGNFMYFLAKL	$RhVEGF_{165}$	3
V5.2	NGYEIEWYSWVTHGMY	$RhVEGF_{165}$	3
V5.10	FVGGWLVPEDERLYPE	$RhVEGF_{165}$	2
V40	PEPEVRLSPPGHIQSL	$RhVEGF_{165}$	1

with the receptor at the concentration of $1x10^9$cfu/ml and was chosen for further studies. To further verify the selectivity of phage clone V5.2 to Flt-1 receptor, its binding was further analyzed by the above-described ELISA assay using two other human endothelial receptors, human low affinity VEGF receptor 1 (KDR), and Intracellular adhesion molecule 1 (hICAM-1), that were also fused with human IgG Fc fragments (anti-Fc-AB), as well as with an irrelevant protein, human anti-Fc-AB. The results presented in Figure 3.3 showed that no significant interaction of the V5.2 phage with KDR and other control proteins was observed. V5.2 phage did exhibit some affinity to murine analog of Flt-1 (mFlt-1) but did not interact with the murine analog of KDR receptor (Flk-1) (Figure 3.3). These results clearly indicate that the phage clone V5.2 specifically binds to the hFlt-1 receptor at the site of the hVEGF.

3.4 Binding of synthetic peptide SP5.2 to hFlt-1 receptor

In order to further confirm the specifity of the V5.2, a linear peptide (SP5.2) labelled with fluorescein at its N-ternimus, was synthesised with the sequence corresponding to that of V5.2 phage nucleotide insert. The Flt-1 binding activity of peptide SP5.2 was analyzed by ELISA (by an assay similar to that used for the phage binding except that the fluorescence of the SP5.2 peptide was measured to evaluate its binding with the Flt-1/Fc chimera immobilized on the surface of 96-well plates). A non-relevant control scrambled peptide of sequence NGSAIAASSAVTHGMS was included as a negative control. The results from Figure 3.4A clearly indicated that the Flt-1 specific binding of peptide SP5.2 was dose-dependent and required about 40 μM concentrations to achieve half-maximum value compared to the binding with the control peptide, that did not show any significant binding. The binding specificity of the SP5.2 peptide to Flt-1 was further reconfirmed by a displacement assay system using the V5.2 phage (1x1010 cfu/ml) as a competitor to SP5.2. The results, presented in Figure 3.4B, clearly show that, phage clone V5.2 inhibited the binding of the SP5.2 peptide to 25%, whereas, a non-selected phage library used at the same concentration as a control did not affect the peptide binding significantly.

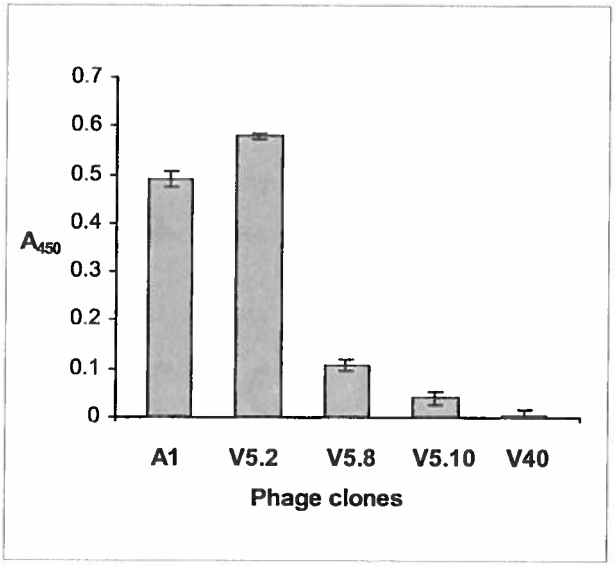


Figure 3.2 Comparison of phage clones binding to Flt-1 receptor by ELISA. The binding affinity of each selected clone was tested by ELISA. The V5.2 phage clone demonstrated the strongest binding with the Flt-1 receptor at a concentration of $1x10^9$cfu/ml.

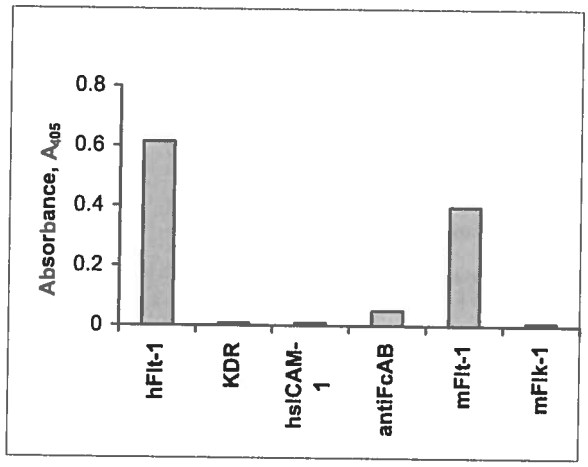
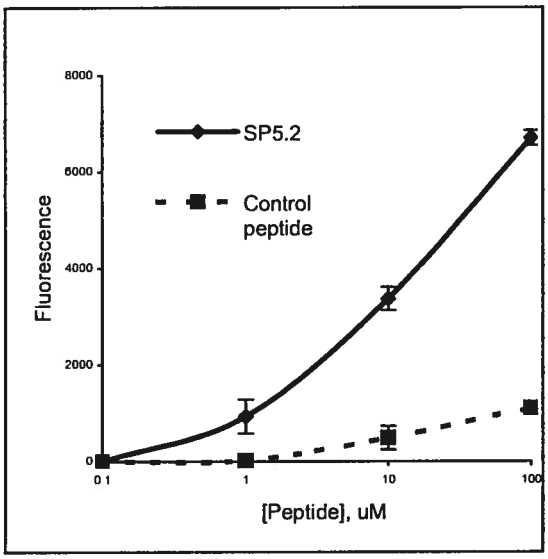


Figure 3.3 Specificity of V5.2 phage to human and murine Flt-1 receptor. Selectivity of V5.2 phage to Flt-1 receptor was analyzed by ELISA. Two other human endothelial receptors, KDR and ICAM-1 were used, as well as other an irrelevant protein, human anti-Fc-AB. V5.2 showed significant interaction to human Flt-1 and its murine analog, but no significant interaction with KDR and other control proteins was observed.

(A)



(B)

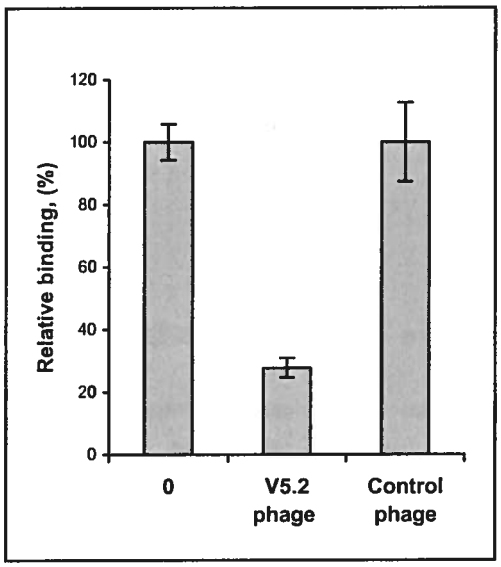


Figure 3.4 Binding of fluorescein labelled SP5.2 peptide to immobilized Flt-1 receptor and competition with V5.2 phage.

(A) A linear peptide was synthesized with the sequence corresponding to that of V5.2 phage clone peptide insert. The SP5.2 was labelled with fluorescein. The fluorescence was assayed to evaluate the binding activity. The Flt-1 specific binding was dose-dependant and required 40μM to achieve half-maximum value. *(B)* The binding specificity of SP5.2 peptide to Flt-1 was further confirmed in displacement assay using V5.2 phage as a competitor to SP5.2. A non-selected phage library was used as a control. V5.2 phage inhibited the binding of SP5.2 peptide to 25%, however, the control did not affect the binding.

3.5 Identification of a common motif by mutational analysis of amino acid (a.a.) residues of the peptide SP5.2

To analyze the functional importance of various a.a. in the SP5.2 peptide, 12 oligonucleotides each with a single point mutation with a particular a.a. coding triplet replaced with GCT, alanine coding triplet in the SP5.2 coding oligonucleotides sequence of V5.2 phage, were synthesized at Supratek Pharma, and cloned using M13 phage. These clones were designed specifically to have an a.a. substitution or deletions in areas believed to be responsible for receptor binding (Table 3.2). The phage-receptor binding affinity of these mutated clones to Flt-1 receptor was measured by ELISA. The results presented in Figure 3.5, show that the mutant phages with replacement of a.a. N1, E4, E6, I5, Y8, W10 and Y16 to alanine significantly decreases the receptor binding ability to the phage expressing SP5.2, whereas mutations at positions W7, S9, T12, H13 and M15 had little or no effect. From the result of these experiments a consensus motif for SP5.2 peptide binding could be deduced as to be NXXEIEXYXWXXXXXY.

3.6 Peptide SP5.2 blocks the interaction between VEGF and Flt-1

The ability of synthetic SP5.2 peptide and V5.2 phage to inhibit VEGF binding to Flt-1 was analyzed by competitive receptor binding assay, using $5x10^9$ cfu/ml V5.2 phage or 30μM fluorescein labelled SP5.2 peptide and various concentrations of rhVEGF (1pM to 100nM), and immobilized rhFlt-1/Fc chimera. As shown in figure 3.6, both SP5.2 peptide and V5.2 phage competed with VEGF for the receptor binding, and the inhibition of peptide SP5.2 binding was half-maximum at 5nM of VEGF. However, a ten times higher concentration of VEGF was required to achieve a 50% inhibition of phage V5.2 interaction with Flt-1 receptor. Considering the multi-valency of V5.2 phage (3 - 5 copies of the P3 protein, in which the insert is located, displayed on each M13 phage filaments), these results clearly indicate that the synthetic SP5.2 peptide had a comparable but lower binding potency to that of the phage-displayed peptide.

Table 3.2 Peptide sequences of different mutations of V5.2 phage insert at specific amino acid (a. a.) sites.

Single point mutation in the SP5.2 coding oligonucleotides sequence of V5.2 phage where an amino acid coding triplet replaced with GCT were synthesized and cloned using M13 phage at Supratek Pharma.

a.a. sites / Phage clones	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
V5.2	N G Y E I E W Y S W V T H G M Y
N1/A	A G Y E I E W Y S W V T H G M Y
E4/A	N G Y A I E W Y S W V T H G M Y
I5/A	N G Y E A E W Y S W V T H G M Y
E6/A	N G Y E I A W Y S W V T H G M Y
W7/A	N G Y E I E A Y S W V T H G M Y
Y8/A	N G Y E I E W A S W V T H G M Y
S9/A	N G Y E I E W Y A W V T H G M Y
W10/A	N G Y E I E W Y S A V T H G M Y
T12/A	N G Y E I E W Y S W V A H G M Y
H13/A	N G Y E I E W Y S W V T A G M Y
M15/A	N G Y E I E W Y S W V T H G A Y
Y16/A	N G Y E I E W Y S W V T H G M A

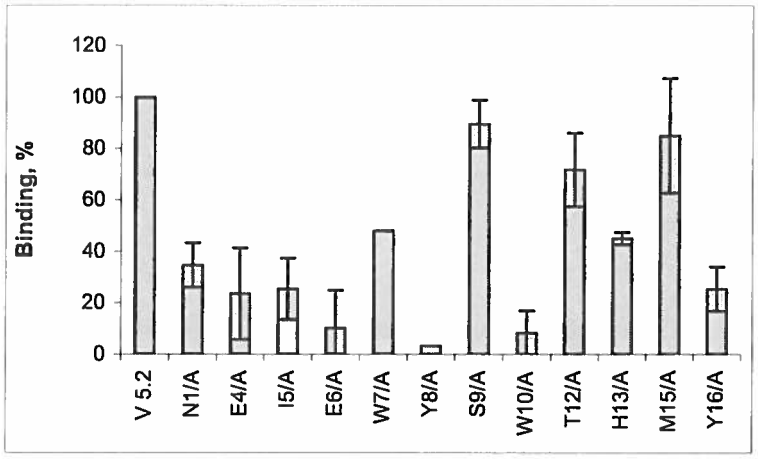


Figure 3.5 Analysis of the binding affinity of the V5.2-mutant phages to the immobilized Flt-1 receptor.

Single point mutations in V5.2 phage insert were produced. ELISA assay was used to determinutese their binding affinity. This assay showed that the exchange of N1, E4, E6, I5, Y8, W10, and Y16 to Alanine significantly decreases the binding to Flt-1, however, other mutations, W7, S9, T12, H13 and M15 had little or no effect.

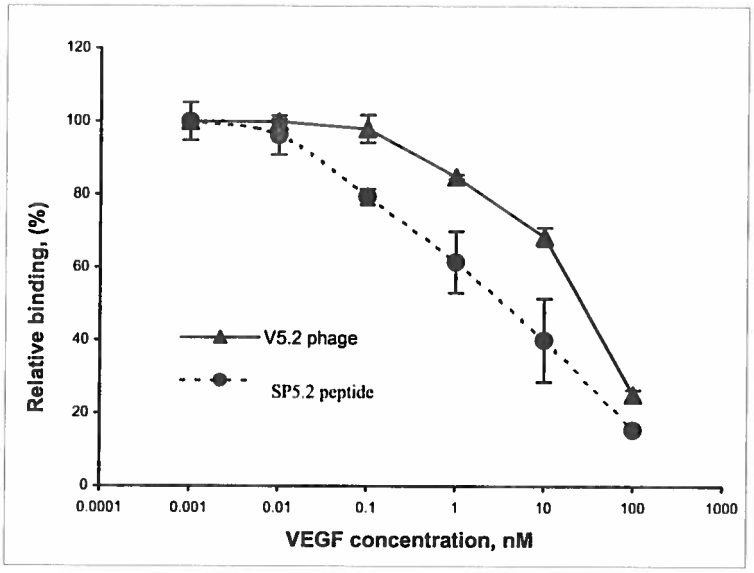


Figure 3.6 Inhibition of phage V5.2 and peptide SP5.2 binding to Flt-1 receptor by VEGF.

$5x10^9$cfu/ml of V5.2 phage or 30μM fluorescein labelled peptide SP5.2 and various concentrations of human recombinant VEGF (1pM-100nM). Those samples were added to wells with immobilized Flt-1 receptor. Both peptide SP5.2 and Phage V5.2 competed with VEGF for receptor binding. The half-maximum inhibition was detected at the presence of 5nM VEGF for SP5.2 peptide and 50nM for V5.2 phage.

3.7 Peptide SP5.2 binds specifically with Flt-1 expressing HCEC

The binding of the fluorescein-labelled peptide SP5.2 to Flt-1-expressing and non-expressing cells was analyzed. The Flt-1 expression in the primary HCEC grown in the presence of 10ng/ml of rhVEGF was confirmed by immunocytochemical assay detection (Figure 3.7A, B). Neuroblastoma ShyY cell line, in which Flt-1 was not detected by immunocytochemistry (Figure 3.7F, G), was used as a negative control. Fluorescein-labelled SP5.2 bound to VEGF-stimulated HCEC in a concentration-dependent manner (Figure 3.7C, D, E), as determined by fluorescent microscopy. Internalization of the receptor-ligand complex was also seen in some cells. No bound fluorescence was detected in Flt-1-negative ShyY cells at SP5.2 peptide concentration of 50μM (Figure 3.7H).

3.8 Peptide SP5.2 inhibits various VEGF mediated events in *in vitro* systems

Effect of peptide SP5.2 on VEGF mediated events was studied in 4 different *in vitro* systems, on proliferating endothelial cells , HUVEC and HCEC, on angiogenesis in HCEC, on the cell permeability of HCEC monolayers and migration of HCEC cells.

3.8.1 Peptide SP5.2 specifically inhibits the VEGF mediated proliferation of endothelial cells, HUVEC and HCEC

To determine whether the peptide SP5.2 could inhibit proliferation of vascular endothelial cells HUVEC were stimulated with 10ng/ml $rhVEGF_{165}$ and then treated with various concentrations of peptide SP5.2 for 48 hours, and DNA synthesis rate of the treated cells was determined as a measure of cell proliferation. The results, presented in Figure 3.8A, demonstrated that the peptide inhibits proliferation of HUVEC in a dose-dependent manner, with an IC_{50} of 5μM.

In order to establish that the inhibition activity of peptide SP5.2 was not only restricted to HUVECs, inhibition assays were performed using another endothelial cell culture, HCEC. The proliferation of HCEC was induced by a set of different growth factors: $rhVEGF_{165}$ (20ng/ml), PIGF (100ng/ml), bFGF (10ng/ml), and the DNA synthesis in the presence of SP5.2 peptide and a non-target control peptide NGSAIAASSAVTHGMS was followed (Figure 3.8B). At the concentration of 10μM the peptide SP5.2 completely inhibited VEGF and PIGF-induced HCEC proliferation. However, no effect on bFGF induced HCEC proliferation was detected, suggesting clearly that the effect of the peptide

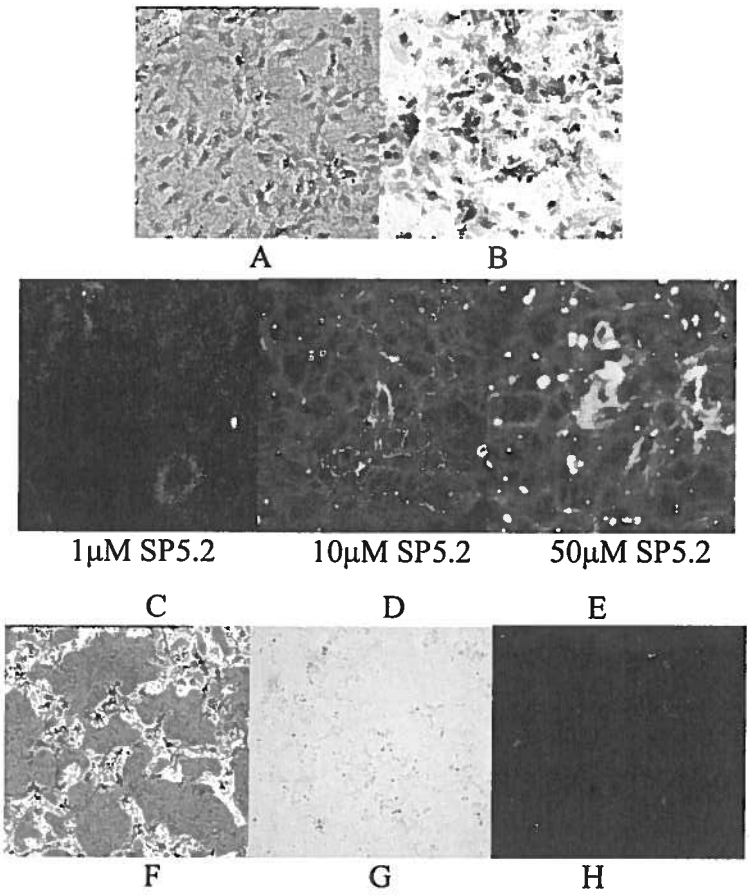
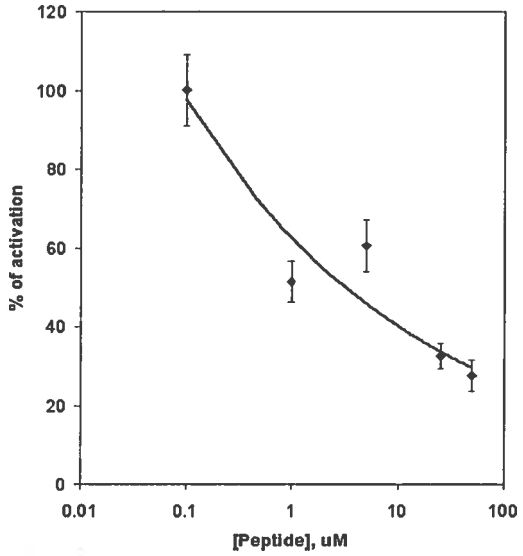


Figure 3.7 Binding of fluorescent SP5.2 peptide to Flt-1 expressing cells.
A) Phase contrast image of HCEC in culture. B) Immunocytochemical staining for Flt-1 receptor in HCEC. C-E) Binding of fluorescently-labeled SP5.2 to HCEC. SP5.2 was added to HCEC at 1μM (C), 10μM (D) and 50μM (E) for 30 minutes, media were aspirated, replaced with clear M199, and cells were observed under fluorescent microscope. F-G) Control experiments using neuroblastom ShyY cell line. (F) phase contrast micrograph of ShyY cells; G) Immunocytochemical detection for Flt-1 receptor in ShyY cells; H) Binding of fluorescently-labeled SP5.2 (50μM; 30 minutes) to ShyY cells. Immunocytochemical detection procedures are as described in Materials and Methods section.

A)



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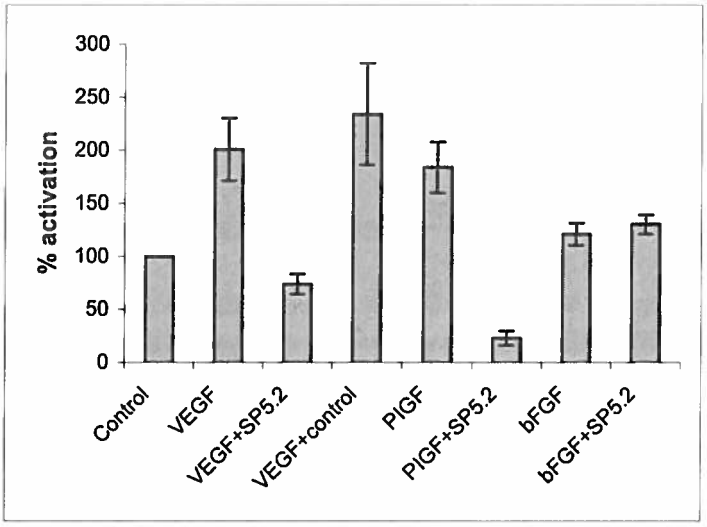


Figure 3.8 Inhibition of HUVEC and HCEC proliferation by peptide SP5.2.
(A) HUVEC were stimulated with 10ng/ml rhVEGF$_{165}$ and then treated with various concentrations of peptide SP5.2 for 48 hours. It was demonstrated that the peptide inhibits endothelial cell proliferation in a dose-dependant manner with an IC_{50} of 5μM. *(B)* HCEC cells were induced by different growth factors, rhVEGF$_{165}$ (20ng/ml), PIGF (100ng/ml) and bFGF (10ng/ml). At the concentration of 10μM the SP5.2 peptide completely inhibited VEGF and PIGF induced proliferation, however, it had no effect on bFGF induced proliferation.

is due to Flt-1 receptor. At the same concentration, the control peptide did not change the HCEC proliferation.

3.8.2 Peptide SP5.2 specifically inhibits the VEGF induced angiogenesis (capillary like-tube formation) in HCEC

At the concentration of 10μM, the peptide was found to inhibit VEGF-induced formation of capillary-like tubes by HCEC grown in the extracellular matrix, Matrigel^{ITM} (Figure 3.9A). SP5.2 also inhibited capillary like tubes formed by HCEC exposed to media conditioned by human glioblastoma cells, known to contain high levels of VEGF (Naganuma et *al.*, 2003), in a concentration-dependent manner (Figure 3.9B.). In a separate experiment human primary astrocytes were exposed to hypoxia and the medium conditioned by hypoxic astrocytes was applied to HCEC grown in Matrigel. SP5.2 was shown to inhibit capillary tube formation induced by hypoxic ACM.

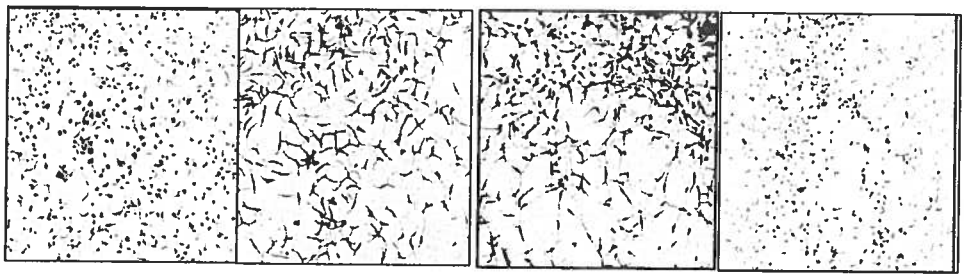
3.8.3 Peptide SP5.2 specifically inhibits the VEGF mediated increase in the permeability of HCEC monolayers

A prolonged (24 hours) exposure of HCEC monolayers to VEGF was found to increase permeability of endothelial monolayers for the paracellular diffusion marker, sodium fluorescein due to a disrupting effect of the growth factor on the tight junctions formed by HCEC in monolayers. This effect of VEGF was attenuated on basolateral and apical surfaces when SP5.2 peptide was added to the cells at a concentration of 5μM (Figure 3.10).

3.8.4 Peptide SP5.2 specifically inhibits the VEGF-mediated migration of HCEC

Migration (chemotaxis) of calcein-AM labelled HCEC is induced by several stimuli including $VEGF_{165}$, IL-8 and glioblastoma-conditioned media (serum-free DME conditioned for 48-72 hours) was tested in the absence or presence of SP5.2 peptide or scrambled peptide using a ChemoTx#101-5 assembly as previously described (Junger et *al.*, 1993). VEGF induced increased migration of HCEC across the filter that was significantly reduced by SP5.2 but not by control scrambled peptide (Figure 3.11). Peptide SP5.2 did not reduce migration of HCEC induced by IL-8 (Figure 3.11).

(A)



(B)

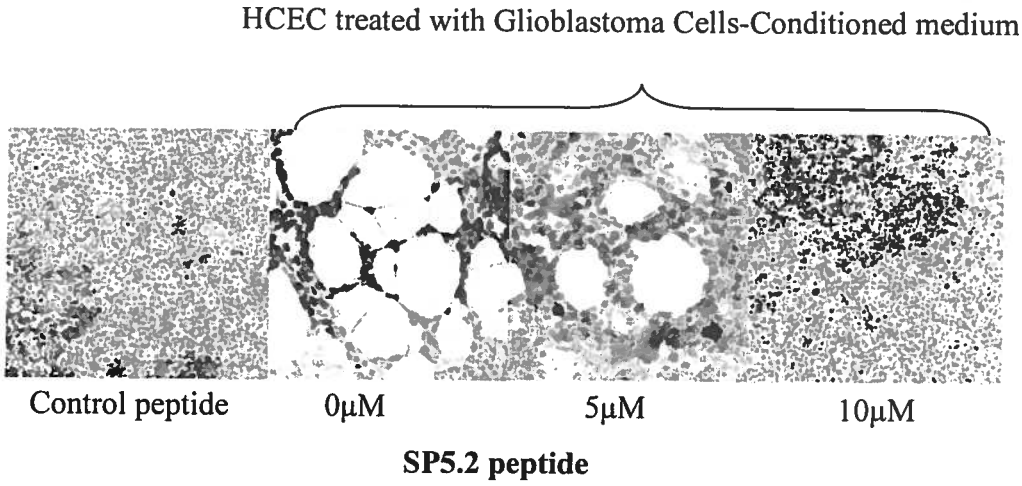


Figure 3.9 Inhibition of VEGF and tumor induced angiogenesis by SP5.2 peptide. HCEC were seeded on a semi-solid basement membrane matrix, Matrigel and exposed to serum-free media with various additions. *(A)* Cells were stimulated with $VEGF_{165}$ and then treated with SP5.2 or scrambled control peptide. SP5.2 inhibited capillary-like tubes formation grown on the matrix. *(B)* Cells were exposed to human glioblastoma cells and then treated with SP5.2 or a scrambled control peptide. SP5.2 also inhibited capillary-like tubes formation in a concentration-dependant manner.

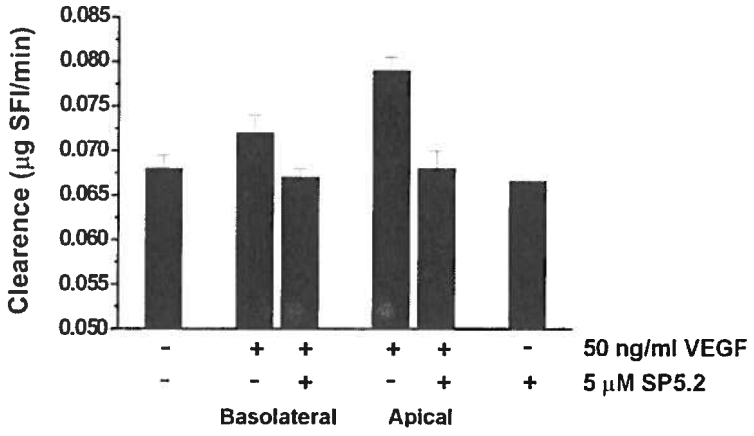


Figure 3.10 Inhibition of VEGF mediated permeability of blood brain barrier using HCEC model by SP5.2 peptide.

A 24 hours exposure of HCEC monolayers to $VEGF_{165}$, was found to increase permeability of monolayers, due to a known disrupting effect of $VEGF_{165}$ on the tight junctions formed by HCEC. The addition of 5µM of SP5.2 attenuated this function of VEGF.

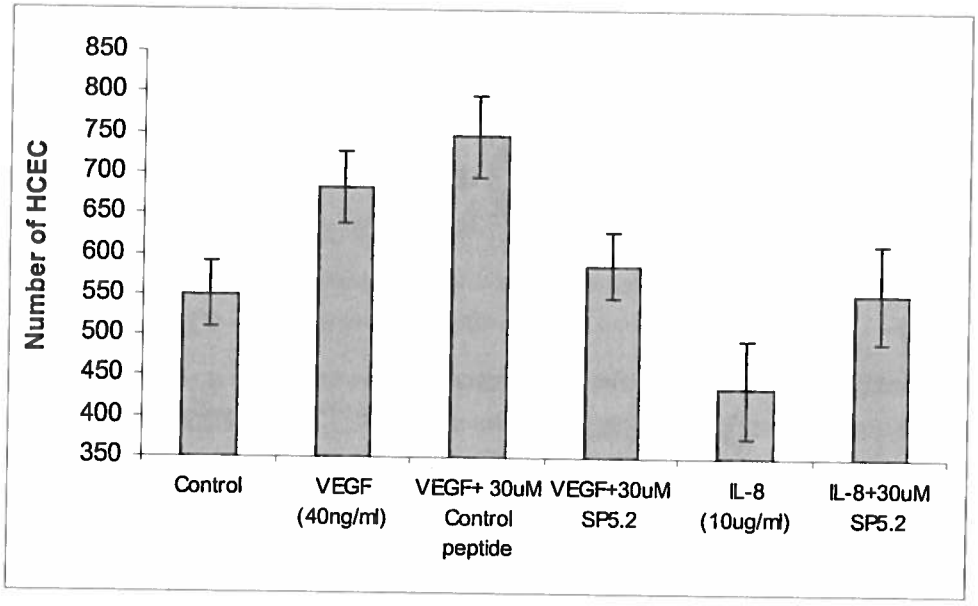


Figure 3.11 Inhibition of VEGF chemotaxis of HCEC by peptide SP5.2 peptide. Migration (chemotaxis) of calcein-AM labelled HCEC induced by several stimuli including $VEGF_{165}$ and IL-8 was tested in the absence or presence of SP5.2 peptide or control scrambled peptide using a ChemoTx#101-5 assembly. VEGF induced increased migration of HCEC across the filter that was significantly reduced by SP5.2 and not the control or IL-8.

3.9 SP5.2-β-gal fusion protein binds to Flt-1 receptor

To explore a possibility of using SP5.2 sequence as a targeting moiety in a recombinant protein form, a fusion protein, SP5.2-β-gal, expressing the peptide sequence on the N-terminus of β-galactosidase and $(His)_6$ on the C-teminus was produced and purified by His-tag affinity chromatography at Supratek Pharma, was assayed for binding to Flt-1. The affinity and selectivity of the recombinant protein SP5.2-ß-gal, for Flt-1 receptor was determined by ELISA assay using recombinant ß-gal as control. The results produced in Figure 3.12 showed that SP5.2-□-gal fusion protein not only binds to Flt-1 receptor with an apparent K_D of 200nM, but had about 200-fold higher binding potency compared to the synthetic peptide SP5.2. Furthermore, the recombinant SP5.2-ß-gal did not show any significant interaction with the KDR receptor (Figure 3.13), indicating that the recombinant protein keeps its specificity as well. The results suggest that SP5.2 can potentially and effectively be used as a targeting element for genetically constructed therapeutic proteins to be delivered into specific Flt-1-rich compartments such as tumour endothelium, inflammation sites or damaged blood brain barrier sites.

3.10 SP5.2-Peroxidase conjugated peptide binds to Flt-1 receptor

The affinity of the SP5.2 peptide fused to a therapeutic entity was also tested. To this end, SP5.2 peptide that was chemically conjugated with a therapeutic protein with peroxidase activity produced by N-succinimidyl 3-(2-pyridylthio)propionate (SPDP) as a heterobifunctional cross-linking agent at Supratek Pharma was used in binding assays. The analysis of the resulted conjugated product has been shown to contain between 3 and 5 molecules of SP5.2 peptide per molecule of peroxidase. The Flt-1 binding activity of the conjugate was then analyzed by evaluating the peroxidase activity on the substrate ABTS. Peroxidase conjugated with Cystein (Cys-peroxidase conjugate) was used as a negative control. SP5.2 peroxidase conjugate demonstrated concentration-dependant specific binding to rhFlt-1, while no detectable binding was observed with the Cys-peroxidase conjugate (Figure 3.14). However, the half-maximum binding concentration of 0.1μM was 400-fold lower compared to that of the non-conjugated SP5.2 peptide (Figure 3.4A).

The results clearly suggest that SP5.2 can potentially be linked to therapeutic entities, such as proteins, and perhaps to small drug molecules, as well as liposomes, micelles,

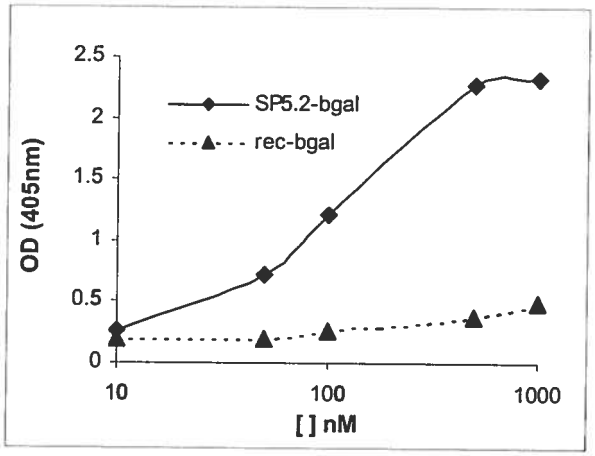


Figure 3.12 Binding affinity of SP5.2-β-gal to Flt-1 receptor.
Using ELISA assay, different concentrations of SP5.2-β-gal was used as well as recombinant β-gal which is used as a control. SP5.2-β-gal binds to Flt-1 receptor with a K_d of 200nM, which is 200-fold higher binding potency compared to synthetic fluorescein labelled SP5.2.

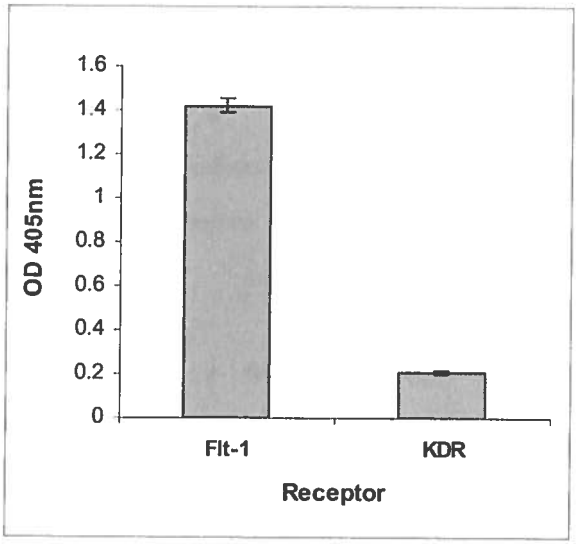


Figure 3.13 Binding specificity of SP5.2-β-gal to Flt-1 receptor.
Using ELISA assay with two different receptors, Flt-1 and KDR, SP5.2-β-gal showed no significant interaction to KDR receptor at a concentration of 50μM, which is 7 fold lower than Flt-1.

nanoparticles and other particulate delivery vehicles.

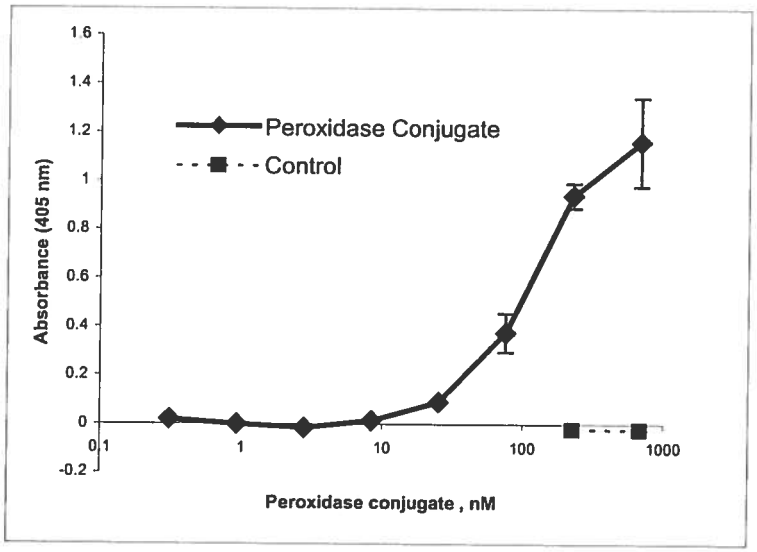


Figure 3.14 Binding of SP5.2 peroxidase conjugate to Flt-1 receptor.
SP5.2 was chemically conjugated with peroxidase by using SPDP. The Flt-1 binding activity of the conjugate was analyzed by evaluating the peroxidase activity using its substrate ABTS. Peroxidase conjugated to cysteine was used as a negative control. SP5.2 peroxidase conjugate demonstrated concentration dependant specific binding to Flt-1, while no detectable binding was observed in the case of the negative control.

CHAPTER 4: Discussion

Previous studies have shown that anti-angiogenic therapy is a promising approach for the treatment of cancer (Bisacchi et *al.*, 2003; Turetschek et *al.*, 2003; Sheng et *al.*, 2003; Shinkaruk et *al.*, 2003). Vascular Endothelial growth factor (VEGF) and its receptors are the focus of intense interest because of their role in several biological processes that involve angiogenesis. VEGF has also been shown to play an important role in development of vascular leakiness, blood-brain barrier disruption, brain edema in brain tumors (Puduvalli et *al.*, 2000) and cerebral ischemia (Schoch et *al.*, 2002). VEGF activities are mediated through its binding to two high affinity receptors Flt-1 and KDR. VEGF-Flt-1 receptor system plays an important role in the stimulation of angiogenesis including tumor angiogenesis. This makes Flt-1 an important target for anti-angiogenic drugs. Indeed, blocking the interaction between VEGF and Flt-1 has been shown to promote the regression of murine and human tumors (Broggini et *al.*, 2003; Hiratsuka et *al.*, 2002).

Phage display is a potential enabling technology in drug development that promises to have benefit in the development of therapeutics, targeting many disorders including cancer. It is also useful in screening for novel high affinity ligands and their receptors. We report here the identification of a novel VEGF antagonist peptide, SP5.2, by screening of a phage-displayed peptide library.

Phage-expressed peptide, capable of binding to Flt-1 receptor, was identified after five rounds of biopanning against Flt-1 immobilized on magnetic particles using a 16mer peptide phage display library. At the end of the selection, five different types of phage clones were identified as being capable of binding to immobilized Flt-1 as established by the ELISA assay. The most efficient of the 5 clones, phage V5.2 expressing the peptide SP5.2 (NGYEIEWYSWVTHGMY) was chosen for further analysis, and was chemically synthesized. Chemically synthesized SP5.2 peptide was found to bind selectively to Flt-1, and not to other endothelial receptors such as ICAM-1, Flk-1 and KDR. However there was a significant and selective cross interaction with the murine analog of Flt-1. To identify functionally important amino acids in the SP5.2 sequence, alanine scan was used and the binding potencies of the obtained mutated phages were analyzed by ELISA. Based on these results a critical structural motif of the SP5.2 peptide was identified as N X X E I E X Y X

W X X X X X Y (X represents amino acid residues that are not involved in the Flt-1 binding).

Further, *in vitro* cell-based experiments clearly demonstrated that SP5.2 interacts with Flt-1 positive cells such as HUVEC and HCEC, and inhibits a variety of VEGF mediated events including early angiogenesis, proliferation, vascular permeability and chemotaxis. All the inhibitory effects observed were sequence specific, and selective with respect to VEGF compared to other growth factors. In the experiments with bFGF, instead of VEGF, peptide SP5.2 did not exhibit any inhibitory activities. Peptide SP5.2 was also shown to antagonize PIGF, which is known to interact with Flt-1 receptor, similar to VEGF, and to share more than 50% homology to VEGF (Thomas K, 1996).

Most of the *in vitro* studies aiming to establish functional role of the Flt-1 and KDR receptors have led to a general agreement that the high affinity receptor Flt-1 is mainly implicated in the early stages of VEGF induced angiogenesis, such as, modulation of cell motility and adhesion in the endothelial tissue, while KDR, a low affinity receptor, is more involved later in endothelial proliferative response (Matsumoto et *al.*, 2002; Mayr-Wohlfart et *al.*, 2002; Hong et *al.*, 2001). However, it has recently been demonstrated that Flt-1 and KDR can form heterodimers in which Flt-1 may participate in the regulation of proliferative response to VEGF (Shinkaruk et *al.*, 2003). Interestingly, in our study, we have observed that SP5.2, while being a selective inhibitor of Flt-1 mediated events such as capillary like tube formation, vascular permeability and chemotaxis, also suppress VEGF mediated proliferation that is mainly attributed to KDR function. This is in agreement with the results from a recent study that demonstrated that Flt-1 and KDR could form heterodimers in which Flt-1 may participate in the regulation of proliferative response to VEGF (Shinkaruk et *al.*, 2003). Therefore, the effects seen, most likely, have been through its interaction with KDR upon heterodimer formation (Shinkaruk et *al.*, 2003). However, further studies are required for more detailed mechanistic understanding of this anti-proliferative effect of SP5.2.

The synthetic form of SP5.2 when presented as a single molecule demonstrated a micromolar potency in a variety of assays in the present study. It is well known that peptides identified during phage display library screening often show a much lower

potencies, both *in vitro* and *in vivo*, when synthesized as individual compounds (Ferrieres et *al.*, 2000; Balass et *al.*, 1993). The two main reasons suggested for this effect are the following: (i): small synthetic peptides most often do not have sufficient conformational rigidity and integrity that in the case of phage display is provided by its filament protein in which the peptide in inserted; (ii): The phage particles display multiple copies of the inserted peptides (from 3 to 5 peptide in the case of P3 protein based libraries), which provides the phage with a higher avidity compared to the synthetic peptide due to cooperative effect of multi-site interaction with the target. One potential way to restore, or even increase, the reduced activity of a ligand is to attach its multiple copies to a structurally rigid moiety such as a protein, liposome, polymer, etc. (Kuehne et *al.*, 2001; Li et *al.*, 2003; Lee et *al.*, 2003). To explore a possibility of increasing potency of SP5.2, two derivatives of this molecule were produced. The first one was a recombinant protein, fusion of SP5.2 with β-gal, in which SP5.2 was present as a single copy at the N-terminus of the enzyme that was used as a carrier protein. The second derivative, which was constructed at Supratek, was a chemical conjugate of SP5.2 to a protein with therapeutic activity, peroxidase, with three to five copies of the peptide. The resulted products, not only exhibited the selective affinity of SP5.2, but also showed considerably higher binding potencies with Flt-1 compared to the original synthetic peptide. The apparent dissociation constants established in the Flt-1 binding assay were 200nM (200-fold lower compared to SP5.2) for the fusion with a single copy of the peptide, but 100nM (400-fold higher compared to the peptide) in the case of the chemically linked polyvalent product. These results suggest that SP5.2 can potentially be used as an efficient targeting component either with its own beneficial anti-angiogenic effects as well as in combination with other therapeutic protein, under exploration in the on going studies in our laboratories. These include combining SP5.2 with therapeutic proteins such as cytokines in a targeted genetic construct, as well as combining this peptide with polymeric micelles loaded with a cytotoxic drug.

Conclusion

The work described in this thesis identified a novel peptide, SP5.2, with potential anti-angiogenic activity. This peptide was selected against the extracellular domain of Flt-1 receptor using a phage display library screening. Several studies were performed to characterize the binding affinity of this peptide to Flt-1 receptor as well as its ability to selectively inhibit several VEGF mediated events in cultured endothelial cells. In addition, the potential of using SP5.2 as a targeting agent, in combination with other therapeutic or diagnostic biomolecules for targeted or functionalised delivery, was also explored by a synthetic and a genetically constructed recombinant conjugates of the peptide in the reporter proteins, β-galactosidase and peroxidase.

In summary, this work has clearly demonstrated that the newly discovered peptide SP5.2 is an effective Flt-1 antagonist. To our knowledge this is the first report on the identification of an Flt-1 antagonist peptide. We believe that this molecule and its derivatives may have utility in clinical applications that might include cancer biotherapy and other VEGF related diseases.

References

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Annex 1

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