VASCULAR ENDOTHELIAL GROWTH FACTOR'S ANGIOGENIC ROLE IN TUMOR GROWTH AND METASTASIS

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Abstract

Angiogenesis and vasculogenesis are two very important processes in the development and maintenance of mammalian health. All structures of the body (human or animal) need certain essential elements in order to live thrive and maintain. The angiogenic role is to supply and support tissue with ample vasculature, thus providing a route of access for the transportation of essential nutrients and the removal of waste in a sustained fashion. Just like normal tissue, tumorogenic tissue is no exception; neoplastic tissue has the same nutritional requirements which must be supported via vascularization.

Vascular endothelial growth factor (VEGF) has been shown to be a key mediating factor in the underlying cascade of chemical events leading to angiogenesis, which makes it a very important precursor molecule for early neoplasia detection. The overall purpose of this study was to establish circulatory baseline VEGF levels in healthy dog models. Baseline levels of VEGF in plasma will aid as a model in detection, comparison and evaluating of disease progression in sled dogs.

There were significant differences between male and female dogs and exercising males and exercising females. A significant factor affecting baseline levels was gender. In addition there is some data which suggest that breed may play a role in baseline VEGF levels.

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AIP	Angiogenic Inhibitory Protein
ANG-1	Angiopoietin Receptor -1
ANG-2	Angiopoietin Receptor-2
ANG-3	Angiopoietin Receptor-3
ASM	Angiogenic Stimulatory Molecule
AT1	Angiostatin Receptor
CAM	Cell Adhesion Molecule
ChMA	Chick Embryo Chorioallantoic Membrane Assay
CMA	Corneal Micropocket Assay
EC	Endothelial Cell
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
ETB	Endothelin Receptor
aFGF	Acidic Fibroblast Growth Factor
bFGF	Basic Fibroblast Growth Factor
Flt-1	Fetal Liver Transferase Receptor 1
Flt-4	Fetal Liver Transferase Receptor 4
Flk-1	Fetal Liver Kinase Receptor 1

FN	Fibronectin
FNR	Female Non-Runner
FR	Female Runner
GCSF	Granulocyte Colony-Stimulating Factor
GF	Growth Factor
HCPA	Hamster Cheek Pouch Assay
HGF/SF	Hepatocyte Growth Factor/ Scatter Factor
Ig	Immunoglobulin
KDR	Kinase Domain Receptor
MMP	Matrix Metalloproteinase
MNR	Male Non-Runner
MP	Metalloproteinase
MR	Male Runner
NR	Non-Runner
PD-ECGF	Platelet Derived-Endothelial Cell Growth Factor
PDGF	Platelet Derived Growth Factor
R	Runner
SCRAM	Surface Cell Receptor Amobile Molecule
sFlt-1	Soluble Fetal Liver Transferase Receptor 1
S-K	Split- Kinase (Domain)
STM	Signal Transduction Molecule
TGF	Transforming Growth Factor

TKR	Tyrosine Kinase Receptor
TM	Transmembrane
αTNF	Alpha Tumor Necrosis Factor
uPA	Urokinase-Type Plasminogen Activator
VEGF	Vascular Endothelial Growth Factor
VEGFR-1	Vascular Endothelial Growth Factor Receptor 1
VEGFR-2	Vascular Endothelial Growth Factor Receptor 2
VEGFR-3	Vascular Endothelial Growth Factor Receptor 3
VPF	Vascular Permeability Factor

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As with any endeavor of significance, this was a collaborative effort which would not be complete without the mention of those individuals who have made the path I travel less arduous. Foremost, I am very thankful for the support of Dr. Larry Duffy. He has been a constant source of encouragement for me through academic advisement for both my undergraduate and graduate degrees. In addition, and perhaps most importantly he has been an avid supporter of my future academic goals in medical school, and for that I am most appreciative to have had Dr. Duffy as an advisor, professor and friend.

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The department of Chemistry and Biochemistry has been a home for me for the past six years, nurturing me academically, supporting me via gainful employment and introduced

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Doug passed away just days ago and his passing is a shock to all who knew him, but I would like to share a part of Doug that stays clear in my head everyday. Doug once told me that passionate people always find a way. He said that if you need something done and you think you do not have time or the energy to do it, find the busiest person you know who has no time in their schedule to spare and ask them for help, and they will find a way to help. Doug always found time for myself and others.

DEDICATED IN LOVING MEMORY TO DOUGLAS SCHAMEL (1949-2005)

Chapter 1

INTRODUCTION

Angiogenesis and vasculogenesis are two processes important to developing mammals. These two processes are mediating factors within the embryonic cardiovascular system, which is the first organ to undertake morphological development (Risau and Flamme, 1995). Vasculogenesis is the term used to describe the vascular development during embryogenesis. During vasculogenesis, vascular plexus formation (capillary bed development) arises from angioblasts of progenitor endothelial cells (ECs), which differentiate into mesodermal embryonic tissue. Following differentiation, angiogenic factors activate and refine and/or remodel the unmodulated vasculature which is, at this stage, just budding and sprouting tubular endothelial rolls formed via secondary angiogenesis. The vascular modeling forms these rolls into fully developed and functional vessels which increase in size over time (Risau, 1997). Once vascular maturation is achieved, smooth muscle cells, called pericytes, migrate and adhere to the outer basal membrane of the matured vessel. The pericytes serve as stabilizers for the developed vasculature.

The developing angiogenic vasculature responds to perturbation of the growing embryo's demand for oxygen, glucose and other nutrients. Angiogenesis in matured or adult vasculature also responds to other sources of perturbation, specifically the metabolic needs of tissue and organs, including perturbations of hypoxia and ischemia. Excessive or insufficient angiogenesis (unregulated) plays a major role in the pathogenesis of many diseases: rheumatoid arthritis, blindness (ocular disease and hypertrophy), complication with AIDS patients, stroke, heart disease, ulcers scleroderma and infertility in women (Folkman, 1995; Griffioen and Molema, 2000; Griffioen et al., 1998; Molema and Griffioen, 1998). Vasculogenesis and angiogenesis both are biological processes which are predicated on many different chemical factors: cytokines, cell adhesion molecules (CAMs), components of the extracellular matrix (ECM), surface cell receptors, various proteins and angiogenic/mitogenic growth factors (Risau, 1997).

1.1 Proteins and Their Role in Cellular Signaling

Proteins are in many respects the main chemical mediators of biological systems. The body uses protein for storage, structural support, enzymatic catalyzation of both anabolic and catabolic metabolism, cell to cell signaling, muscle movement, transportation and elimination of cellular waste products and immunological defense of invading foreign substances. The importance of proteins and their function in biological systems is apparent from the Greek derivation of protein, *proteios*, meaning "first place" (Campbell

et al., 1999). A brief overview of various proteins and the roles they play in biological systems is displayed in Table 1.1.

Table 1.1. Protein functions within biological systems and their respective roles (Adapted from Campbell et al..1999).

TYPE OF PROTEIN	BIOLOGICAL FUNCTION	EXAMPLE
Structural	Support: cellular and noncellular	Collagen and elastin provide structural framework for connective tissue. Keratin provides the framework for hair and skin.
Storage	Amino acid storage	Ovalbumin is an amino acid source used for developing embryos. Casein in breast milk is the major source of protein utilized by infant mammals.
Transport	Transportation of chemicals	Hemoglobin in the blood transports oxygen form the lungs to various parts of the body.
Hormonal	Coordination of systemic activities.	Insulin secreted from the pancreas regulates the systemic concentration of glucose in the blood.
Cell Signaling	Cell to cell chemical communication.	Neurotransmitters bind to receptors of nearby nerve cells which propagates stimuli along to other regions of the body. Acetylcholine is a common neurotransmitter.
Contractile	Movement	Actin and myosin are contractile proteins which are responsible for the movement of muscles. Other contractile proteins are responsible for the movement of cilia and flagella.
Immunoglobins	Defense against foreign substances	Antibodies are responsible for the main defense against foreign bacteria and viruses.
Enzymes	Selective acceleration of chemical Reactions.	Urease selectively catalyzes the breakdown of urea for excretion in the urine.

Winner of the Nobel Prize in 1971, Earl W. Sutherland was the pioneer of our basic understanding of cellular events which most modern cellular signaling research is based. At Vanderbilt University, Sutherland and his colleagues studied the cellular elucidation of how epinephrine depolymerizes glycogen stores within hepatocytes and skeletal muscles for the release of glucose to be used for energy. For simplicity, Sutherland characterized cellular signaling events into three basic events: (1) cellular signal reception, (2) signal transduction or propagation and (3) the cellular response to the signal reception.

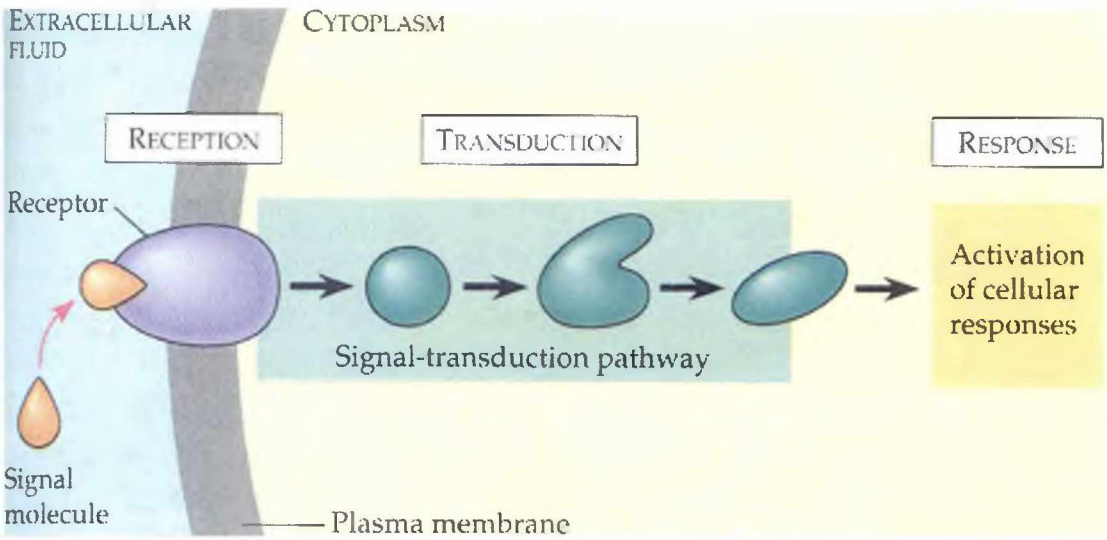

Figure 1.1. Overview of basic cellular events involved in cellular signaling. Cellular events can be broken down into three major events: reception, transduction and cellular response. Reception involves the binding of a signal molecule that causes a conformational change in the adhesion surface cell receptor. The conformational change causes a cascade of chemical interactions (transduction) involving numerous molecules; each molecule brings about a conformational change in the next molecule. The last molecule in the pathway is the molecule which elicits or propagates the cellular response (Adapted from Campbell et al., 1999).

As previously described in Table 1.1, there are many areas within protein chemistry pertaining to biological systems. The remainder of this chapter will focus on cellular signaling and protein functions pertaining to neovascularization during embryogenesis and angiogenesis. These processes serve as a molecular framework for the understanding of normal and abnormal cellular mechanisms involving vasculature.

1.2 Angiogenesis

The term angiogenesis was first used in 1787 by a British surgeon, Dr. John Hunter (Encyclopedia Britannica, 2003 ed.), who was explaining the blood vessel growth in reindeer antlers. Angiogenesis is literally taken to mean the forming of new blood vessels (capillaries) or neovascularization. "Angi"- translated from Greeks meaning vessel and – "genesis" refers to the creation or forming of a given entity. Angiogenesis is a complex process involving many signaling pathways leading to the neovascularization or revascularization of specific targeted areas or tissue. It is at the tissue level that the role of angiogenesis is made known. All structures of the body (human or animal) have to have certain essential elements in order to live, thrive and maintain. The angiogenic role is to supply and support the vasculature in order to provide a route of access for the transportation of essential elements and the removal of waste in a sustained fashion. Without a properly functioning vasculature, tissue is subjected to various insults such as hypoxia and ischemia, both of which lead to a cascade of deleterious effects on the surrounding milieu. This phenomenon is apparent in the study of embryology.

Angiogenesis in the developing embryo and fetus is referred to as vasculogenesis (Risau and Famme, 1995), the forming of the body's vascular system. The internal infrastructure is essential in vasculogenesis in order for the developing embryo and fetus to grow and develop while receiving the proper balance of "nutrition" brought in via the vascular system. Embryonic waste is filtered out through the same vascular system.

Angiogenesis is an essential part of a developing embryo; however, this does not hold true in adults. Just like a developing embryo, adults need the same network of vasculature to bring in oxygen, glucose, nutrients and to remove waste products. The difference is that the adult vascular system has reached maturation and is, thus, functioning at a normal level without the added neovascularization that angiogenesis employs. As adults, angiogenesis is only initiated under certain instances of insults or threats to the normoxic functioning of the body, such as with insults of hypoxia, ischemia, neoplasia or focal tissue insult (Folkman, 1995; Breier, 2000). All insults are inducers of angiogenesis and follow the same or similar signal transductive cascades of activation.

The underlying processes involved with the formation of neovascularization or vasculogenesis occurs in a well orchestrated cascade of events initiated at the molecular level and terminated at the macroscopic level. Diseased (tumorogenic) or injured tissue (laceration) release various angiogenic and mitogenic proteins as a direct response to the insult. The same angiogenic and mitogenic proteins are released during embryonic

development *in utero*. These proteins, collectively called growth factors, diffuse out from their source of origin into the surrounding tissue. Once diffused, these growth factors migrate and recognize surface cell receptor amobile molecules (SCRAMs), which are located on the outer surface of endothelial cells (ECs). The ECs line the outer portion of nearby preexisting vasculature: blood vessels, capillary beds, veins and arteries (dependent upon location). The binding of growth factors to their respective SCRAM sites on ECs is a regio-specific process. As a result of the growth factor and SCRAM synergism, each growth factor recognizes only one or two specific SCRAMs. Once binding has occurred between the growth factor and the SCRAM, the EC becomes activated. The activation of ECs is the result of a signal transductive process that relays from the ECs surface to the nucleus, and as a result, the ECs cellular machinery induces production of various enzymes called metalloproteinases (Brown and Giavazzi, 1995; Brown and Hudlicka, 2003). Metalloproteinases are a select group of protease enzymes which attack and degrade the basel basement membrane of ECs. This chemical degradation includes the digestion of specialized cells called pericytes (Folkman and Shing 1992), which line the outer layer of ECs. Resting nonproliforative ECs are tightly controlled in a state of quiescent. In other words, pericytes thwart additional EC proliferation (Antonelli-Orlidge et al., 1989; Denekamp and Hill, 1991). The degradation results in small tears or holes in the vessel where the endothelial lining has been compromised.

In addition to signaling the production of the metalloproteinase, the nucleus has received signals for the ECs to proliferate. The now active proliferation of ECs leads to the sprouting and intussusceptive buds of ECs from the degraded holes in the parent vessel (Figure 1.2; Risau, 1997). Specialized proteases called matrix metalloproteinase or MMPs are produced to dissolve intruding tissue in the path of sprouting angiogenic buds. The buds of ECs start to compile into stacks or rolls forming small vesicle tubes. The tissue matrix which has been dissolved by MMPs starts to form back around the neovascular tubes. The newly formed tube vesicles form loops and connect with other neovascular tubes, thus establishing a circulatory pathway. The newly formed vasculature is now anchored and stabilized by the smooth muscle cell pericytes. These cells again provide support and protection from further degradation by MMPs. The end result is a neovascular pathway through which blood, and consequently, oxygen, glucose and nutrients can flow; these events are schematically summarized in figure 1.2.

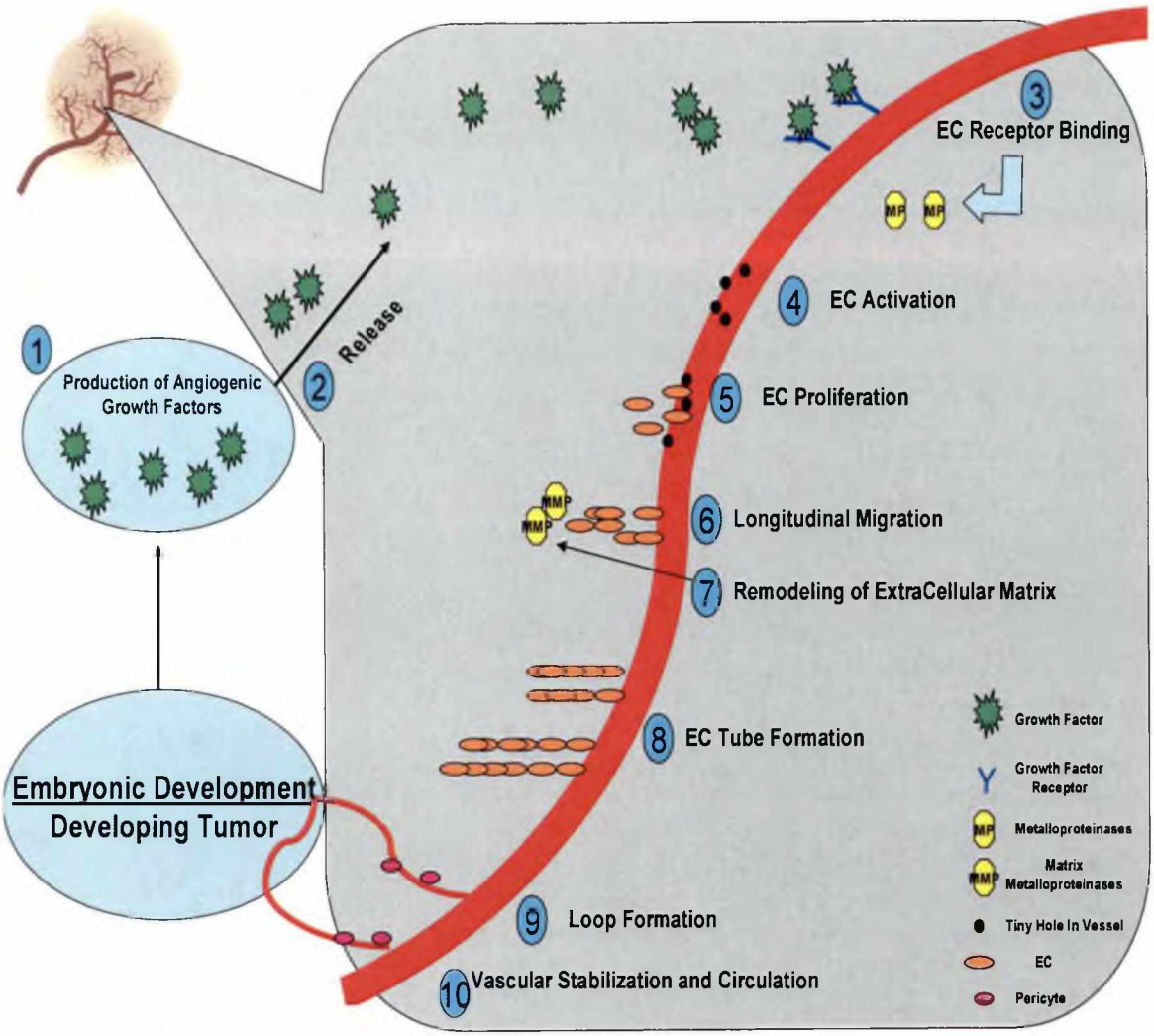

Figure 1.2. Generalized cascade of events leading to the formation of new vasculature. Normal embryonic development and abnormal tumor development both utilize similar cascading events in the development and maturation of their supporting vasculature. The events of development are as follow: (1) Release of angiogenic proteins or growth factors (GFs) which diffuse out into the surrounding tissue. (2) The GFs are released where they recognized certain GF receptors (SCRAMs). (3) Once GFs bind to their respective receptors, they activate ECs, which cause intracellular signaling from the cell's surface to the nucleus. The signaling initiates production of various enzymes called metalloproteinases (MPs). (4) The MPs degrade the outer basement membrane of existing blood vessels, thus leaving tiny holes in the vessel walls. (5) Mitogenic ECs begin to

proliferate and dissolve through the tiny holes made by the MPs, where they migrate forward. (6) The sprouting vesicle of ECs is pulled forward via specialized enzymes or integrins (avb3 and avb5). (7) Remodeling of the extra cellular matrix is mediated by matrix metalloproteinases (MMPs). The MMPs degrade the extra cellular matrix so the newly forming vesicles have room to grow. The matrix quickly adheres back around the forming vesicle after vesicle extension. (8) ECs role and stack on top of one another and start to form tubes. (9) Newly formed tubes hook up and grow into other tubes near by. They form a loop with one another through which blood can begin to circulate. (10) The newly formed vasculature is now stabilized by specialized muscle cells called pericytes.

There are many stimulators which initiate the events of angiogenesis. Most of these stimulators are specific proteins called growth factors which were referred to previously. The idea of specific proteins mediating the events of angiogenic growth was first proposed by Judah Folkman back in 1971 (Folkman, 1971). Folkman proposed that if specific proteins (GFs) existed, then those proteins could help in the elucidation of angiogenic abnormalities. It was not until 1984 that the first of these specific angiogenic proteins was isolated and characterized (Shing, 1988; Shing and Klagsbrun, 1984). Since the first characterization, many angiogenic genes and gene products have been isolated, purified and produced via recombinant DNA methodology. This has enabled a plethora of new data characterizing many new molecular angiogenic stimuli. These angiogenic stimulatory molecules (ASM) can be divided into seven major groups of classification: (1) growth factors, (2) proteases, (3) trace elements or metals, (4) oncogenes, (5) cytokines, (6) molecules involved in cellular signal transduction (STMs), and (7) endogenous angiogenic inducers (Table 1.2).

Table 1.2. Endogenous Growth Factors And Angiogenic Stimulators (Adapted from Brem, 1999).

Growth Factors	Angiogenin Angiotropin Epidermal growth factor (EGF) Fibroblast growth factor: basic and acidic (bFGF and aFGF) Granulocyte colony-stimulating factor (GCSF) Hepatocyte growth factor/ scatter factor (HGF/SF) Platelet-derived growth factor (PDGF) Tumor necrosis factor alpha (αTNF) Vascular Endothelial growth factor (VEGF)
Proteases	Cathepsin Gelatinase A, B Stromelysin Urokinase-type plasminogen activator (uPA)
Trace Element: Metal	Copper
Oncogenes	c-myc ras c-src v-raf c-jun
Signal Transductive Enzymes	Thymidine phosphorylase Farnesyl transferase Geranylgeranyl transferase
Cytokines	Interleukin-1 Inerleukin-6 Interleukin-8
Endogenous Angiogenic Inducers	Alpha v Beta 3 integrin Angiopoietin-1 Angiostatin II (AT1 receptor) Endothelin (ETB receptor) Erythropoietin Hypoxia and Ischemia (perturbation) Nitric oxide synthase Platelet-activating factor Prostaglandin E Thrombopoietin

All of the previously mentioned categories or angiogenic stimulators (Table 1.2) are currently under study at numerous laboratories (Brem, 1999). Among these categories, growth factors seem to be the most popular due to their dual role of embryonic angiogenesis and angiogenic malignancies. The most widely studied growth factors are: acidic and basic fibroblast growth factor (aFGF and bFGF), vascular endothelial growth factor (VEGF) which is sometimes called vascular permeability factor (VPF), platelet derived-endothelial cell growth factor (PD-ECGF), alpha and beta transforming growth factor (αTGF and βTGF), angiogenin, and alpha tumor necrosis factor (αTNF). A detailed list of these polypeptides and their respective functions is listed in Table 1.3.

Table 1.3. Angiogenic Polypeptides: Structure and Biological Function (Adapted from Y. Shing and J. Folkman, 1992).

Growth Factors	MW	EC Mitogenicity	Angiogenic Biological Activities and Functions
bFGF aFGF	18kDa 16.4kDa	Yes Yes	• Mitogenicity found in many different cell types (Burgess and Maciag 1986; Folkman and Klagsbrun, 1987; Gospodarowicz, 1990; Klagsbrun and D'Amore, 1991; Rifkin and Moscatelli, 1989; Thomas, 1987). • Binds to heparan sulfate proteoglycan (Vlodavsky et al., 1987; Baird and Ling, 1987) and copper (Shing, 1988). • Stimulates EC migration and tube formation (Montesano et al., 1986). • Stimulates protease production and plasminogen activator (Moscatelli et al., 1986; Presta et al., 1986). • Induces embryogenesis (Kimmelman and Kirschner, 1987; Slack et al., 1987).
VEGF/VPF	45kDa	Yes	• Proliferatory activation which is highly specific for ECs (Ferrara and Henzel, 1989). • Induces secretory proteins (Conn et al, 1990; Myoken et al., 1991; Rosenthal et al., 1990) and increases vascular permeability (Connolly et al., 1989). • Mediates plasminogen activation and inhibition in ECs (Pepper et al., 1991). • Structurally homologous to PDGF (Conn et al., 1990; Keck et al., 1989; Tischer et al., 1991).
PD-ECGF	45kDa	Yes	• Stimulates DNA synthesis in ECs and induces chemotaxis (Ishikawa et al., 1989).

Table 1.3. Continued

α-TGF	5.5kDa	Yes	• Switches normoxic ECs to transformed phenotype (Derynck, 1990). • Recognizes and binds to EGF receptor (Derynck, 1990).
Angiogenin	14.1kDa	None established	• EC formation of diacylglycerol (Bicknell and Vallee, 1988) and secretion of prostacylin (Bicknell and Vallee, 1989) via phospholipase C and phospholipase A2 activation, respectively. • Ribonucleolytic activity which is essential for neovascularization (Shariro et al., 1986; St. Clair et al., 1987).
α-TGF	25kDa	No	• Binds to copper (Roberts and Sporn, 1990). • Stimulation of extracellular matrix production (Roberts and Sporn, 1990). • Monocytic chemotatability (Roberts and Sporn, 1990).
β-TNF	55kDa	No	• ECs stimulation of bFGF and its secretion (Okmaura et al., 1991). • Monocytic chemotatability (Beutler and Cerami, 1986). • Macrophage activation (Beutler and Cerami, 1986).

All polypeptide growth factors and angiogenic stimulator data were determined via chick embryo choriallantoic membrane assay (ChMA), corneal micropocket assay (CMA) and/or hamster cheek pouch assay (HCPA). For further review and assay protocols see the following references: bFGF (Montesano et al., 1986; Shing et al., 1985), aFGF (Lobb et al., 1985; Thomas et al., 1985), VEGF/VPF (Connolly et al., 1989; Kimmelman and Kirschner, 1987; Leung et al., 1989), PD-ECGF (Ishikawa et al., 1989), α-TGF

(Schreiber et al., 1986), Angiogenin (Fett et al., 1985), β-TGF (Roberts et al., 1990), α-TNF (Frater-Schroder et al., 1987; Leibovich et al., 1987).

1.3 Vascular Endothelial Growth Factor (VEGF/VPF)

Vascular endothelial growth factor (VEGF) (Ferrara and Henzel, 1989), also referred to as vascular permeability factor (VPF) (Senger et al., 1983) or vasculotropin (Plouet et al., 1989), has very specific properties with respect to ECs. VEGF is a heparin-binding polypeptide glycoprotein with mitogenic and angiogenic ECs specificity. In addition, VEGF has an enhanced ability for vascular permeability specific for ECs. VEGF recognizes and binds to specific SCRAMs located on the outer membrane of ECs. VEGF is found throughout the body in many different cell types. In normal tissue VEGF has been found in activated macrophages (Fava et al., 1994), keratinocytes (Brown et al., 1992a), renal glomerular visceral epithelium (Brown et al., 1992b; Iijima et al., 1993), smooth muscle cells (Ferrara et al., 1991), hepatocytes (Monacci et al., 1993), mesangial cells (Brown et al., 1992b; Iijima et al., 1993), leydig cells (Shweiki et al., 1993), embryonic fibroblast, bronchial and choroid plexus epithelium (Breier et al., 1992; Pertovaara et al., 1994). In addition, VEGF has been shown to play important roles in varying types of tumors (Connolly, 1991; Ferrara et al., 1992; Neufeld et al., 1992; Schott and Morrow., 1993).

1.3.1 Vascular Endothelial Growth Factor: Structure

Structurally VEGF is a homodimeric polypeptide which occurs in at least five different isoforms in humans (Breier, 2000; Veikkola et al., 1999): $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ respectively (also resported as VEGF-A, VEGF-B, VEGF-C, VEGF-D and VEGF-E). All five isoforms are derived through alternate mRNA splicing variants of a single gene (Breier, 2000; Veikkola and Alitalo, 1999). Besides their amino acid sequence, these isoforms differ primarily in their heparin binding which may also affect their extracellular diffusion rates (Breier, 2000). $VEGF_{145}$ or VEGF-A is the founding member of the VEGF family of growth factors (Figure 1.3).

Figure 1.3. Structure of the dimeric form of $VEGF_{145}$/VEGF-A. Helices as shown in pink, beta sheets are shown in yellow/orange and loop regions are shown in white. Three dimensional analysis was conducted using RasMol® computer simulation.

VEGF shows structural and amino acid sequence homology with placental growth factor (PlGF) and transforming growth factor (TGF), which are growth factors having mitogenic and angiogenic activities. However, VEGF and PlGF are more closely related to one another as compared to TGF due to their ability to bind specific tyrosine kinase receptors (TKRs).

1.3.2 VEGF Tyrosine Kinase Receptors

As previously stated, angiogenic and mitogenic cellular activities are mediated by many different cellular molecules (Breier, 2000; Risau, 1997), and specifically growth factors and their cellular receptors (Risau, 1997). There is ample evidence that suggests that endothelial-specific tyrosine kinase receptors and their ligands mediate these complex biological processes (Breier, 2000; Neufeld et al., 1999). VEGF binds several TKRs: VEGF receptor one or fetal liver transferase one (VEGFR-1 or Flt-1), VEGF receptor two or fetal liver kinase one or kinase domain receptor (VEGFR-2 or Flk-1/KDR), VEGF receptor three or fetal liver transferase four (VEGFR-3 or Flt-4) and neuropilin (Figure 1.4). In addition to the VEGF signaling pathway, another signaling pathway has been found to be governed by angiopoietin and its family of receptors: Ang-1, Ang-2, Ang-3 and the Tie2 receptor (Figure 1.4).

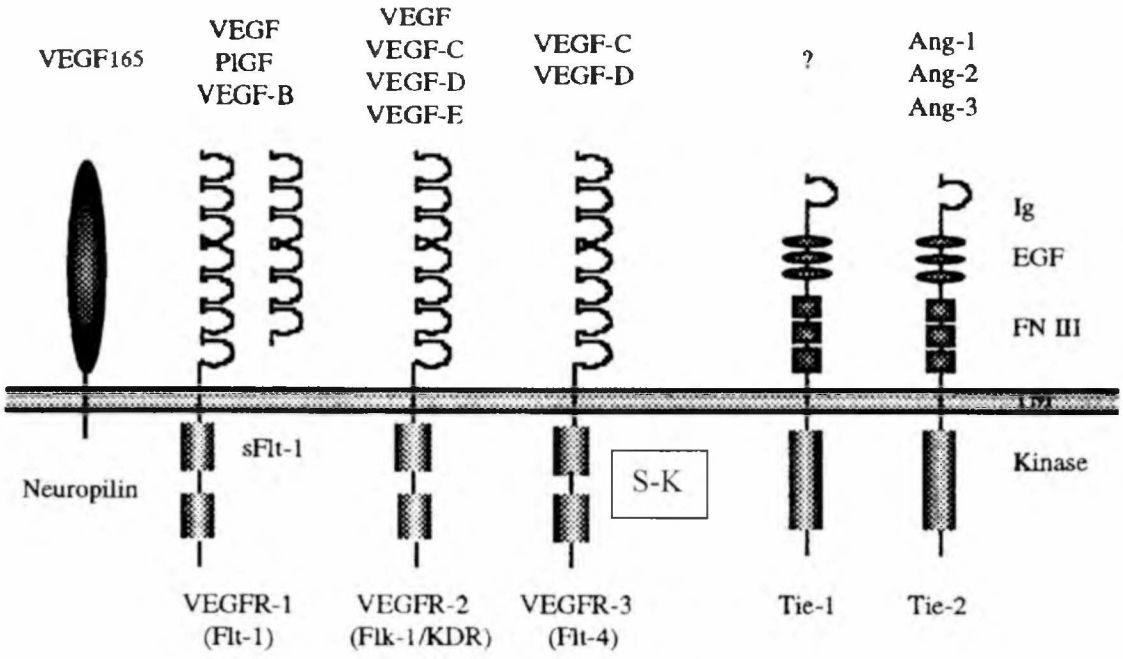

Figure 1.4. Receptors involved in angiogenic and mitogenic cascade activation, ECs signaling system (Adapted from Breier et al., 1997; Breier, 2000). The VEGF family of receptors are characterized by an extracellular immoglobulin-like domain (Ig) which makes these receptors soluble (sFlt-1) during laboratory extraction. The Ig domain is comprised of seven Ig loops exposed in the cytosol of the extracellular membrane. Separated only by the transmembrane (TM), is the split-kinase (S-K) domain embedded within the EC. In contrast to the VEGF receptors, the Angiopoietin receptors (Tie-1 and Tie-2, respectively) have only one Ig extracellular domain followed by three epidermal growth factor-like (EGF) repeats and three fibronectin-like domains (FN). Across the TM from these domain lies only a single kinase domain embedded in the EC. Neuropilin is not structurally related to either of the VEGF or the Angiopoietin receptors. Neuropilin seems to only have binding interactions with the $VEGF_{165}$ isomer (Breier et al., 1997; Breier, 2000).

Gene expression studies have been instrumental in the current understanding of the VEGF and angiopoietin EC receptor system during embryonic development. It appears that VEGF (VEGF-A or $VEGF_{145}$) is essential for the early development of vasculature

(Breier, 2000). Various studies, using knockout mice have revealed that the development of vasculature is lethally thwarted in mice lacking a single VEGF allele, ultimately leading to death around day ten (Carmeliet et al., 1996; Ferrara et al., 1996). These studies concluded that numerous embryonic processes are adversely affected; the formation of the dorsal aorta (large-vessel vasculature), vascular remodeling of the yolk sac and the secondary sprouting of capillaries form the dorsal aorta. In addition Carmeliet and Ferrara and their colleagues (1996) found that the inactivation of individual VEGF receptors resulted in embryonic death during mid gestation. They determined that these receptors act in a very distinct manner. Each receptor has a different function with respect to binding and biological activity, leading to phenotypic specificity. For example, the mice which were engineered to be deficient in the Flk-1 receptors showed a dramatic failure of vasculogenesis, EC differentiation and hematopoiesis (Shalaby et al., 1995). In contrast Fong et al. (1995) found that Flt-1 deficient mouse embryos sustained abnormally enlarged vasculature resulting from hyperstimulatory angioblast activity. Numerous studies indicate that the Flk-1 receptor is the main VEGF receptor governing signaling in ECs, and that the Flt-1 receptor's tyrosine kinase domain appears to be dispensable during embryonic development (Hiratsuka et al., 1998), suggesting its main function is ligand binding in ECs. VEGFR -2/Flk-2 has displayed evidence that it may play a role during axonal sprouting and neuronal development during vasculogenesis and also following hypoxic or ischemic insults (Jin et al., 2000; Sondell et al., 1999; Terman et al., 1992; Walternberger et al., 1994).

Angiopoietin receptors have similar functions analogous to VEGF with a few exceptions. Mice lacking the Tie2 receptor displayed underdeveloped vasculature leading to embryonic death (Sato et al., 1995; Suri et al., 1996). The Ang-1 and Tie2 deficient mice showed normal embryonic vascular development, indicating that the Ang-1/Tie2 signaling system is downstream with respect to the VEGF receptor system (Breier, 2000). The Ang-1/Tie2 receptor system, however, does show areas of malicious activity. For example, Koblizek and colleagues (1997) found that Ang-1/Tie2 knockout mice displayed no evidence of cardial trabeculation, yolk sac remodeling or sprouting of capillaries in neuronal tubes. Thus, suggesting that cardial trabecubation is the result of the Ang-1/Tie2 involvement in perivascular cardiac cellular recruitment. It has previously been shown that Ang-1 is involved with ECs formation of sprouts *in vitro* (Koblizek et al., 1997). VEGF receptors along with Ang-1 appear to act synergistically during angiogenesis, but this relationship does not hold true during vasculogenesis. The significance of the Tie-1 receptor is yet to be established.

1.4 VEGF's Role in Pathogenesis

As discussed in previous sections, angiogenesis is the sprouting and migration of capillary vessels and is the primary means through which the brain and other organs become vascularized. All of this takes place in embryogenesis and under certain normal conditions in adult tissue growth. For example, meticulously controlled angiogenesis takes place in wound healing and in the female reproductive cycle. In contrast to normal

angiogenesis, there are many malignancies which are mediated via angiogenesis and mitogenic proteins. Angiogenesis is characterized by the constant proliferation of ECs (Plate et al., 1994). Unregulated EC proliferation is a prominent characteristic of many disease processes, including but not limited to: proliferative retinopathy, rheumatoid arthritis, psoriasis, colon polyps, hemangiomas and breast cancer. Angiogenesis must be kept under tight biological control in order to avoid abnormal pathogenesis. Hyperangiogenic activity and hypoangiogenic activity can both lead to deleterious pathological events *in vivo* (Figure 1.5).

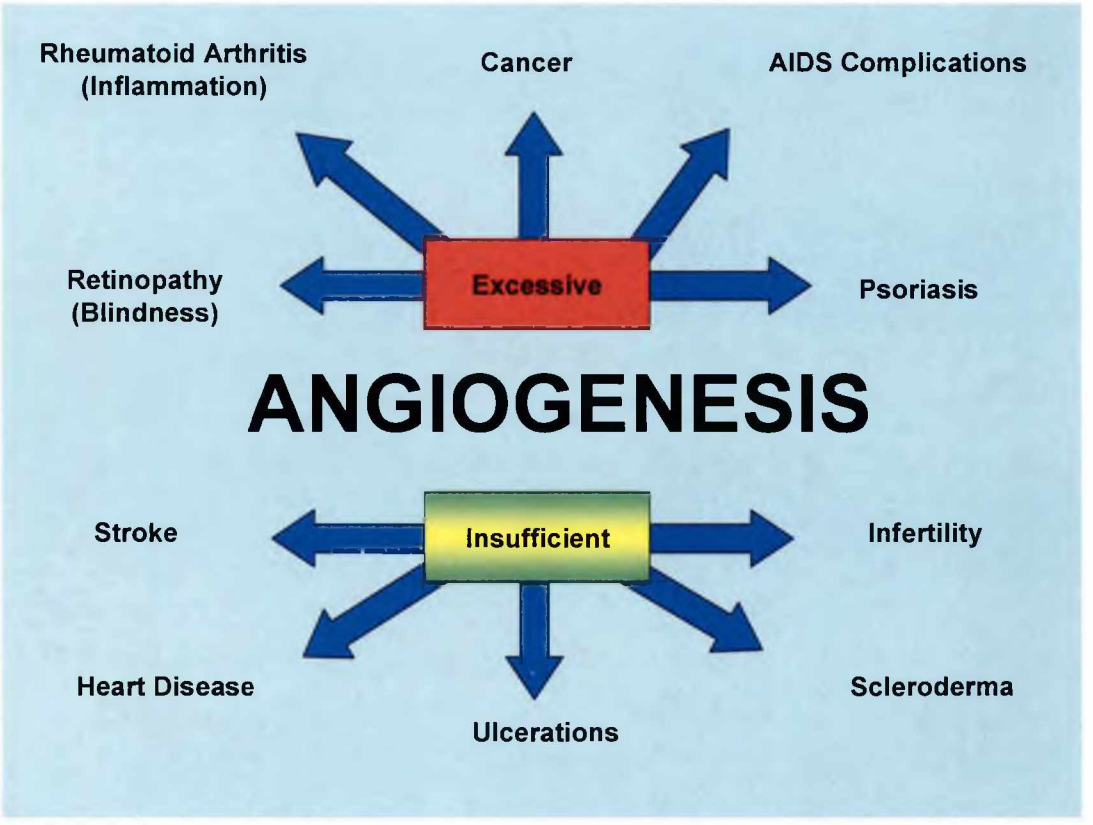

Figure 1.5 Pathological processes (abnormal) which are angiogenic dependant.

Tissue damage after reperfusion of hypoxic or ischemic tissue during myocardial infarction is angiogenic dependant in that angiogenesis is inadequate. In many other diseases angiogenesis is excessive such as solid state tumors and hematological tumors. Atherosclerosis, restenosis (cardiovascular disease), Crohn's Disease and rheumatoid arthritis (chronic inflammatory processes), diabetes (diabetic retinopathy), psoriasis, endometriosis and adiposity; all are characterized by uncontrolled angiogenesis (Griffioen et al., 1998; Griffioen and Molema, 2000; Molema and Griffioen, 1998).

1.5 Neoplasia and Tumors

The onset of many tumors appears to be mediated by genes encoding various mitogenic and angiogenic growth factors, such as VEGF. These genes also coordinate induction of other genes in EC to encode the respective SCRAM growth factor receptor proteins (Plate et al., 1994). These pathways appear to be regulated via paracrine mechanisms in two of the VEGF family of TKRs; VEGFR-1(Flt-1) and VEGFR-2 (Flk-1/KDR), respectively (Plate et al., 1994).

Tumors exists as small conglomerations of ECs during their early stage of pathogenesis, which at this point, the tumor can take on the form of malignant or benign pathology. The point at which the tumor cells switch to the malignant phenotype is a major area of research and to date has not been fully characterized. Tumor cells can remain in a nonproliferatory state for weeks or even years (weeks in mice and years in humans).

During this "dormant" time period, tumor growth is restricted to only a few mm^3. At some point the tumor cells develop vasculature which is sufficient for prolonged survival, but not for the rapid malignant growth of the parent tumor which is needed to metastasize to distant organs of the body (Folkman and Shing, 1992). The mechanisms mediating these events are not clear and previous publications rest on the assumption that tumor cells simply release angiogenic factors. This assumption is no longer tenable (Folkman and Shing, 1992). While the previous assumption is certainly true, it is but one of many different events mediating tumor angiogenesis. Furthermore, the elucidation as to how angiogenic factors such as VEGF and bFGF are released is still poorly understood. One major underlying unexplained phenomena is how the release of angiogenic factors is balanced with the release of angiogenic inhibitors. For example, thrombospondin is an angiogenic inhibitory protein (AIP) secreted by normal cells. This endogenous protein has previously been shown to be under tight control and down-regulation during tumorogenesis (Bouck, 1990; Rastinejad et al., 1989). Such control may be under the local control of tumor suppressor genes. However, even under control via gene expression, a shift of balance or new mutation must occur in order for a stagnant tumor cell to turn into an active proliferatory phenotype. This idea is supported by recent findings that tumor cells simultaneously secrete plasminogen activators (proteases) and their counterparts (plasminogen inhibitors or AIPs). This relationship is a key regulatory process which controls proteolysis and ultimately suppresses or fosters the growth of angiogenesis leading to tumor growth and metastasis. Steiner (1992) proposed the

following mechanisms for the angiogenic switch to the proliferatory phenotype (Figure 1.6).

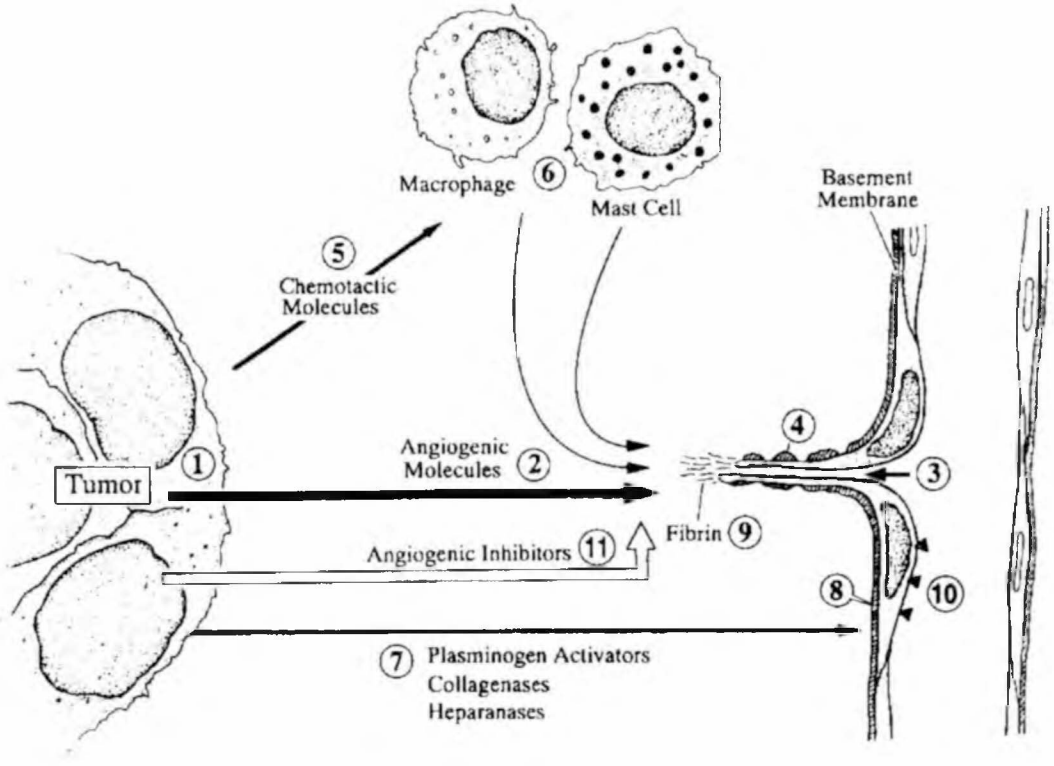

Figure 1.6 Proposed mechanisms for the switch to angiogenic phenotype in tumor pathogenesis. (1) Various angiogenic molecules are exported out of the cell: VEGF, PDGF, bFGF and other mitogenic growth factors. (2) These angiogenic molecules must navigate and migrate through tissue in order to reach the surrounding vasculature. Heparan sulfate plays a role in the stabilization of many growth factor's migratory process. (3) Some of the migratory growth factors stimulate endothelial cell migration and chemotaxis more efficiently than DNA synthesis. (4) Growth factors and other angiogenic factors release collagenases and plasminogen activators from endothelial cells which degrade the basement membrane of the parent venule. These proteases are responsible for "clearing" the way for endothelial sprouting and budding. (5) In addition to the release of angiogenic growth factors, tumors may also employ macrophages and mast cells which can foster angiogenesis; (6) it is here that macrophages can release angiogenic molecules (α-TNF) which in turn recruit more macrophages. (7) Tumor cells also have the ability to secrete various proteases which attack the basement membrane. (8) bFGF is stored in the extracellular membrane and can be mobilized for the membrane degradation. (9) VEGF is an angiogenic factor and is also referred to as a vascular permeability factor (VPF) which cause increase permeability in the surrounding capillary

beds, which may lead to fibrin leakage into the extracellular space (Dovorak et al., 1988). (10&11) Inhibition of angiogenic inhibitory factors must be regulated and adequately suppressed before angiogenic molecules can diffuse and initiate neovascularization.

Many laboratories are working with the manipulation of the varying factors mediating angiogenesis. There are currently numerous late stage clinical trials involving angiogenic inhibitors for cancer therapy and there are clinical trials involving angiogenic inducers involving myocardial infarction and stroke patients. These therapies and their unique approach to cellular regulation have excellent promise for becoming useful medical modalities.

The overall purpose of my study was to establish circulatory baseline VEGF levels in healthy dog models. It is my hope that baseline levels of VEGF in plasma will aid as a comparative model for disease and other angiogenic abnormalities in this animal.

Chapter 2

MATERIAL AND METHODS

2.1 Animal Models

The Institute of Animal Use and Care Committee at the University of Alaska Fairbanks approved this study. The animals utilized in this study were racing sled dogs owned and under the supervision of Dr. Arleigh Reynolds. The animals were housed at the Nestle Purina® Research Facility located in Salcha, Alaska. Each animal was tethered to a 2m chain attached to individual houses; each animal had access to his/her own food and water. Ambient temperature during sampling was between -23°C to -15°C. Twenty animals were sampled and placed into categories of runners (R), non-runners (NR), male runners and non-runners (MR and MNR) and female runners and non-runners (FR and FNR), respectively(Table 2.1). Ages of the animals ranged from less than 1 year to just over 11 years. A total of 12 non-neutered males and 8 non-spayed females comprised the two groups. All animals were healthy at the time of sampling and were free of prescribed medication except for one animal (Marvin) who received Thyroxin® 1.2 mg B.I.D. for hypothyroidism.

Table 2.1. Animals used in the study listed with their age, sex, breed and grouping, respectively.

ANIMAL NAME	GROUP	AGE (yrs)	SEX	BREED
Brown	R	5	M	Alaskan Sled Dog
Mocha	R	2	M	Alaskan Sled Dog
Boney	R	2	M	Alaskan Sled Dog
Bruce	R	2	M	Alaskan Sled Dog
Nigel	R	2	M	Alaskan Sled Dog
Finnmark	R	1	M	Alaskan Sled Dog
Tromso	R	1	M	Alaskan Sled Dog
Dori	R	2	F	Alaskan Sled Dog
Peach	R	2	F	Alaskan Sled Dog
Hera	R	6	F	Alaskan Sled Dog
AVERAGE		2.5	70%M	
Rambo	NR	12	M	Alaskan Sled Dog
Jose	NR	1	M	Alaskan Sled Dog
Apollo	NR	1	M	Alaskan Sled Dog
Sully	NR	2	M	Alaskan Sled Dog
Marvin	NR	9	M	Alaskan Sled Dog
Roz	NR	2	F	Alaskan Sled Dog
Pin	NR	4	F	Alaskan Sled Dog
Celia	NR	2	F	Alaskan Sled Dog
Lucy	NR	12	F	Alaskan Sled Dog
Twister	NR	4	F	Alaskan Sled Dog
AVERAGE		4.9	50%M	

*All animal ages are rounded to the nearest whole number

2.2 Animal Activity Levels

Control dogs remained tethered to their houses and activity levels varied with the individual dog. Runner dogs were exercised daily for approximately 0.5 h.

2.3 Blood Sampling

Sampling of dogs was conducted at the Nestle Purina® Research Facilities. All dogs were subjected to cephalic venipuncture using a 21G X 3/4 winged infusion set by Terumo® in connection with 12cc syringes. Approximately 10ml of whole blood was collected from each animal and placed into 12cc herparinized vacutainer tubes. The samples were placed on ice and transported to the biochemistry laboratory at the University of Alaska Fairbanks Natural Sciences Facility where they were subjected to centrifugation at 1000Xg for 15min. Plasma was collected and transferred into freezer vials and stored at -70°C until they were analyzed.

2.4 Biochemical Analysis

The biochemical analysis of Vascular Endothelial Growth Factor was conducted at the University of Alaska Fairbanks Natural Science Facility biochemistry laboratory. A commercial available ELISA Quanikine® human VEGF Immunoassay was used from R&D Systems® (Cat# DVE00, Lot 225301). The assay was utilized to quantitatively evaluate circulatory levels of VEGF in all of the sampled animals. The Procedure supplied by R&D Systems® was followed (Cat# DVE00). A summary of assay protocol is provided in figure 2.1. For the entire blood collection, all samples were analyzed using one assay kit. This approach was utilized to reduce variability and reagent related

differences, therefore comparisons were only made between samples which were collected at the same time and analyzed at the same time using one assay. Ages of the subjects animals were all rounded to the nearest whole number (Table 3.1-3.8).

Table 2.2. Intra-assay and Inter-assay precision and variation among samplings in humans.

	INTRA-ASSAY PRECISION			INTER-ASSAY PRECISION		
Sample	1	2	3	1	2	3
n	20	20	20	40	40	40
Mean (pg/ml)	53.7	235	910	64.5	250	1003
STDEV	3.6	10.6	46.2	5.7	17.4	61.7
CV (%)	6.7	4.5	5.1	8.8	7	6.2

Three human samples of known concentration were tested twenty times on one plate to assess intra-assay precision. Three samples of known concentration were tested in forty separate assays to assess inter-assay precision (DVE00).

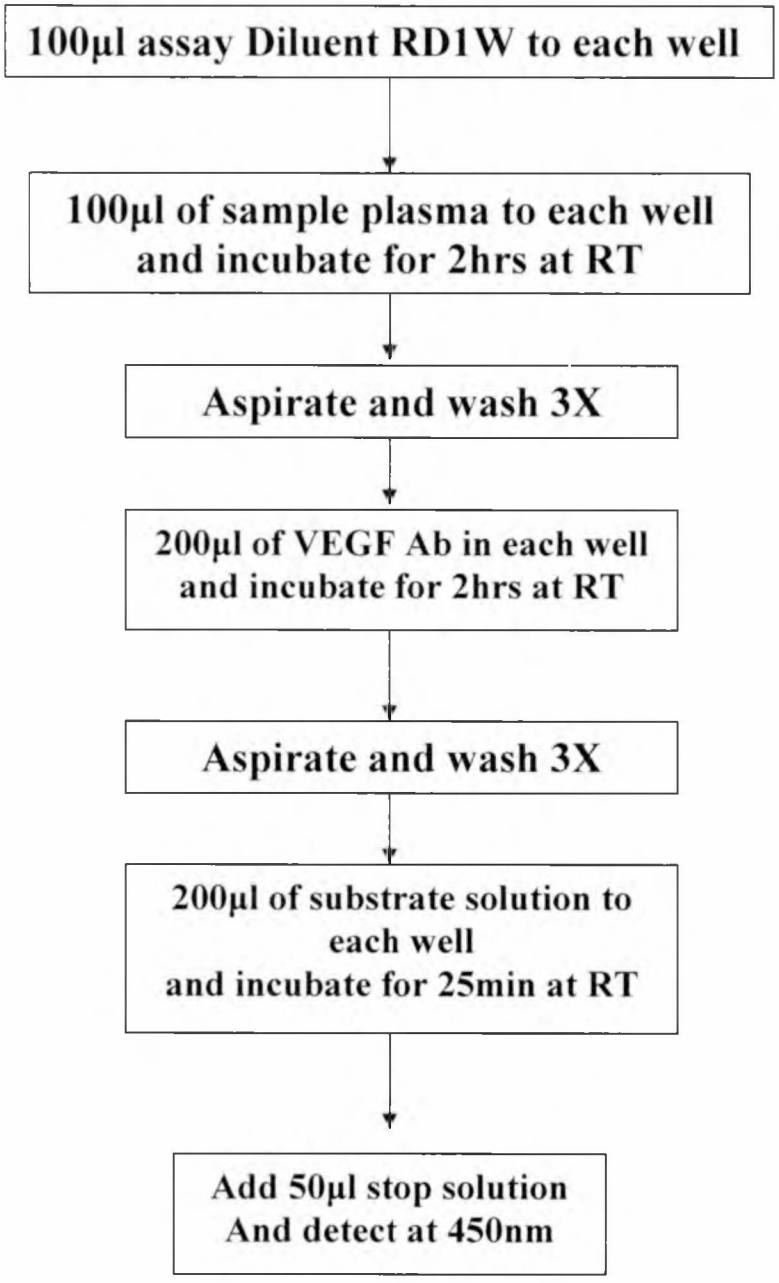

Figure 2.1. Overall schematic of VEGF assay protocol and detection for ELISA Quanikine® human VEGF Immunoassay from R&D Systems® (Cat# DVE00, Lot 225301).

Optical density (OD) of each sample was determined using Benchmark® Microplate Reader from BioRad®. The VEGF concentrations for each sample were extrapolated using a standard curve (Figure 2.2) developed from known concentrations of VEGF; the standard samples were human VEGF and assayed in parallel with the canine samples; all samples (including standards) were run in duplicate.

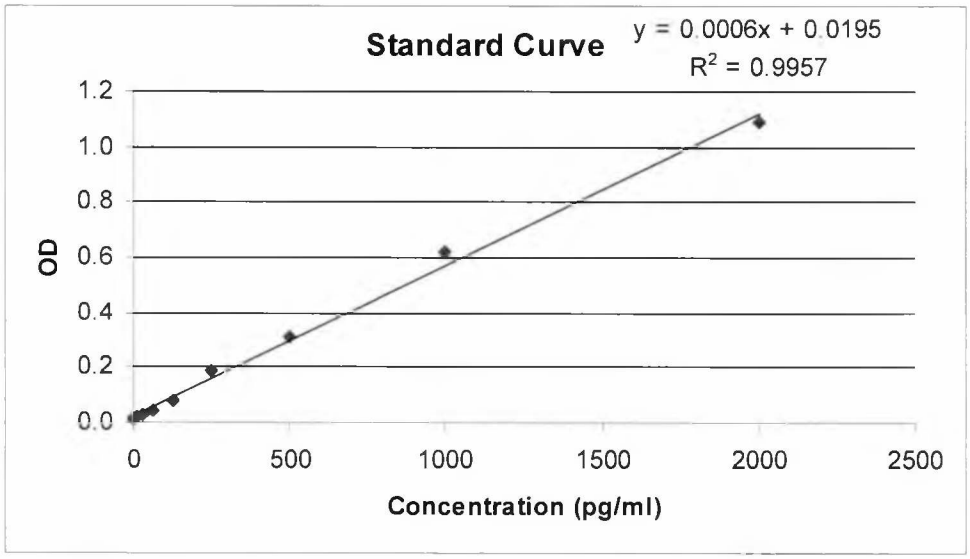

Figure 2.2. Standard curve used for concentration calculations

2.5 Statistical Analysis

Analysis of variance was used to evaluate any statistical differences between the groups. The F test was use to determine if significant differences were present within standard deviations. No significant differences between standard deviations. Since there were no significant differences in the standard deviation, a simplified Student's T test was utilized to evaluate group data. Significant differences were observed between two groups (Male vs. Female and Male runner vs. Female runners), the remaining groups displayed no significant differences.

Chapter 3

RESULTS

In order to establish baseline levels for VEGF in healthy dogs, we analyzed plasma samples to establish concentrations and then analyzed established grouping within the studies overall sample size. The groupings were as follow: Runners, Non-runners, Male and Female, respectively. The various groupings were evaluated using analysis of variance and differences between the dog groupings were considered to be significant at $p \leq 0.05$.

3.1 Overall Sampling of Healthy Dogs

Overall mean VEGF levels for all dogs analyzed were higher (19 pg/ml) than originally anticipated; however, a consistent pattern is observed within the VEGF concentrations.

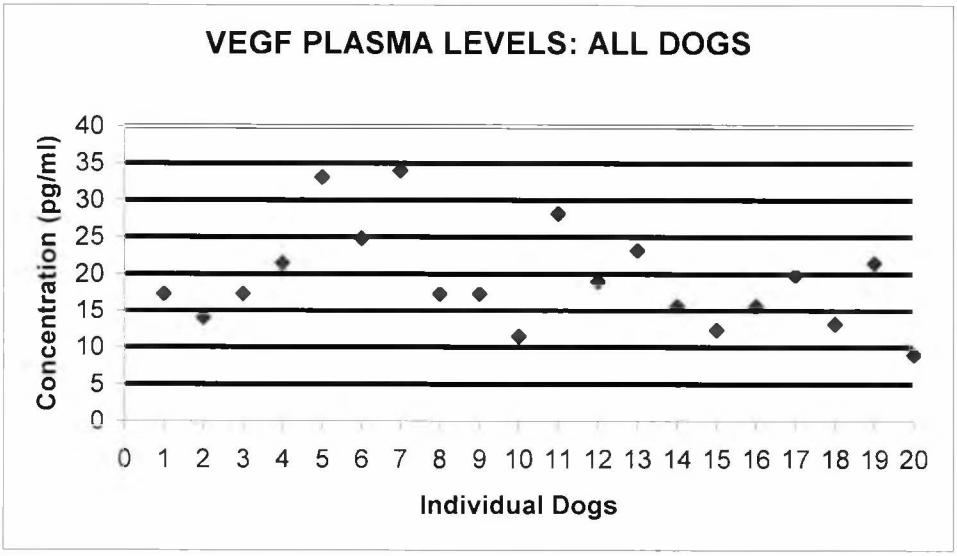

Figure 3.1. VEGF Plasma levels in all dogs.

While there are various clusters of VEGF levels for the dogs within the overall group, there appears to be a wide range of concentrations among dogs. These clusters and separations, which are clearly displayed when looking at the mean and standard deviation, will be analyzed further in coming sections in order to elucidate any additional information regarding sub-grouping comparison and significance.

Table 3.1. Values (pg/ml) of VEGF plasma levels for each dog involved in the overall grouping.

GROUP	AGE	SEX	BREED	CONCENTRATION
ALL SAMPLES				
Brown	4	M	Sled Dog	17.33
Mocha	2	M	Sled Dog	14.00
Boney	2	M	Sled Dog	17.33
Bruce	2	M	Sled Dog	21.50
Nigel	2	M	Sled Dog	33.17
Finnmark	1	M	Sled Dog	24.83
Tromso	1	M	Sled Dog	34.00
Dori	2	F	Sled Dog	17.33
Peach	2	F	Sled Dog	17.33
Hera	5	F	Sled Dog	11.50
Rambo	12	M	Sled Dog	28.17
Apollo	1	M	Sled Dog	19.00
Jose	1	M	Sled Dog	23.17
Sully	3	M	Sled Dog	15.67
Marvin	9	M	Sled Dog	12.33
Roz	2	F	Sled Dog	15.67
Pin	4	F	Sled Dog	19.83
Celia	2	F	Sled Dog	13.17
Lucy	12	F	Sled Dog	21.50
Twister	4	F	Sled Dog	9.00
AVERAGE	3.7	60%M		19.29
STDEV	3.41			6.75

3.2 Males vs. Females

Male and female dogs (both runners and non-runners) were compared. Male dogs displayed higher levels of VEGF as compared to the female. The difference between the two groups was statistically evaluated and found to be significant at $p \leq 0.05$ (Table 3.2).

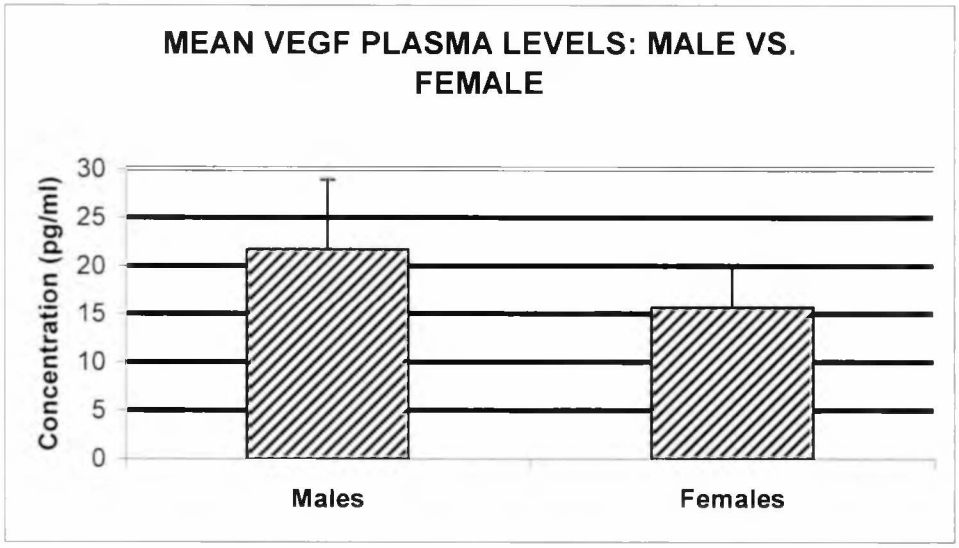

Figure 3.2. Means and standard deviations of VEGF plasma levels in male (running and non-running) and female (running and non-running) dogs.

Table 3.2. Values (pg/ml) of VEGF plasma levels for both male and female dogs.

GROUP	AGE	SEX	BREED	CONCENTRATION
MALES				
Brown	4	M	Sled Dog	17.33
Mocha	2	M	Sled Dog	14.00
Boney	2	M	Sled Dog	17.33
Bruce	2	M	Sled Dog	21.50
Nigel	2	M	Sled Dog	33.17
Finnmark	1	M	Sled Dog	24.83
Tromso	1	M	Sled Dog	34.00
Rambo	12	M	Sled Dog	28.17
Apollo	1	M	Sled Dog	19.00
Jose	1	M	Sled Dog	23.17
Sully	3	M	Sled Dog	15.67
Marvin	9	M	Sled Dog	12.33
AVERAGE	3.3	100%M		21.71
STDEV	3.5			7.17
GROUP	**AGE**	**SEX**	**BREED**	**CONCENTRATION**
FEMALE				
Dori	2	F	Sled Dog	17.33
Peach	2	F	Sled Dog	17.33
Hera	5	F	Sled Dog	11.50
Roz	2	F	Sled Dog	15.67
Pin	4	F	Sled Dog	19.83
Celia	2	F	Sled Dog	13.17
Lucy	12	F	Sled Dog	21.50
Twister	4	F	Sled Dog	9.00
AVERAGE	4.1	100%		15.67
STDEV	3.4			4.23

3.3 Runners vs. Non-Runners

Comparison of the exercising animals, both males and females, showed slightly elevated levels (Table 3.3) in the exercising group. However, the elevated levels of the exercising group were not statistically significant. The slight elevation in the runners could be due to the increased metabolic demand of exercise or the larger number in the exercising group.

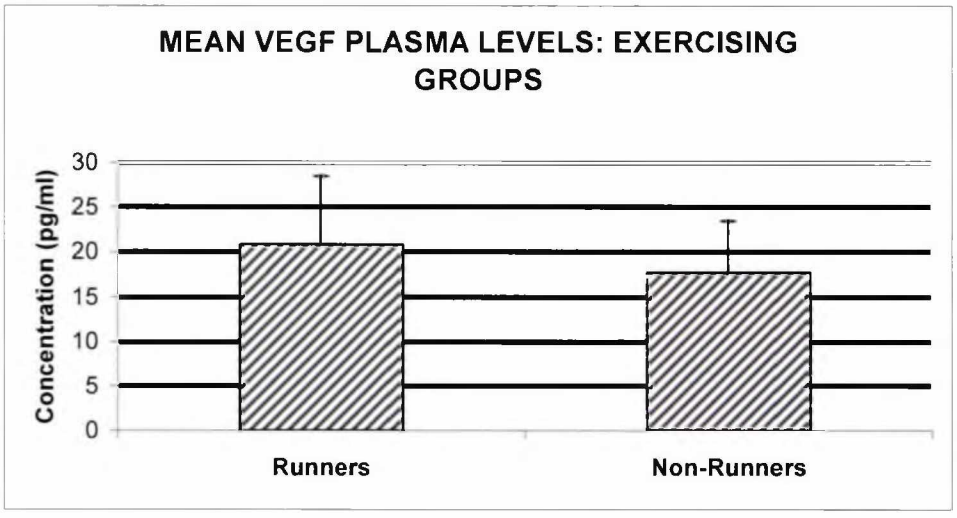

Figure 3.3. Means and standard deviations of VEGF levels for exercising and non-exercising dogs (male and female).

Table 3.3. Values (mg/pg) of VEGF levels for exercising (runners) and non-exercising (non-runners) dogs.

GROUP	AGE	SEX	BREED	CONCENTRATION (pg/ml)
RUNNERS				
Brown	4	M	Sled Dog	17.33
Mocha	2	M	Sled Dog	14.00
Boney	2	M	Sled Dog	17.33
Bruce	2	M	Sled Dog	21.50
Nigel	2	M	Sled Dog	33.17
Finnmark	1	M	Sled Dog	24.83
Tromso	1	M	Sled Dog	34.00
Dori	2	F	Sled Dog	17.33
Peach	2	F	Sled Dog	17.33
Hera	5	F	Sled Dog	11.50
AVERAGE	2.3	67%M		20.83
STDEV	1.3			7.64
GROUP	**AGE**	**SEX**	**BREED**	**CONCENTRATION**
NON-RUNNERS				
Rambo	12	M	Sled Dog	28.17
Apollo	1	M	Sled Dog	19.00
Jose	1	M	Sled Dog	23.17
Sully	3	M	Sled Dog	15.67
Marvin	9	M	Sled Dog	12.33
Roz	2	F	Sled Dog	15.67
Pin	4	F	Sled Dog	19.83
Celia	2	F	Sled Dog	13.17
Lucy	12	F	Sled Dog	21.50
Twister	4	F	Sled Dog	9.00
AVERAGE	5	50%M		17.75
STDEV	4.4			5.71

3.4 Male Runners vs. Male Non-Runners

Comparison of male exercising and non-exercising dogs were consistent with the overall elevation between running dogs and non-running dogs, displaying a slight elevation (but insignificant) in VEGF plasma levels in the running group (Figure 3.4). It is important to note there is an age variation between the 2 groups with the runners (mean age = 2) being younger than the non-runners (mean age = 5).

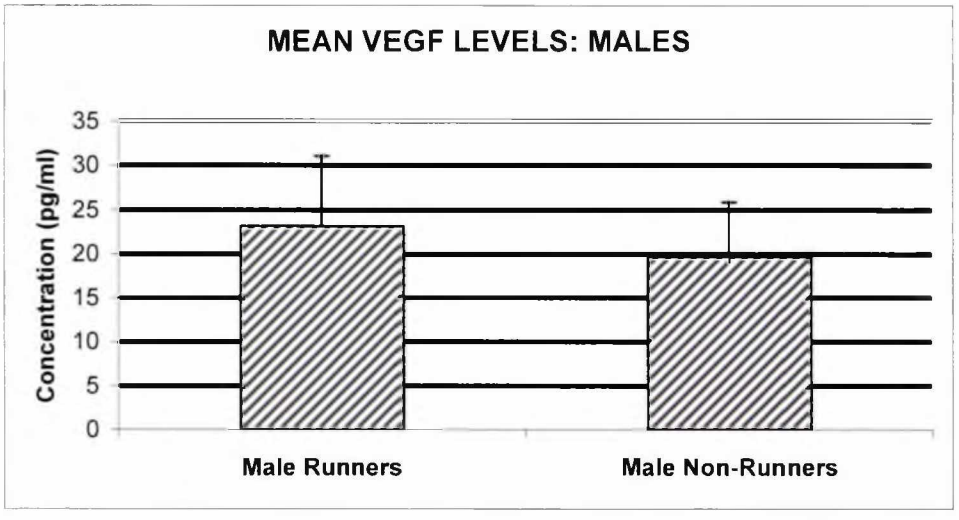

Figure 3.4. Means and standard deviations of VEGF plasma levels of male dogs (both runners and non-runners).

Table 3.4. Values (pg/ml) of VEGF for male dogs who were runners and for male dogs who were non-runners.

GROUP	AGE	SEX	BREED	CONCENTRATION
MALE RUNNERS				
Brown	4	M	Sled Dog	17.33
Mocha	2	M	Sled Dog	14.00
Boney	2	M	Sled Dog	17.33
Bruce	2	M	Sled Dog	21.50
Nigel	2	M	Sled Dog	33.17
Finnmark	1	M	Sled Dog	24.83
Tromso	1	M	Sled Dog	34.00
AVERAGE	2	100%M		23.17
STDEVA	1.0			7.90
GROUP	**AGE**	**SEX**	**BREED**	**CONCENTRATION**
MALE NON-RUNNERS				
Rambo	12	M	Sled Dog	28.17
Apollo	1	M	Sled Dog	19.00
Jose	1	M	Sled Dog	23.17
Sully	3	M	Sled Dog	15.67
Marvin	9	M	Sled Dog	12.33
AVERAGE	5	100%M		19.67
STDEVA	5.0			6.21

3.5 Female Runners vs. Female Non-Runners

Comparison of female runners and non-runners is different from previous patterns of elevated VEGF plasma levels with running dogs having a lower VEGF mean (Figure 3.5). No significant difference was observed upon statistical analysis.

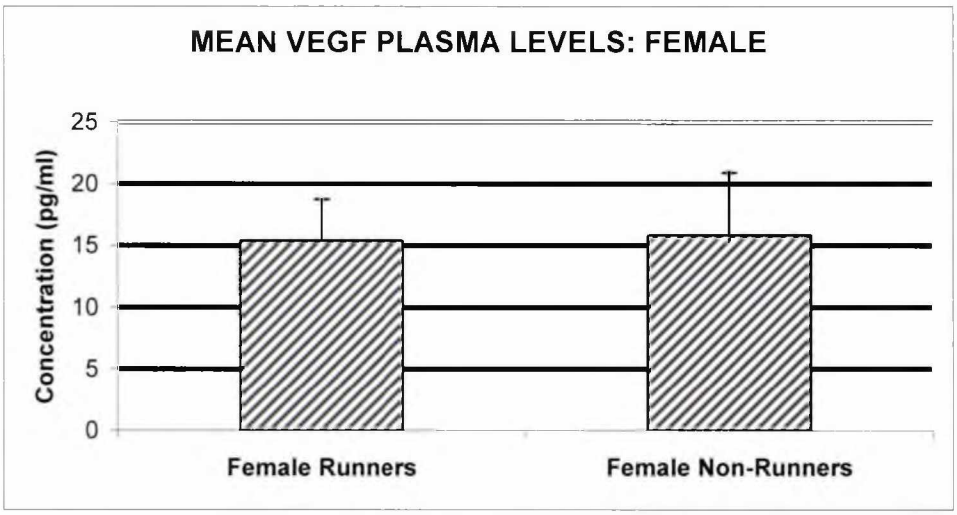

Figure 3.5. Means and standard deviations of VEGF plasma levels of all female dogs (both runners and non-runners).

Table 3.5. Values (pg/ml) of VEGF for all female dogs (both runners and non-runners).

GROUP	AGE	SEX	BREED	CONCENTRATION
FEMALE RUNNERS				
Dori	2	F	Sled Dog	17.33
Peach	2	F	Sled Dog	17.33
Hera	5	F	Sled Dog	11.50
AVERAGE	3	100%F		15.39
STDEV	1.7			3.37
GROUP	**AGE**	**SEX**	**BREED**	**CONCENTRATION**
FEMALE NON-RUNNERS				
Roz	2	F	Sled Dog	15.67
Pin	4	F	Sled Dog	19.83
Celia	2	F	Sled Dog	13.17
Lucy	12	F	Sled Dog	21.50
Twister	4	F	Sled Dog	9.00
AVERAGE	4.8	100%F		15.83
STDEV	4.2			5.05

3.6 Male Runners vs. Female Runners

VEGF levels in male and female runners were consistant with previous data showing male dogs having elevated levels when compared to females (Figure 3.2; Table 3.2). The differences between the male runners and the female runners were considered to be statistically significant at $p \leq 0.05$ (Table 3.6).

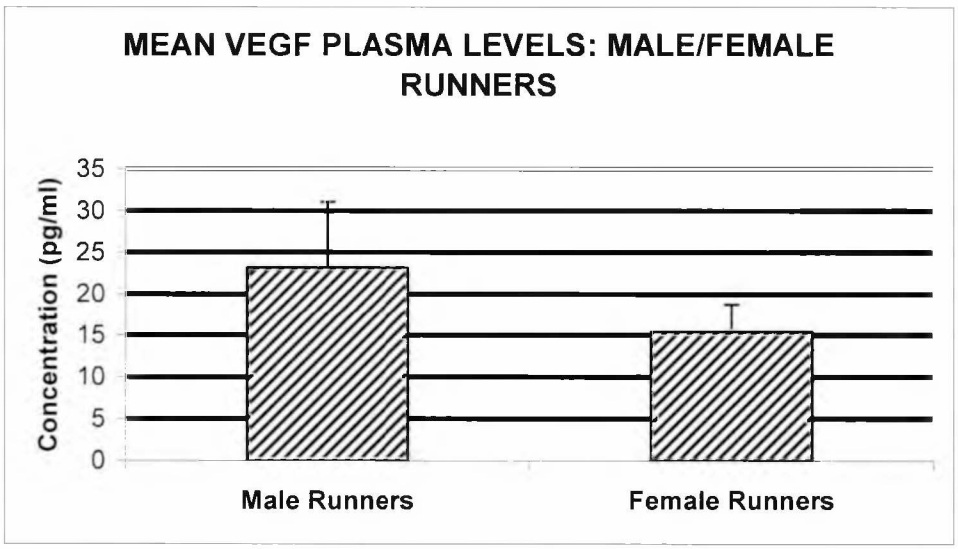

Figure 3.6. Means and standard deviations of VEGF plasma levels in male and female running dogs.

Table 3.6. Values (pg/ml) of VEGF for male and female running dogs.

GROUP	AGE	SEX	BREED	CONCENTRATION
MALE RUNNERS				
Brown	4	M	Sled Dog	17.33
Mocha	2	M	Sled Dog	14.00
Boney	2	M	Sled Dog	17.33
Bruce	2	M	Sled Dog	21.50
Nigel	2	M	Sled Dog	33.17
Finnmark	1	M	Sled Dog	24.83
Tromso	1	M	Sled Dog	34.00
AVERAGE	2	100%M		23.17
STDEVA	1.0			7.91
GROUP	**AGE**	**SEX**	**BREED**	**CONCENTRATION**
FEMALE RUNNERS				
Dori	2	F	Sled Dog	17.33
Peach	2	F	Sled Dog	17.33
Hera	5	F	Sled Dog	11.50
AVERAGE	3	100%F		15.39
STDEVA	1.7			3.37

3.7 Male Non-Runners vs. Female Non-Runners

VEGF levels for male and female non-running dogs were consistant with previous data (Figure 3.6; Table 3.6), having lower levels compared to running dogs, and female dogs displaying lower levels than the males. However, the differences were not statistically determined to be significant (Table 3.7). In this group, age does not appear to be a factor.

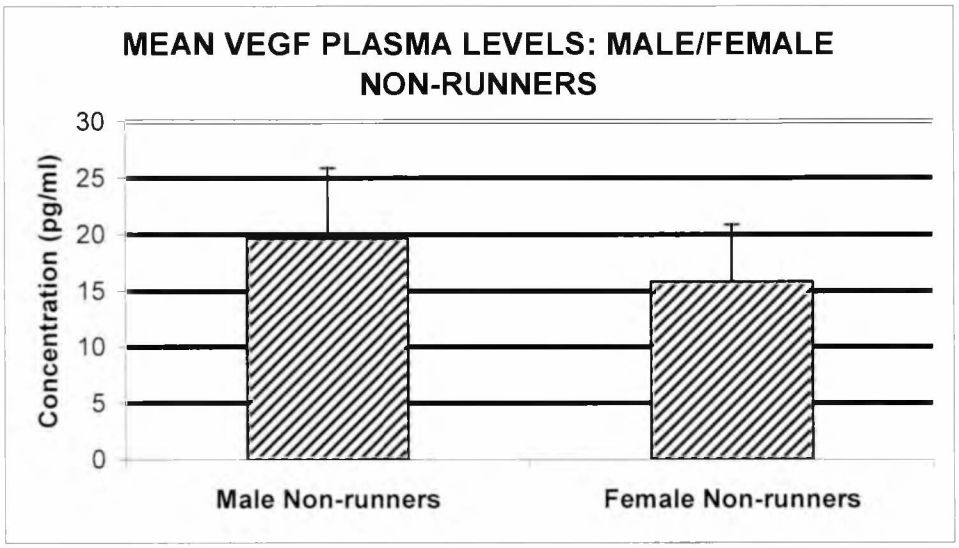

Figure 3.7. Means and standard deviations of VEGF plasma levels of male and female non-running dogs.

Table 3.7. Values (pg/ml) of VEGF for both male and female non-running dogs.

GROUP	AGE	SEX	BREED	CONCENTRATION
MALE NON-RUNNERS				
Rambo	12	M	Sled Dog	28.17
Apollo	1	M	Sled Dog	19.00
Jose	1	M	Sled Dog	23.17
Sully	3	M	Sled Dog	15.67
Marvin	9	M	Sled Dog	12.33
AVERAGE	5.2	100%M		19.67
STDEVA	5.0			6.22
GROUP	**AGE**	**SEX**	**BREED**	**CONCENTRATION**
FEMALE NON-RUNNERS				
Roz	2	F	Sled Dog	15.67
Pin	4	F	Sled Dog	19.83
Celia	2	F	Sled Dog	13.17
Lucy	12	F	Sled Dog	21.50
Twister	4	F	Sled Dog	9.00
AVERAGE	4.8	100%F		15.83
STDEVA	4.2			5.05

3.8 Healthy Dogs vs. Dog With Tumor

Samples from four healthy female non-running beagles were compared with one dog diagnosed with have a mammary gland tumor. While sample size is certainly an issue when evaluating data, the following figure is provided solely for further discussion on future research directions. The VEGF levels are lower than observed in sled dogs suggesting that baselines for individual breeds are needed for medical diagnosis. The mean for the healthy female dogs was 5 pg/ml (n=4).

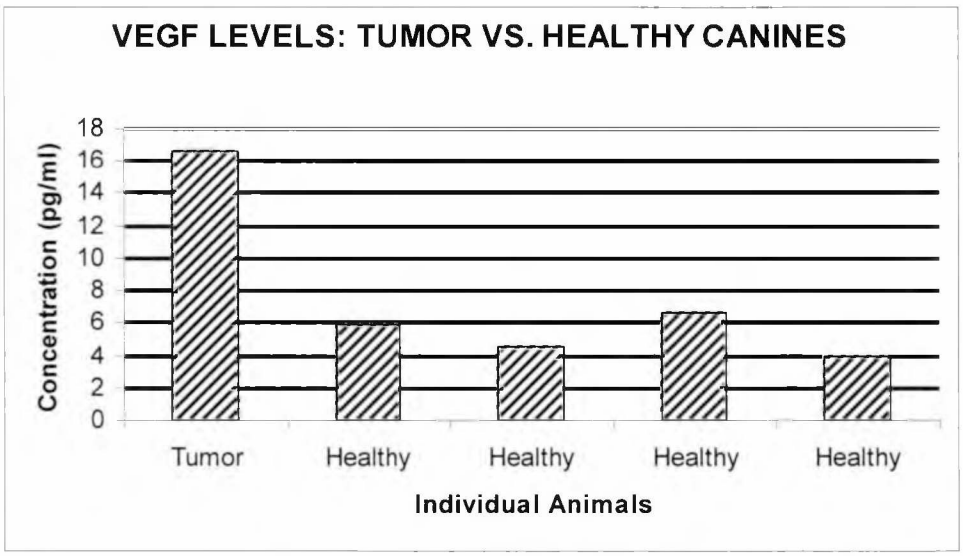

Figure 3.8. Values (pg/ml) of four healthy (non-running) dogs with one dog diagnosed with a vascular tumor.

Chapter 4

DISCUSSION AND FUTURE DIRECTION

Circulatory VEGF was readily detectable in all of the dogs sampled. In this study the mean VEGF concentration was 19 pg/ml (Table 3.1), with the lowest detected concentration being 9 pg/ml and the highest concentration being 34 pg/ml (Table 3.1).

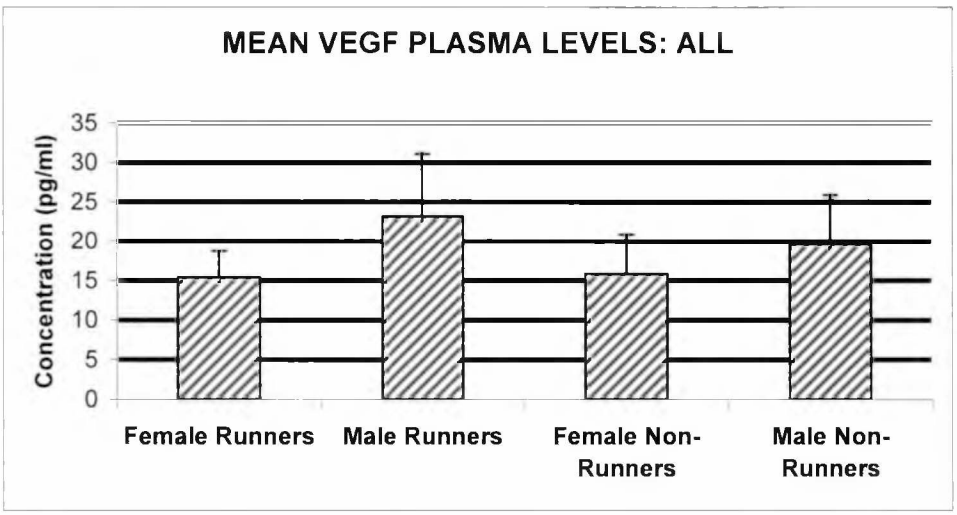

Figure 4.1. Overall comparison of means and standard deviations for all male and female runners and non-runners.

The males of the study displayed an overall higher mean elevation in VEGF levels compared to the females; likewise, the runners had a higher mean concentration than non-runners. To elucidate any significance, the groupings were broken down into categories of runners, non-runners, female. male, male runners and non-runners and female runners

and non-runners, respectively. However, these various groups led to differences in mean age and gender. These factors must be considered when interpreting the data.

4.1 Runners vs Non-Runners

The mean VEGF concentrations for the two groups were 20.8 pg/ml for the runners and 17.8 pg/ml for the non-runners, respectively. Concentrations ranged from 34 pg/ml to 9 pg/ml for both groups (Figure 3.3; Table 3.3). Although the sample size (n=12; Table 3.3) for the two groups was the same, the inconsistency of average age and gender within the samplings may explain why the difference was not statistically significantly. It is interesting to note that these elevated levels of VEGF are consistent with the findings of Kraus et al. (2004), where he and colleagues reported differences in VEGF levels in endurance athletes (human marathon runners) when compared to the sedentary individuals.

4.2 Male Runners vs Male Non-Runners

The mean VEGF concentrations for the male runners and non-runners was 23.2 pg/ml and 19.7 pg/ml (Figure 3.4; Table 3.4), respectively. The range for the male runners was as low as 14 pg/ml and as high as 34 pg/ml (Table 3.4). The non-runners display a similar range with levels of 12.3 to 28.2 pg/ml (Table 3.4). Sample representation was consistent

with n=7 for the running males and n=5 for the non-running males. Upon statistical evaluation, no significant difference was found between the two groups.

4.3 Female Runners vs Female Non-Runners

The female runners and non-runners definitely displayed lower levels of VEGF plasma concentrations when compared to the males. As a group, both runners and non-runners, the females displayed fairly consistent VEGF levels (Figure 3.5; Table 3.5). The running group had a mean concentration of 15.4 pg/ml and the non-running group mean concentration was 15.8 pg/ml. This is the opposite of expectations that the running groups should have higher VEGF concentrations when compared to the non-runners. The running group had a sample size of n=3 while the non-running group n=5. It is interesting to note that the two highest concentrations contributing to the higher mean in the non-runners were the oldest dogs in the grouping (Table 3.5); however, no significant difference correlation VEGF levels and age was observed. Statistical evaluation between female runners and non-runners did not reach significance.

4.4 Male vs Female

Male dogs when compared to the female dogs seemed to have the second greatest VEGF difference between the various groupings analyzed. Mean VEGF levels for the males was 21.7 pg/ml while the female levels were much lower, 15.7 pg/ml (Figure 3.2; Table 3.2). Male sampling was n=12 and the female sampling was n=8 (Table 3.2). Male concentrations ranged form 12.3 pg/ml to 34 pg/ml, while female samples ranged in concentration from 9 pg/ml to 21.5 pg/ml (Table 3.5). The differences between the male and female groups were found to be statistically significant at $p \leq 0.05$, and there was little age difference between the two groups.

4.5 Male Runners vs Female Runners

Concentrations for male runners were significantly higher than those of the female runners. The mean male concentration of VEGF was 23.2 pg/ml and the female mean concentration was 15.4 pg/ml (Figure 3.6; Table 3.6). This was the greatest difference observed between groupings but the female sample size was small. The concentrations for the male runners ranged from 14 pg/ml to 34 pg/ml, while the female concentrations ranged from 11.5 pg/ml to 17.3 pg/ml (Table 3.6). The male running sample size was over twice that of the female (Table 3.6) with male n=7 and female n=3. The difference in the mean concentrations between the two groups was found to be statistically

significant at $p \leq 0.05$. Gender needs to be taken into consideration when VEGF levels are being used for health related diagnostics.

4.6 Male Non-Runners vs Female Non-Runners

The non-runners for the male and female groups were similar in their concentrations range: 12.3 pg/ml to 28.2 pg/ml for the males and 9 pg/ml to 21.5 pg/ml (Table 3.7). The mean concentrations for the two groups was only slightly different, 19.7 pg/ml for the males and 15.83 pg/ml for the females (Figure 3.7; Table 3.7). Both male and female non-runners had n=5. The difference between the two groups was not statistically significant, possibly due to the lower sample size and the standard deviation of the assay.

4.7 VEGF Baseline Levels

There is a wealth of physiological research establishing that skeletal muscle capillary density is an important factor when evaluating athletic maximum exercise capacity. Furthermore, it has long been established that endurance athletes, such as marathon runners and cyclists, have higher muscle capillary supplies when compared to individuals whom exercise modestly or not at all. Recent reports by Kraus and colleagues (2004) suggest that exercise-induced angiogenesis is mediated in part by the secretion of VEGF via skeletal muscle cells. Kraus and colleagues (2004) study was conducted using human endurance athletes. They reported a 2-fold increase in the VEGF plasma levels shortly

after exercise when comparing endurance athletes to sedentary individuals. The animals used in our study were all racing sled dogs, some of which were retired and no longer running or actively training; however, at some stage in their life, all the dogs sampled were endurance trained athletes. One would expect a similar relationship in these athletic dogs. In comparison to human athletes, if systemic VEGF levels in human athletes was observable at levels two times that of sedentary individuals one would expect to see an even bigger increase of VEGF shortly after exercising. Maximal sustained metabolic rates of sled dogs have been measured well above those measure rates for human athletes. The small data set on healthy beagles (Figure 3.8) with VEGF levels around 5 pg/ml suggests that size may relate to muscle mass and, in turn, VEGF plasma levels.

To date there is a limited amount of data available concerning canine VEGF in healthy dogs. It is rather surprising considering there are numerous studies of VEGF levels in tumor ridden animals. Most of the studies have concentrated their efforts on changes in VEGF levels in response to radiation and chemotherapy. It is important to note that our study has shown that VEGF levels appear to be dependant upon the gender of the animal and the animal's level of exercise. Establishing baseline levels for both sedentary and exercising animals will provide valuable insight into the further elucidation of how VEGF protein levels respond to insult, as is focal limb ischemia, wound healing, menstruation and neoplasia.

4.8 VEGF As A Diagnostic Tool

VEGF has been widely shown in human and animal (mostly rat) studies to increase in response to a developing growth or tumor. It is certainly plausible that detection of increased VEGF levels in the blood stream could lead to an early suspicion of tumor growth and presence, or at least, suggest further analysis to investigate whether or not a growth is present. Having this information at the beginning stages of disease would lead to early treatment and management of canine cancer.

CHAPTER 5

LITERATURE CITED

Antonelli-Orlidge, A., Saunders, K.B., Smith, S.R. and D'Amore, P.A. (1989) An activated form of transforming growth factor beta is produced by cocultures of endothelial cells and pericytes. *Proc Natl Acad Sci U S A*, **86**, 4544-4548.

Baird, A. and Ling, N. (1987) Fibroblast growth factors are present in the extracellular matrix produced by endothelial cells in vitro: implications for a role of heparinase-like enzymes in the neovascular response. *Biochem Biophys Res Commun*, **142**, 428-435.

Beutler, B. and Cerami, A. (1986) Cachectin and tumour necrosis factor as two sides of the same biological coin. *Nature*, **320**, 584-588.

Bicknell, R. and Vallee, B.L. (1988) Angiogenin activates endothelial cell phospholipase C. *Proc Natl Acad Sci U S A*, **85**, 5961-5965.

Bicknell, R. and Vallee, B.L. (1989) Angiogenin stimulates endothelial cell prostacyclin secretion by activation of phospholipase A2. *Proc Natl Acad Sci U S A*, **86**, 1573-1577.

Bouck, N. (1990) Tumor angiogenesis: the role of oncogenes and tumor suppressor genes. *Cancer Cells*, **2**, 179-185.

Breier, C. and Lisch, H.J. (1984) [Distinct increase of plasma concentrations of high-density lipoprotein 2 and post-heparin lipolytic activity by constant moderate alcohol intake]. *Schweiz Med Wochenschr*, **114**, 1930-1932.

Breier, C., Lisch, H.J., Drexel, H. and Braunsteiner, H. (1984) [Atherosclerosis in familial hypercholesteremia possibly induced by defective HDL]. *Schweiz Med Wochenschr*, **114**, 288-291.

Breier, G. (2000) Angiogenesis in embryonic development--a review. *Placenta*, **21 Suppl A**, S11-15.

Breier, G. (2000) Endothelial receptor tyrosine kinases involved in blood vessel development and tumor angiogenesis. *Adv Exp Med Biol*, **476**, 57-66.

Breier, G., Albrecht, U., Sterrer, S. and Risau, W. (1992) Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Development*, **114**, 521-532.

Breier, M., Somogyvari, K. and Gyarmathy, F. (1973) [The use of tissue adhesive in experimental bladder surgery]. *Orv Hetil*, **114**, 2492-2493.

Brem, S. (1999) Angiogenesis and Cancer Control: From Concept to Therapeutic Trial. *Cancer Control*, **6**, 436-458.

Brown, L.F., Berse, B., Tognazzi, K., Manseau, E.J., Van de Water, L., Senger, D.R., Dvorak, H.F. and Rosen, S. (1992) Vascular permeability factor mRNA and protein expression in human kidney. *Kidney Int*, **42**, 1457-1461.

Brown, L.F., Yeo, K.T., Berse, B., Yeo, T.K., Senger, D.R., Dvorak, H.F. and van de Water, L. (1992) Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. *J Exp Med*, **176**, 1375-1379.

Brown, M.D. and Hudlicka, O. (2003) Modulation of physiological angiogenesis in skeletal muscle by mechanical forces: involvement of VEGF and metalloproteinases. *Angiogenesis*, **6**, 1-14.

Brown, P.D. and Giavazzi, R. (1995) Matrix metalloproteinase inhibition: a review of anti-tumour activity. *Ann Oncol*, **6**, 967-974.

Burgess, W.H. and Maciag, T. (1989) The heparin-binding (fibroblast) growth factor family of proteins. *Annu Rev Biochem*, **58**, 575-606.

Campbell, N.A., Reece, J.B. and Mitchell, L.G. (1999) Biology 5th ED., Benjamin/Cummings, an imprint of Addsion Wesley Longman, Inc. 2725 Sand Hill Road, Menlo Park, CA 94025.

Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W. and Nagy, A. (1996) Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature*, **380**, 435-439.

Conn, G., Bayne, M.L., Soderman, D.D., Kwok, P.W., Sullivan, K.A., Palisi, T.M., Hope, D.A. and Thomas, K.A. (1990) Amino acid and cDNA sequences of a vascular endothelial cell mitogen that is homologous to platelet-derived growth factor. *Proc Natl Acad Sci U S A*, **87**, 2628-2632.

Conn, G., Soderman, D.D., Schaeffer, M.T., Wile, M., Hatcher, V.B. and Thomas, K.A. (1990) Purification of a glycoprotein vascular endothelial cell mitogen from a rat glioma-derived cell line. *Proc Natl Acad Sci U S A*, **87**, 1323-1327.

Connolly, D.T. (1991) Vascular permeability factor: a unique regulator of blood vessel function. *J Cell Biochem*, **47**, 219-223.

Connolly, D.T., Heuvelman, D.M., Nelson, R., Olander, J.V., Eppley, B.L., Delfino, J.J., Siegel, N.R., Leimgruber, R.M. and Feder, J. (1989) Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *J Clin Invest*, **84**, 1470-1478.

Connolly, D.T., Olander, J.V., Heuvelman, D., Nelson, R., Monsell, R., Siegel, N., Haymore, B.L., Leimgruber, R. and Feder, J. (1989) Human vascular permeability factor. Isolation from U937 cells. *J Biol Chem*, **264**, 20017-20024.

Denekamp, J. and Hill, S. (1991) Angiogenic attack as a therapeutic strategy for cancer. *Radiother Oncol*, **20 Suppl 1**, 103-112.

Derynck, R. (1990) Transforming growth factor-alpha. *Mol Reprod Dev*, **27**, 3-9.

Dvorak, H.F., Nagy, J.A., Dvorak, J.T. and Dvorak, A.M. (1988) Identification and characterization of the blood vessels of solid tumors that are leaky to circulating macromolecules. *Am J Pathol*, **133**, 95-109.

Fava, R.A., Olsen, N.J., Spencer-Green, G., Yeo, K.T., Yeo, T.K., Berse, B., Jackman, R.W., Senger, D.R., Dvorak, H.F. and Brown, L.F. (1994) Vascular permeability factor/endothelial growth factor (VPF/VEGF): accumulation and expression in human synovial fluids and rheumatoid synovial tissue. *J Exp Med*, **180**, 341-346.

Ferrara, N., Carver-Moore, K., Chen, H., Dowd, M., Lu, L., O'Shea, K.S., Powell-

Braxton, L., Hillan, K.J. and Moore, M.W. (1996) Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*, **380**, 439-442.

Ferrara, N. and Henzel, W.J. (1989) Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochem Biophys Res Commun*, **161**, 851-858.

Ferrara, N., Winer, J. and Burton, T. (1991) Aortic smooth muscle cells express and secrete vascular endothelial growth factor. *Growth Factors*, **5**, 141-148.

Ferrara, N., Winer, J. and Henzel, W.J. (1992) Pituitary follicular cells secrete an inhibitor of aortic endothelial cell growth: identification as leukemia inhibitory factor. *ProcNatl Acad Sci U S A*, **89**, 698-702.

Fett, J.W., Strydom, D.J., Lobb, R.R., Alderman, E.M., Bethune, J.L., Riordan, J.F. and Vallee, B.L. (1985) Isolation and characterization of angiogenin, an angiogenic protein from human carcinoma cells. *Biochemistry*, **24**, 5480-5486.

Folkman, J. (1971) Tumor angiogenesis: therapeutic implications. *N Engl J Med*, **285**, 1182-1186.

Folkman, J. (1995) Angiogenesis in cancer, vascular, rheumatoid and other disease. *Nat Med*, **1**, 27-31.

Folkman, J. and Klagsbrun, M. (1987) Angiogenic factors. *Science*, **235**, 442-447.

Folkman, J. and Shing, Y. (1992) Angiogenesis. *J Biol Chem*, **267**, 10931-10934.

Fong, G.H., Rossant, J., Gertsenstein, M. and Breitman, M.L. (1995) Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature*, **376**, 66-70.

Frater-Schroder, M., Risau, W., Hallmann, R., Gautschi, P. and Bohlen, P. (1987) Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth *in vitro*, is angiogenic in vivo. *Proc Natl Acad Sci U S A*, **84**, 5277-5281.

Gospodarowicz, D. (1990) Fibroblast growth factor and its involvement in developmental processes. *Curr Top Dev Biol*, **24**, 57-93.

Gospodarowicz, D., Abraham, J.A. and Schilling, J. (1989) Isolation and characterization of a vascular endothelial cell mitogen produced by pituitary-derived folliculo stellate cells. *Proc Natl Acad Sci U S A*, **86**, 7311-7315.

Griffioen, A.W., Barendsz-Janson, A.F., Mayo, K.H. and Hillen, H.F. (1998) Angiogenesis, a target for tumor therapy. *J Lab Clin Med*, **132**, 363-368.

Griffioen, A.W. and Molema, G. (2000) Angiogenesis: potentials for pharmacologic intervention in the treatment of cancer, cardiovascular diseases, and chronic inflammation. *Pharmacol Rev*, **52**, 237-268.

Hiratsuka, S., Minowa, O., Kuno, J., Noda, T. and Shibuya, M. (1998) Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc Natl Acad Sci U S A*, **95**, 9349-9354.

Iijima, K., Yoshikawa, N., Connolly, D.T. and Nakamura, H. (1993) Human mesangial cells and peripheral blood mononuclear cells produce vascular permeability factor. *Kidney Int*, **44**, 959-966.

Ishikawa, F., Miyazono, K., Hellman, U., Drexler, H., Wernstedt, C., Hagiwara, K., Usuki, K., Takaku, F., Risau, W. and Heldin, C.H. (1989) Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor. *Nature*, **338**, 557-562.

Jin, K.L., Mao, X.O. and Greenberg, D.A. (2000) Vascular endothelial growth factor: direct neuroprotective effect in *in vitro* ischemia. *Proc Natl Acad Sci U S A*, **97**, 10242-10247.

Keck, P.J., Hauser, S.D., Krivi, G., Sanzo, K., Warren, T., Feder, J. and Connolly, D.T. (1989) Vascular permeability factor, an endothelial cell mitogen related to PDGF. *Science*, **246**, 1309-1312.

Klagsbrun, M. and D'Amore, P.A. (1991) Regulators of angiogenesis. *Annu Rev Physiol*, **53**, 217-239.

Koblizek, T.I., Runting, A.S., Stacker, S.A., Wilks, A.F., Risau, W. and Deutsch, U. (1997) Tie2 receptor expression and phosphorylation in cultured cells and mouse tissues. *Eur J Biochem*, **244**, 774-779.

Kraus, R.M., Stallings, H.W., 3rd, Yeager, R.C. and Gavin, T.P. (2004) Circulating plasma VEGF response to exercise in sedentary and endurance-trained men. *J Appl Physiol*, **96**, 1445-1450.

Leibovich, S.J., Polverini, P.J., Shepard, H.M., Wiseman, D.M., Shively, V. and Nuseir, N. (1987) Macrophage-induced angiogenesis is mediated by tumour necrosis factor-alpha. *Nature*, **329**, 630-632.

Leung, D.W., Cachianes, G., Kuang, W.J., Goeddel, D.V. and Ferrara, N. (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science*, **246**, 1306-1309.

Lobb, R.R., Alderman, E.M. and Fett, J.W. (1985) Induction of angiogenesis by bovine brain derived class 1 heparin-binding growth factor. *Biochemistry*, **24**, 4969-4973.

Molema, G. and Griffioen, A.W. (1998) Rocking the foundations of solid tumor growth by attacking the tumor's blood supply. *Immunol Today*, **19**, 392-394.

Monacci, W.T., Merrill, M.J. and Oldfield, E.H. (1993) Expression of vascular permeability factor/vascular endothelial growth factor in normal rat tissues. *Am J Physiol*, **264**, C995-1002.

Montesano, R., Vassalli, J.D., Baird, A., Guillemin, R. and Orci, L. (1986) Basic fibroblast growth factor induces angiogenesis in vitro. *Proc Natl Acad Sci U S A*, **83**, 7297- 7301.

Moscatelli, D., Presta, M. and Rifkin, D.B. (1986) Purification of a factor from human placenta that stimulates capillary endothelial cell protease production, DNA synthesis, and migration. *Proc Natl Acad Sci U S A*, **83**, 2091-2095.

Myoken, Y., Kayada, Y., Okamoto, T., Kan, M., Sato, G.H. and Sato, J.D. (1991) Vascular endothelial cell growth factor (VEGF) produced by A-431 human epidermoid carcinoma cells and identification of VEGF membrane binding sites. *Proc Natl Acad Sci U S A*, **88**, 5819-5823.

Neufeld, G., Cohen, T., Gengrinovitch, S. and Poltorak, Z. (1999) Vascular endothelial growth factor (VEGF) and its receptors. *Faseb J*, **13**, 9-22.

Neufeld, G., Tessler, S., Gitay-Goren, H., Cohen, T. and Levi, B.Z. (1994) Vascular endothelial growth factor and its receptors. *Prog Growth Factor Res*, **5**, 89-97.

Okamura, K., Sato, Y., Matsuda, T., Hamanaka, R., Ono, M., Kohno, K. and Kuwano, M. (1991) Endogenous basic fibroblast growth factor-dependent induction of collagenase and interleukin-6 in tumor necrosis factor-treated human microvascular endothelial cells. *J Biol Chem*, **266**, 19162-19165.

Pepper, M.S., Ferrara, N., Orci, L. and Montesano, R. (1991) Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. *Biochem Biophys Res Commun*, **181**, 902-906.

Pepper, M.S., Montesano, R., Orci, L. and Vassalli, J.D. (1991) Plasminogen activator inhibitor-1 is induced in microvascular endothelial cells by a chondrocyte-derived transforming growth factor-beta. *Biochem Biophys Res Commun*, **176**, 633-638.

Pepper, M.S., Vassalli, J.D., Orci, L. and Montesano, R. (1992) Proteolytic balance and capillary morphogenesis in vitro. *Exs*, **61**, 137-145.

Pertovaara, L., Kaipainen, A., Mustonen, T., Orpana, A., Ferrara, N., Saksela, O. and Alitalo, K. (1994) Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells. *J Biol Chem*, **269**, 6271-6274.

Plate, K.H., Breier, G. and Risau, W. (1994) Molecular mechanisms of developmental and tumor angiogenesis. *Brain Pathol*, **4**, 207-218.

Plouet, J., Schilling, J. and Gospodarowicz, D. (1989) Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT-20 cells. *Embo J*, **8**, 3801-3806.

Plouet, J., Schilling, J. and Gospodarowicz, D. (1989) Isolation and characterization of a newly identified endothelial cell mitogen produced by AtT-20 cells. *Embo J*, **8**, 3801-3806.

Presta, M., Moscatelli, D., Joseph-Silverstein, J. and Rifkin, D.B. (1986) Purification from a human hepatoma cell line of a basic fibroblast growth factor-like molecule that stimulates capillary endothelial cell plasminogen activator production, DNA synthesis, and migration. *Mol Cell Biol*, **6**, 4060-4066.

Rastinejad, F., Polverini, P.J. and Bouck, N.P. (1989) Regulation of the activity of a new inhibitor of angiogenesis by a cancer suppressor gene. *Cell*, **56**, 345-355.

Rifkin, D.B. and Moscatelli, D. (1989) Recent developments in the cell biology of basic fibroblast growth factor. *J Cell Biol*, **109**, 1-6.

Risau, W. (1997) Mechanisms of angiogenesis. *Nature*, **386**, 671-674.

Risau, W. and Flamme, I. (1995) Vasculogenesis. *Annu Rev Cell Dev Biol*, **11**, 73-91.

Roberts, A.B., Flanders, K.C., Heine, U.I., Jakowlew, S., Kondaiah, P., Kim, S.J. and Sporn, M.B. (1990) Transforming growth factor-beta: multifunctional regulator of differentiation and development. *Philos Trans R Soc Lond B Biol Sci*, **327**, 145-154.

Roberts, A.B., Sporn, M.B., Assoian, R.K., Smith, J.M., Roche, N.S., Wakefield, L.M., Heine, U.I., Liotta, L.A., Falanga, V., Kehrl, J.H. and et al. (1986) Transforming

growth factor type beta: rapid induction of fibrosis and angiogenesis in vivo and stimulation of collagen formation *in vitro*. *Proc Natl Acad Sci U S A*, **83**, 4167-4171.

Rosenthal, R.A., Megyesi, J.F., Henzel, W.J., Ferrara, N. and Folkman, J. (1990) Conditioned medium from mouse sarcoma 180 cells contains vascular endothelial growth factor. *Growth Factors*, **4**, 53-59.

Sato, T.N., Tozawa, Y., Deutsch, U., Wolburg-Buchholz, K., Fujiwara, Y., Gendron- Maguire, M., Gridley, T., Wolburg, H., Risau, W. and Qin, Y. (1995) Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature*, **376**, 70-74.

Schott, R.J. and Morrow, L.A. (1993) Growth factors and angiogenesis. *Cardiovasc Res*, **27**, 1155-1161.

Schreiber, A.B., Winkler, M.E. and Derynck, R. (1986) Transforming growth factor-alpha: a more potent angiogenic mediator than epidermal growth factor. *Science*, **232**, 1250-1253.

Senger, D.R., Galli, S.J., Dvorak, A.M., Perruzzi, C.A., Harvey, V.S. and Dvorak, H.F. (1983) Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science*, **219**, 983-985.

Senger, D.R., Van de Water, L., Brown, L.F., Nagy, J.A., Yeo, K.T., Yeo, T.K., Berse, B., Jackman, R.W., Dvorak, A.M. and Dvorak, H.F. (1993) Vascular permeability factor (VPF, VEGF) in tumor biology. *Cancer Metastasis Rev*, **12**, 303-324.

Shalaby, F., Rossant, J., Yamaguchi, T.P., Gertsenstein, M., Wu, X.F., Breitman, M.L. and Schuh, A.C. (1995) Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature*, **376**, 62-66.

Shapiro, R., Riordan, J.F. and Vallee, B.L. (1986) Characteristic ribonucleolytic activity of human angiogenin. *Biochemistry*, **25**, 3527-3532.

Shing, Y. (1988) Heparin-copper biaffinity chromatography of fibroblast growth factors. *J Biol Chem*, **263**, 9059-9062.

Shing, Y., Folkman, J., Haudenschild, C., Lund, D., Crum, R. and Klagsbrun, M. (1985) Angiogenesis is stimulated by a tumor-derived endothelial cell growth factor. *J Cell Biochem*, **29**, 275-287.

Shing, Y., Folkman, J., Sullivan, R., Butterfield, C., Murray, J. and Klagsbrun, M. (1984) Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. *Science*, **223**, 1296-1299.

Shing, Y.W. and Klagsbrun, M. (1984) Human and bovine milk contain different sets of growth factors. *Endocrinology*, **115**, 273-282.

Shweiki, D., Itin, A., Neufeld, G., Gitay-Goren, H. and Keshet, E. (1993) Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis. *J Clin Invest*, **91**, 2235-2243.

Slack, J.M., Darlington, B.G., Heath, J.K. and Godsave, S.F. (1987) Mesoderm induction in early Xenopus embryos by heparin-binding growth factors. *Nature*, **326**, 197-200.

Slack, J.M., Darlington, B.G., Heath, J.K. and Godsave, S.F. (1987) Mesoderm induction in early Xenopus embryos by heparin-binding growth factors. *Nature*, **326**, 197-200.

Sondell, M., Sundler, F. and Kanje, M. (2000) Vascular endothelial growth factor is a neurotrophic factor which stimulates axonal outgrowth through the flk-1 receptor. *Eur J Neurosci*, **12**, 4243-4254.

St Clair, D.K., Rybak, S.M., Riordan, J.F. and Vallee, B.L. (1987) Angiogenin abolishes cell-free protein synthesis by specific ribonucleolytic inactivation of ribosomes. *Proc Natl Acad Sci U S A*, **84**, 8330-8334.

Steiner, R. (1992) Angiogenesis--historical perspective. *Exs*, **61**, 1-3.

Suri, C., Jones, P.F., Patan, S., Bartunkova, S., Maisonpierre, P.C., Davis, S., Sato, T.N. and Yancopoulos, G.D. (1996) Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell*, **87**, 1171-1180.

Terman, B.I., Dougher-Vermazen, M., Carrion, M.E., Dimitrov, D., Armellino, D.C., Gospodarowicz, D. and Bohlen, P. (1992) Identification of the KDR tyrosine kinase as a receptor for vascular endothelial cell growth factor. *Biochem Biophys Res Commun*, **187**, 1579-1586.

Thomas, K.A. (1987) Fibroblast growth factors. *Faseb J*, **1**, 434-440.

Thomas, K.A., Rios-Candelore, M., Gimenez-Gallego, G., DiSalvo, J., Bennett, C., Rodkey, J. and Fitzpatrick, S. (1985) Pure brain-derived acidic fibroblast growth factor is a potent angiogenic vascular endothelial cell mitogen with sequence homology to interleukin 1. *Proc Natl Acad Sci U S A*, **82**, 6409-6413.

Tischer, E., Mitchell, R., Hartman, T., Silva, M., Gospodarowicz, D., Fiddes, J.C. and Abraham, J.A. (1991) The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem*, **266**, 11947-11954.

Veikkola, T. and Alitalo, K. (1999) VEGFs, receptors and angiogenesis. *Semin Cancer Biol*, **9**, 211-220.

Vlodavsky, I., Folkman, J., Sullivan, R., Fridman, R., Ishai-Michaeli, R., Sasse, J. and Klagsbrun, M. (1987) Endothelial cell-derived basic fibroblast growth factor: synthesis and deposition into subendothelial extracellular matrix. *Proc Natl Acad Sci U S A*, **84**, 2292-2296.

Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M. and Heldin, C.H. (1994) Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem*, **269**, 26988-26995.