DISSERTATION

TUMOR-ASSOCIATED NEOVASCULARIZATION: INNATE IMMUNITY AND THERAPEUTIC INTERVENTION

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY DEBRA A. KAMSTOCK ENTITLED TUMOR ASSOCIATED NEOVASCULARIZATION: INNATE IMMUNITY, REGULATION, AND THERAPEUTIC INTERVENTION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

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Tumor growth and metastasis are dependent on the development of a tumor-associated blood supply. Abrogation of this vascular network would result in tumor stasis. Conventional cancer therapy has traditionally targeted neoplastic cells directly, however, since the recognition of this critical vascular component, research efforts have incorporated a significant focus on the pathogenesis of tumor-associated vascular development. It has been established that this process is dependent on the intricate balance between pro- and anti-angiogenic factors in the tumor microenvironment and a significant number of these factors have since been identified. The mechanisms which ultimately control or shift the balance of these factors, recognized as the angiogenic switch, have been extensively studied yet remain to be clearly elucidated. Further understanding of these mechanisms is critical to the future development of diagnostic assays for early detection of cancer as well as for both preventative and therapeutic intervention strategies. Preventative therapy would inhibit initiation of the angiogenic switch while intervention strategies could shift the environment back to an anti-angiogenic state or inhibit the effects of the pro-angiogenic factors.

Studies presented here sought to further understand the role of endogenous angiogenic factors in the physiological tumor-bearing state, more specifically focusing on the anti-angiogenic effects of type I and II interferons. To evaluate these potential effects, intradermal tumors were induced in type I and II interferon receptor knock out (IFN-R -/-) mice with transplanted syngeneic methylcholanthrene-induced sarcoma cells. Tumor growth rates and tumor microvessel density (MVD) in both type I and II IFN-R -/- mice exceeded those of wild type (wt) mice, thus supporting an endogenous anti-angiogenic role of type I and II interferons. Characterization of tumor infiltrating leukocytes revealed increased numbers of dendritic cells (DCs) in tumors from IFN-R-/- mice suggesting these interferons may elicit their antivascular effects, at least in part, by inhibiting DC differentiation or recruitment to the tumor microenvironment. Initial experiments evaluating circulating endothelial progenitor cells (CEPs) demonstrated a decrease in CEPs in IFNR-/- mice over the course of tumor development suggesting these interferons may also elicit antivascular effects by inhibiting recruitment of progenitor cells to the tumor microenvironment.

Additional studies consisted of clinical trials in canines with spontaneously occurring soft tissue sarcoma. The first of the two studies sought to determine if intravenous gene therapy utilizing plasmid DNA encoding the anti-angiogenic, collagen-derived protein endostatin complexed with cationic liposomes (Cationic Liposome-DNA Complex - CLDC) could induce anti-angiogenic and anti-tumor effects. Dogs enrolled in the study were treated with either endostatin encoded or irrelevant DNA. Serial tumor biopsies were obtained to evaluate tumor MVD, transgene expression, and infiltrative

leukocytes while patients were monitored clinically for tumor responses. Ultimately, > 50% of dogs in each group 1) demonstrated a significant decrease in tumor MVD at one or more time points over the course of treatment and 2) had an objective tumor response or stable disease. Additionally, transgene expression was not detected in tumor tissue from endostatin treated dogs. These results suggested that intravenous infusions of CLDCs may elicit antiangiogenic and antitumor effects in a gene independent manner.

The second clinical trial sought to determine the antiangiogenic and antitumor effects of intradermal xenovaccination with recombinant human vascular endothelial growth factor (rhVEGF). Nine dogs were immunized over a 16-week period. Effects of immunization on the induction of anti-canine and anti-human VEGF antibodies, circulating plasma VEGF levels, tumor MVD, and tumor growth were assessed. The vaccine was well tolerated by all dogs and resulted in the induction of both anti-human and anti-canine VEGF antibodies in dogs which remained in the study long enough to receive five or more vaccinations (n=4). Of these four dogs, 3 had a decrease in circulating VEGF and a >50% reduction in tumor volume, while 2 dogs had a decrease in tumor MVD. Results of this pilot study suggest that multiple immunizations with xenogeneic VEGF in dogs may be a safe and effective therapy for inhibition of tumor growth and angiogenesis.

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DEDICATION

To the laboratory animals and canine clinical patients for whom without this work could not have been possible.

To the researchers around the world who are dedicated and driven by a true passion to continue to understand carcinogenesis and seek out improved methods for prevention, early detection, and therapeutic intervention for the betterment of both human and veterinary health.

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To my friends, colleagues, and office mates for their many ears and shoulders.

To Boomer, Angel, Morgan, and Daisy.

What you believe you will achieve -

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INTRODUCTION

Cancer and its dependence on neovascularization

Cancer is the second leading cause of human deaths in the United States preceded only by heart disease. In dogs, it is the leading cause of both morbidity and mortality. Cancer can be defined as a group of diseases which is characterized by uncontrolled growth and spread of abnormal cells. Both the "growth" and "spread" of these abnormal cells are critically dependent on a tumor-associated vascular network.[1,2] It is this vasculature which provides the neoplastic cells and their environment with the necessary oxygen and nutrients, allows for removal of metabolic wastes, and provides an avenue by which neoplastic cells can enter the circulation and travel to distant sites (metastasis).[3,4] While the field of tumor-associated neovascularization (more commonly referred to as tumor angiogenesis which will be discussed below) predominantly focuses on the production of new blood vessels, it should be noted that "vasculature" encompasses both lymphatics and blood vessels. It is the blood vessels which carry the necessary oxygen and nutrients to the tumor site, however, it is important to recognize that both blood vessels and lymphatics can and do provide routes for metastasis.

Mechanisms of tumor-associated neovascularization

Most commonly, the literature refers to the newly developed, tumor-induced blood supply as "tumor angiogenesis". Angiogenesis however more specifically and accurately refers to the sprouting of new capillaries from a pre-existing vascular network.[5-8] The development of a tumor-associated blood supply can develop by one of two mechanisms or, more likely, a combination thereof. One, as indicated, is angiogenesis, while the second is vasculogenesis, the formation of new vessels from bone marrow derived endothelial progenitor cells.[9-12] Vasculogenesis is thus the initial mechanism by which our vasculature forms during embryologic development and, at times, is further subclassified into natal or post-natal vasculogenesis.[13] It is important to be cognizant of the existence of both mechanisms however, to maintain consistency with the literature and for ease of comprehensiveness of this dissertation, the bulk of the work within will refer, in general, to angiogenesis unless specifically speaking of recruited endothelial progenitor cells associated with post-natal vasculogenesis.

Regulation of tumor angiogenesis

Tumor-induced angiogenesis is a multistep process of marked complexity involving, at the foundation, components of, and interactions between, the neoplastic cells, the tumor microenvironment, endothelial cells of nearby vessels, bone marrow-derived endothelial progenitor cells (hemangioblasts), and, as has been recognized more

recently, cells from the host immune system.[4,6,11,14] Angiogenic factors, receptors, and stimuli regulating angiogenesis have been, and continue to be identified while the mechanisms by which these elements interact and induce their effects remain to be clearly elucidated.[8,15-19]

The current paradigm for the induction of angiogenesis relates to the "angiogenic switch" which refers to the point in time at which the intricate balance between pro- and anti-angiogenic factors falls in favor of the pro-angiogenic molecules.[20-22] At this point, angiogenesis is initiated and the tumor is said to have assumed an angiogenic phenotype.[23] What, however, is at the core of initiating this switch? At present, a primary recognized stimulus is hypoxia. [24-26] Central areas of avascular tumors will become hypoxic and undergo necrosis as the distance from the peritumoral blood vessels exceeds the area limits for diffusion of necessary oxygen and nutrients. It is accepted that this hypoxic environment initiates the upregulation of hypoxia inducible factor-1alpha (HIF-1α).[27-30] HIF-1α functions as a transcription factor for vascular endothelial growth factor (VEGF) where it binds to the hypoxia response element (HRE) in the promoter region of *vegf*.[31-33] VEGF was one of the first recognized pro-angiogenic factors and remains at the forefront of angiogenesis regulation.[34-36] Thus, VEGF is a hypoxia-induced factor secreted by neoplastic cells which serves as both a critical initiator and regulator of the angiogenic process.

While VEGF has been determined to be a multi-functional protein, which will be discussed in more detail below, the earliest stages of angiogenesis are reported to be

VEGF-induced vasodilatation and increased vascular permeability of pre-existing capillaries, or post-capillary venules.[21,37,38] This allows for extravasation of plasma proteins which provide a foundation for activated endothelial cells to migrate. Of note is the earlier identification of vascular permeability factor (VPF) which was later determined to be the same protein as VEGF.[39-44] Hence, VEGF in the literature is sometimes denoted VEGF/VPF. Following these initial steps, VEGF functions as both an endothelial cell mitogen and chemotactic factor, eliciting its effects through endothelial cell tyrosine kinase receptor activity (predominantly through VEGF receptor 2 [VEGF-R2], and to a lesser degree VEGF receptor 1[VEGF-R1]) inducing both proliferation and migration of endothelial cells respectively.[45,46] Other factors (i.e. fibroblast growth factors [FGFs], platelet derived growth factor [PDGF], epidermal growth factor [EGF]) capable of eliciting similar functions do exist, yet VEGF remains at the forefront of tumor angiogenesis.[7,36]

Following and simultaneous to these initial steps, numerous other factors and receptors function in continued degradation of the extracellular matrix, tube formation, recruitment of pericytes, and vascular remodeling including Angiopoietin 1 and 2 (ANG-1, ANG-2) which interact with endothelial cell specific tyrosine kinase receptor TIE-2.[37,47,48] More specifically ANG-1 is involved in vascular stabilization where ANG-2 inhibits this stabilization and promotes continued vascular remodeling.[7] With regard to vasculogenesis, ANG-1, VEGF, and placental growth factor (PlGF) along with stromal derived growth factor (SDF-1) and granulocytic-monocytic colony stimulating factor (GM-CSF), also induce mobilization of the

bone-marrow derived endothelial progenitor cells which are then recruited to the site of angiogenesis and incorporated into the newly developing vasculature. [11,49,50]

Other methods contributing to tumor angiogenesis have also been recognized which include vasculogenic mimicry, the formation of vascular-like networks by the tumor cells themselves which have been shown to express endothelium associated genes.[51-54] Similarly and more recently, tumor associated macrophages, dendritic cells, and CD45+ leukocytes have also been shown to take on an endothelial cell phenotype and be incorporated into the developing tumor associated vasculature.[55-59]

Relevance of mechanistic elucidation of tumor-associated neovascularization

While the work within does not, and in no way could, address all of the angiogenic factors, receptors, and mechanisms discussed above, it is important to demonstrate and relay the networking and cascade of processes involved in angiogenesis as any step or combination of steps could be exploited as a therapeutic target. Additionally, it reveals the investigatory progress of dissecting and deciphering the process of tumor angiogenesis which has provided us with our current understanding and demonstrates the significance of ongoing research in this area. Recognition and comprehension of these mechanisms is also paramount for initiating therapies specific for the tumor vasculature so as not to deleteriously disrupt normal physiological angiogenesis necessary for conditions such as wound healing, pregnancy, normal reproductive tissue function, and development of collateral circulation.

The process of angiogenesis is the same in physiological and pathological conditions however, in normal physiological processes new vessels form under a tightly regulated balance of pro- and anti-angiogenic factors resulting in rapidly maturing vessels, stabilization, and quiescence. In contrast, tumor associated vessels develop in an environment of angiogenic factor chaos where the appropriate balance between the pro- and anti-angiogenic factors have been lost.[60] This vasculature fails to stabilize or become quiescent, undergoes constant growth, and is further characterized by disorganization with lack of definitive venules, arterioles, and capillaries. Additionally, vessels are irregularly shaped, tortuous, dilated, can have dead ends, may be patent or latent, and are frequently leaky and hemorrhagic with poorly associated pericytes.[61] These features however allow the tumor vasculature to be distinguished from normal vascular networks. In addition to these morphologically distinct characteristics, phenotypical differences in tumor-associated endothelial cells when compared to normal endothelium have also been identified.[62] Recognition of these distinct differences between normal and tumor vasculature assists in providing potential targets for tumor directed anti-angiogenic therapies.

Therapeutic intervention

Conventional cancer therapy has traditionally targeted the neoplastic cells directly, employing methods such as chemotherapy and radiation therapy. Anti-cancer vaccine strategies with the goal of inducing an immune response against tumor

associated antigens have also been and remain to be extensively explored yet again focus on direct tumor cell targeting. With the advent of tumor angiogenesis and recognition of its paramount role in tumor growth and metastasis, research in cancer therapy has sharply shifted to focus on this tumor-associated vasculature as an indirect means of inducing neoplastic cell death and / or inhibiting growth and mestastasis.[63,64]

As the mechanisms and factors involved in the angiogenic process continue to be unveiled, potential targets for anti-angiogenic therapy grow proportionately. Methods by which these targets can be exploited are many. At the foundation of angiogenesis, as discussed, is the "angiogenic switch". Thus, the fundamental concept behind most therapies is to control this switch in the tumor microenvironment. This can be achieved by either increasing the concentration of anti-angiogenic factors or their effects, decreasing the pro-angiogenic factors or their effects, or a combination thereof. Other therapies which exploit the tumor associated vasculature may target the endothelial cells directly ablating any existing vasculature and have been termed vascular targeting agents (VTAs).[65] Some therapies, however, seem to induce both destruction of existing vasculature and inhibition of a developing vasculature thus a clear distinction between these two theoretical mechanisms may not always exist.

Clinical trials discussed within broach both methods of antiangiogenic therapy. The objective of one study was to increase the concentration of the antiangiogenic factor endostatin, via gene therapy, as well as to elicit production of the antiangiogenic

cytokines, interferons (IFN) α/β and γ, via initiation of the innate immune response by cationic liposome DNA complexes (CLDCs). Conversely, the objective of the second study sought to inhibit the effects of the pro-angiogenic factor VEGF via induction of an anti-VEGF humoral response.

Innate immunity and tumor angiogenesis

Innate immunity is recognized as the first line of host defense in response to the presence of a pathogen. Components of the innate immune response include epithelial barriers, phagocytic cells (i.e. macrophages, dendritic cells, neutrophils) NK cells, and circulating plasma proteins of the complement system. Toll like receptors (TLRs) have also been recognized as a critical component of innate immunity.[66] TLRs are membrane proteins expressed on cells of the innate immune response including macrophages, dendritic cells, neutrophils, NK cells, mucosal epithelial cells and endothelial cells. These receptors recognize a variety of microbe-derived molecules (more specifically referred to as pathogen associated molecular patterns, PAMPS) which stimulate the innate immune response against the respective microbe.[67-70] Activation of these receptors initiates a signaling cascade ultimately resulting in the expression of numerous genes including cytokines, adhesion molecules, and microbicidal proteins necessary in the innate immune response.[71,72] While TLRs are not directly addressed in the work within, they are introduced with regard to their ability to induce a potent innate immune response which, as will be

discussed, may elicit antitumor and antiangiogenic activity. Thus, TLRs ultimately can be exploited as a means for cancer therapy.

While the innate immune system is classically recognized for its immediate function in protecting the host from infection, activation of innate immunity results in various biological processes through the production, secretion, and networking of numerous cytokines and chemokines which ultimately can drive the adaptive immune response. Involved in these various biological processes are regulators of angiogenesis.[73] Of note, and relevant to the work within, is the production of the potent antiangiogenic cytokines interleukin-12 (IL-12), IFNγ, and IFNα upon activation of the innate immune response.[74,75] IFNγ is secreted by activated natural killer (NK) cells of the innate immune response. IL-12 is an antiangiogenic protein secreted by both activated macrophages and dendritic cells and is an upstream regulator of IFNγ as it can drive a Th1 response as well as further potentiate NK cell activation and enhance IFNγ production. IFNα is secreted by all cells and, in addition to its direct antiangiogenic effects, is another potent activator of NK cells further inducing production of IFNs α and γ. An additional discussion of the type I (IFNα/β) and type II (IFNγ) interferons is provided below.

Interferons and tumor angiogenesis

Interferons (IFNs) consist of a family of pleotrophic cytokines which were first recognized for their antiviral properties and hence were termed "interferons" for their

ability to interfere with viral replication.[76-79] Since that time, recognized roles of IFNs have expanded to include antiproliferative, immunomodulatory, and more recently antiangiogenic properties.[80-82] The IFN family consists of two main classes, type I and type II, where type I IFNs consist of numerous proteins with similar structural homology and include IFNs- α and β, frequently denoted IFNα/β. IFNα is further subdivided into 13 different subtypes produced from variable mRNA splicing.[83] Of note is that all type I interferons bind to and elicit their effects through a common cell-surface receptor, typeI IFN receptor (IFNα/β-R) which is expressed on virtually all cell types.[84] Conversely, the type II IFN consists only of IFNγ which binds to and elicits its effect through the cell-surface type II IFN receptor (IFNγ-R) expressed predominantly on macrophages, monocytes, B-cells, and endothelial cells.[85] It should also be noted that while type I IFNs are typically produced and secreted by all cell types (especially those with associated viral infection) type II IFN is predominantly produced and secreted by activated natural killer (NK) cells of the innate immune response, NKT-cells, and in larger amounts by effector T-cells following induction of the adaptive immune response.[79] While it has been established that IFNs mediate various biological activities via their ability to induce the expression of hundreds of genes, type I interferons are well recognized for their potent activation of NK cells and upregulation of MHC-I molecules while IFNγ is a potent activator of macrophages and upreguates expression of both MHC-I and MHC-II molecules.[86,87] Significant work elucidating the signal transduction pathways through which these effects are elicited has also been pursued.[88,89]

Since the recognition of the antiangiogenic properties of interferons, applications of these cytokines for anti-cancer therapy have been extensively explored. Investigations have pursued these molecules for use as single agents and in combination therapies and have successfully demonstrated both antitumor and antiangiogenic effects.[90-94] At the onset of the work presented within, reported mechanisms of these interferon-associated antitumor effects included inhibition of tumor cell growth, activation of innate immunity of NK cells and macrophages against tumor cells, induction of tumor-specific CTL response, and inhibition of tumor angiogenesis. What had not been clearly addressed, however, was the endogenous role of interferons in vivo at physiological versus supraphysiological levels for suppression of tumor angiogenesis and tumor progression. The question remained as to whether or not these interferons could or did function naturally in antitumor host immunity or was it that their effects were only significant and recognized when iatrogenically increased. Utilizing an interferon receptor knock out mouse model, work within sought to address this question. Similar and simultaneous research was occurring in other labs globally and, since the onset of this work, reports revealing similar findings to ours have been reported.[95,96]

Cationic lipososme DNA complexes (CLDC) and tumor angiogenesis

Over the last 8-10 years the use of cationic liposome-DNA complexes for intravenous gene delivery has gained significant attention as a potential alternative to systemic viral gene therapy. It has introduced a powerful systemic gene therapy approach

which has proven useful in many *in vivo* applications.[97] While some forms of viral gene therapy have been recognized to be extremely efficient, obstacles which may preclude treatment include the development of a host immune response to the viral vector itself as has been seen with adenovirus-mediated gene delivery.[98]

The advent of CLDCs appeared to provide an alternative means of gene delivery in cancer therapy, however, it has become increasingly evident that even complexes formed with noncoding plasmids can elicit an anti-tumor response. Regardless of transgene expression, CLDCs injected systemically or locally have been shown to elicit potent pro-inflammatory activities resulting in the release of cytokines, inflammatory cell recruitment, and activation of NK cells consistent with an innate immune response.[74,99,100] More specifically, increased cytokines consisted of IL-12, IFNγ and IFNα and were noted to correlate with antitumor activity.[74,100-102] Antitumor responses in the face of CLDC treatment has been reported with and without specific DNA encoded plasmids while reports of enhanced antitumor activity and antiangiogenesis with plasmid encoded DNA has also been reported.[102,103] As has been previously discussed, IL-12, IFNα, and IFNγ all posses antiangiogenic properties and thus may be responsible, at least in part, for the antitumor effects. In addition to the potent immunostimulation of innate immunity, CLDC have also demonstrated an affinity for tumor endothelium and thus may also elicit direct, as well as indirect, effects on the tumor vasculature.[104]

This brief review of cationic liposome DNA complexes (CLDC) has been introduced as CLDCs are a component of treatment in both clinical trials presented within.

VEGF and tumor-associated neovascularization

As previously mentioned, VEGF is a multifunctional protein at the forefront of both initiation and regulation of tumor angiogenesis recognized predominantly for its role as and endothelial cell mitogen and chemotactic factor.[46,105-109] Functions of VEGF however extend far beyond that of its direct effects on endothelial cells. As some of the work within focuses on inhibiting VEGF and its biologic activity and, as all of the work in one way or another involves some component of immunity, I will briefly introduce other identified biological roles of VEGF and how, in addition to the induction of angiogenesis, it may also play a critical role in immune escape by the tumor.

Vascular endothelial growth factor (VEGF) is actually a family of factors consisting of VEGF A-E, placental growth factor (PlGF) and a recently identified tissue specific endothelial growth factor, endocrine gland-derived vascular endothelial growth factor (EF-VEGF).[37,43,110,111] Mediation of angiogenesis is primarily through the effects of VEGF-A which further consists of at least 5 different isoforms formed via alternative exon splicing of the 8 exons which compose the *vegf–a* gene.[112] The more common isoforms consist of 121, 145, 165, 189, and 206 amino acids and are termed $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$ respectively. An additional isoform,

$VEGF_{165}b$, has also been identified and actually functions as an endogenous inhibitor of VEGF by binding to VEGF receptor 2 (VEGF-R2) without the induction of tyrosine kinase (TK) phosphorylation and thus absence of signaling pathway induction and downstream effects.[113] These splice variants are distinguished by their heparin and heparin-sulfate binding ability where the heparin binding domains are encoded for in exons 6 and 7. Isoforms that encode exon 6 (145, 189, 206) are tightly bound to the cell surface whereas those which lack exon 6 are diffusible (165, 121).[114] VEGF165 is the predominant isoform, is the most potent angiogenic stimulator, and can bind to both heparin and the extracellular matrix. When "VEGF" is discussed in the literature, it more specifically refers to VEGF-A and, moreover, implies $VEGF_{165}$ unless otherwise indicated.

While VEGF-A predominantly functions in mediating angiogenesis, VEGF-C and D play a predominant role in lymphangiogenesis. For completeness, VEGF-B is less well understood but, like VEGF-A appears to regulate endothelial cell function while VEGF-E is structurally similar to VEGF-A also acting as potent stimulator of angiogenesis.[115] VEGF family members elicit their effects through respective tyrosine kinase receptors and subsequent activation of signaling cascades. Four VEGF receptors, VEGFR-1 (Flt1), VEGFR-2 (Flk1/KDR), VEGFR-3 (Flt4), and Neuropilin -1 and 2 (NRP 1,2), have been identified. VEGF165 and PlGF bind to NRP1,2 however this receptor does not have a tyrosine kinase domain and signaling pathway via NRPs remains unclear.[116,117] VEGFR-3 is primarily expressed on lymphatic endothelium and is specific for VEGF C and D.[118] VEGFR-2 is the

primary mediating receptor of VEGF signaling, binds all VEGF family members except VEGF B, and this receptor ligand interaction can initiate a number of signaling cascades including the mitogen activated protein kinase (MAPK) pathway, phosphotidyl inositol 3' kinase (PI3-K) pathway, protein kinase C (PKC) pathway and focal adhesion kinase (FAK) pathway, resulting in various outcomes including cell proliferation, migration, survival, and increase gene expression.[45] VEGF-R1 is also involved in angiogenesis, is specific for VEGF A, B, and PlGF, however demonstrates decreased TK activity when compared to VEGFR-2.[119,120]

As previously mentioned, VEGF was originally identified as an endothelial specific growth factor with inductive and regulatory functions for angiogenesis and vascular permeability. It plays an essential role in the normal physiology of embryologic development, wound healing, and the adult reproductive system however also plays a role in various disease states where it can promote negative host effects. (i.e. promote disease). Of relevance in this work is the association of VEGF and cancer.[41] VEGF is secreted by neoplastic cells as well as by stromal cells of the surrounding tumor environment. One method by which VEGF promotes angiogenesis is via its anti-apoptotic paracrine effect on endothelial cells (EC).[121,122] VEGF-mediated EC survival is achieved via its ability to upregulate expression of survivin, an antiapoptotic protein.[123] More recently it has been identified that VEGF also elicits autocrine effects acting as a survival factor for the tumor cells themselves protecting them from stresses such as hypoxia, chemotherapy, and radiotherapy.[124-126]

Although counterintuitive, VEGF has also been described as eliciting both pro-inflammatory as well as immunosuppressive function. VEGF can induce the expression of EC adhesion molecules ICAM-1, VCAM-1, and E-selectin which assist in leukocyte margination, rolling, adhesion, and extravasation in inflammatory responses.[127] While this feature may be desirable and beneficial in wound repair and inflammation (i.e. in response to a pathogen), the presence of infiltrating leukocytes (contrary to previous understanding) may actually be deleterious to the host and promote tumorigenesis. Additionally, VEGF can upregulate the expression of MCP-1 functioning as a monocytic chemotactic factor.[128] Again, this may be beneficial under normal physiological condition but undesirable in neoplasia as tumor associated macrophages have been shown to promote angiogenesis and tumorigenesis by various mechanisms including increased secretion of VEGF, production of MMPs, and even via incorporation into the vasculature taking on an endothelial cell phenotype as previously discussed.[58,129]

For these reasons and more, VEGF has been recognized as a negative prognostic indicator with regard to tumorigenesis. More recently, VEGF has been found to suppress dendritic cell function by inhibiting maturation.[130-132] Inhibition of maturation results in loss of antigen presenting capabilities, the prime function of these cells, by inhibiting expression of necessary co-stimulatory molecules. It has been recognized that VEGF elicits these effects via suppressing DC associated NF-κβ activation, (a primary nuclear transcription factor).[130,133] Similar VEGF-mediated effects on hematopoietic progenitor cells (HPC) have also been identified resulting in

altered or retarded development of multiple cell lineages thus resulting in defective immune induction (perhaps a VEGF mediated mechanism of tumor escape).[134] Similar to macrophages, VEGF has also been implicated in co-opting DCs directly into the vasculature taking on an endothelial cell phenotype.[55,56] VEGF has also been associated with T-cell suppression as well as induction of T-cell anergy.[135] Although not clearly elucidated in the literature, this may be an indirect effect of the inhibition of DC maturation as T-cell activation is dependent on mature and activated dendritic cells or other antigen presenting cells (APCs).

Thus, at present, functional roles of VEGF extend far beyond those elicited directly on and limited to endothelial cells and in general VEGF could be characterized as a mitogenic, chemotactic, pro-angigogenic, pro-inflammatory, and immunosuppressive factor while also promoting tumorigenesis and metastasis in neoplasia. As VEGF functions in both physiological and pathological conditions, its activities are both desirable and undesirable. Continued understanding of VEGF activities and elucidation of the molecular mechanisms by which they occur will provide the foundation to further develop intervention strategies to manipulate and direct these effects in a manner of increased desirability to the host.

Dissertation research

The goal of this research was to further elucidate tumor angiogenesis regulation by the innate immune response and endogenous angiogenic factors in physiologic tumor-

bearing states as well as to explore potential anti-angiogenic therapeutic strategies in an animal model with spontaneous tumor development.

Controlled studies focused on the effects of endogenous type I and II interferons (cytokines produced upon activation of the innate immune response) on tumor angiogenesis under physiological tumor-bearing conditions. We hypothesized that endogenous type I and II interferons function as antiangiogenic factors inhibiting tumor angiogenesis in the tumor environment. Interferon receptor knock out mice were inoculated intradermally with syngeneic methylcholanthrene-induced sarcoma cells for tumor induction. Post-innoculation, tumor growth rates were monitored clinically and serial tail bleeds were performed to assess the presence of circulating endothelial progenitor cells by fluorescence activated cell sorting (FACS). At sacrifice, tumor tissues were obtained for evaluation of microvessel density and tumor infiltrating leukocytes via immunohistochemistry and FACS respectively while blood collection was pursued to evaluate circulating VEGF and bFGF levels by enzyme linked immunosorbent assay (ELISA).

Clinical trials investigated tumor angiogenesis intervention strategies in dogs with de novo soft tissue sarcoma. The first of the two studies sought to evaluate the antiangiogenic and antitumor effects of intravenous gene therapy utilizing plasmid DNA encoded for the antiangiogenic protein endostatin complexed to cationic liposomes, thus forming a cationic liposome DNA complex (CLDC). An irrelevant DNA plasmid liposome complex was utilized as a control. We hypothesized 1) that

intratumoral gene expression of endostatin would inhibit tumor angiogenesis and elicit antitumor activity and 2) that activation of the innate immune response subsequent to CLDC administration would also elicit antiagniogenic and antitumor effects. Tumor responses were monitored clinically over the course of treatment. Tumor biopsies were obtained at various time points and at the end of treatment to evaluate MVD and tumor infiltrating leukocytes via immunohistochemistry and to assess endostatin gene expression by reverse transcriptase polymerase chain reaction (RT-PCR). Plasma and serum samples were obtained prior to and over the course of treatment to evaluate circulating concentrations of VEGF and bFGF by ELISA. To further explore the mechanisms of the noted antitumor and antiangiogenic effects in vivo, in vitro cell survival assays, exposing endothelial cells to various concentrations of CLDC, were also pursued.

The second clinical trial explored the antiangiogenic and antitumor effects of xenogeneic vaccination with recombinant human VEGF (rhVEGF) in tumor-bearing dogs. We hypothesized 1) that xenovaccination with rhVEGF in canines would induce an anti-huVEGF humoral response, 2) that anti-huVEGF antibodies would cross-react with canine VEGF and thus overcome immune tolerance, and 3) that induction of these antibodies would elicit both antiangiogenic and antitumor activity via VEGF neutralization. Tumor responses were monitored clinically over the course of treatment. Tumor biopsies were obtained to evaluate tumor MVD, VEGF expression, IgG deposition, and infiltrating leukocytes by immunohistochemistry.

Antibodies against human and canine VEGF as well as circulating VEGF concentrations were measured in plasma via ELISA.

Details of the above work, results, and conclusions are provided within.

REFERENCES

1. Folkman, J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* **285**, 1182-6 (1971).
2. Folkman, J. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* **82**, 4-6 (1990).
3. Folkman, J. Endothelial cells and angiogenic growth factors in cancer growth and metastasis. Introduction. *Cancer Metastasis Rev* **9**, 171-4 (1990).
4. Papetti, M. & Herman, I.M. Mechanisms of normal and tumor-derived angiogenesis. *Am J Physiol Cell Physiol* **282**, C947-70 (2002).
5. Folkman, J., Merler, E., Abernathy, C. & Williams, G. Isolation of a tumor factor responsible for angiogenesis. *J Exp Med* **133**, 275-88 (1971).
6. Risau, W. Mechanisms of angiogenesis. *Nature* **386**, 671-4 (1997).
7. Carmeliet, P. Mechanisms of angiogenesis and arteriogenesis. *Nat Med* **6**, 389-95 (2000).
8. Tonini, T., Rossi, F. & Claudio, P.P. Molecular basis of angiogenesis and cancer. *Oncogene* **22**, 6549-56 (2003).
9. Asahara, T. et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275**, 964-7 (1997).
10. Asahara, T. et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* **85**, 221-8 (1999).
11. Lyden, D. et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med* **7**, 1194-201 (2001).
12. Rafii, S., Heissig, B. & Hattori, K. Efficient mobilization and recruitment of marrow-derived endothelial and hematopoietic stem cells by adenoviral vectors expressing angiogenic factors. *Gene Ther* **9**, 631-41 (2002).
13. Isner, J.M. & Asahara, T. Angiogenesis and vasculogenesis as therapeutic strategies for postnatal neovascularization. *J Clin Invest* **103**, 1231-6 (1999).
14. Hanahan, D. & Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**, 353-64 (1996).
15. Sullivan, R.C., Shing, Y.W., D'Amore, P.A. & Klagsbrun, M. Use of size-exclusion and ion-exchange high-performance liquid chromatography for the isolation of biologically active growth factors. *J Chromatogr* **266**, 301-11 (1983).
16. Folkman, J. Angiogenesis: initiation and modulation. *Symp Fundam Cancer Res* **36**, 201-8 (1983).
17. Folkman, J., Langer, R., Linhardt, R.J., Haudenschild, C. & Taylor, S. Angiogenesis inhibition and tumor regression caused by heparin or a heparin fragment in the presence of cortisone. *Science* **221**, 719-25 (1983).
18. Baird, A., Esch, F., Bohlen, P., Ling, N. & Gospodarowicz, D. Isolation and partial characterization of an endothelial cell growth factor from the bovine

kidney: homology with basic fibroblast growth factor. *Regul Pept* **12**, 201-13 (1985).
19. Folkman, J. & Shing, Y. Angiogenesis. *J Biol Chem* **267**, 10931-4 (1992).
20. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
21. Carmeliet, P. & Jain, R.K. Angiogenesis in cancer and other diseases. *Nature* **407**, 249-57 (2000).
22. Folkman, J. Incipient angiogenesis. *J Natl Cancer Inst* **92**, 94-5 (2000).
23. Folkman, J. Role of angiogenesis in tumor growth and metastasis. *Semin Oncol* **29**, 15-8 (2002).
24. Liotta, L.A. & Stetler-Stevenson, W.G. Tumor invasion and metastasis: an imbalance of positive and negative regulation. *Cancer Res* **51**, 5054s-5059s (1991).
25. Dulak, J. & Jozkowicz, A. Regulation of vascular endothelial growth factor synthesis by nitric oxide: facts and controversies. *Antioxid Redox Signal* **5**, 123-32 (2003).
26. Wouters, B.G. et al. Modulation of cell death in the tumor microenvironment. *Semin Radiat Oncol* **13**, 31-41 (2003).
27. Liu, Y., Cox, S.R., Morita, T. & Kourembanas, S. Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer. *Circ Res* **77**, 638-43 (1995).
28. Huang, L.E., Gu, J., Schau, M. & Bunn, H.F. Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A* **95**, 7987-92 (1998).
29. Maxwell, P.H., Pugh, C.W. & Ratcliffe, P.J. Activation of the HIF pathway in cancer. *Curr Opin Genet Dev* **11**, 293-9 (2001).
30. Vincent, K.A., Feron, O. & Kelly, R.A. Harnessing the response to tissue hypoxia: HIF-1 alpha and therapeutic angiogenesis. *Trends Cardiovasc Med* **12**, 362-7 (2002).
31. Tsuzuki, Y. et al. Vascular endothelial growth factor (VEGF) modulation by targeting hypoxia-inducible factor-1alpha--> hypoxia response element--> VEGF cascade differentially regulates vascular response and growth rate in tumors. *Cancer Res* **60**, 6248-52 (2000).
32. Blancher, C., Moore, J.W., Talks, K.L., Houlbrook, S. & Harris, A.L. Relationship of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression to vascular endothelial growth factor induction and hypoxia survival in human breast cancer cell lines. *Cancer Res* **60**, 7106-13 (2000).
33. Makino, Y. et al. Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression. *Nature* **414**, 550-4 (2001).
34. Benjamin, L.E. & Keshet, E. Conditional switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. *Proc Natl Acad Sci U S A* **94**, 8761-6 (1997).
35. Jain, R.K. et al. Endothelial cell death, angiogenesis, and microvascular function after castration in an androgen-dependent tumor: role of vascular endothelial growth factor. *Proc Natl Acad Sci U S A* **95**, 10820-5 (1998).

36. Carmeliet, P. VEGF as a key mediator of angiogenesis in cancer. *Oncology* **69 Suppl 3**, 4-10 (2005).
37. Yancopoulos, G.D. et al. Vascular-specific growth factors and blood vessel formation. *Nature* **407**, 242-8 (2000).
38. Bergers, G. & Benjamin, L.E. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* **3**, 401-10 (2003).
39. Senger, D.R. et al. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science* **219**, 983-5 (1983).
40. Senger, D.R., Perruzzi, C.A., Feder, J. & Dvorak, H.F. A highly conserved vascular permeability factor secreted by a variety of human and rodent tumor cell lines. *Cancer Res* **46**, 5629-32 (1986).
41. Dvorak, H.F. et al. Vascular permeability factor/vascular endothelial growth factor: an important mediator of angiogenesis in malignancy and inflammation. *Int Arch Allergy Immunol* **107**, 233-5 (1995).
42. Suzuki, K. et al. Expression of vascular permeability factor/vascular endothelial growth factor in human hepatocellular carcinoma. *Cancer Res* **56**, 3004-9 (1996).
43. Neufeld, G., Cohen, T., Gengrinovitch, S. & Poltorak, Z. Vascular endothelial growth factor (VEGF) and its receptors. *Faseb J* **13**, 9-22 (1999).
44. Dvorak, H.F. Vascular permeability factor/vascular endothelial growth factor: a critical cytokine in tumor angiogenesis and a potential target for diagnosis and therapy. *J Clin Oncol* **20**, 4368-80 (2002).
45. Gille, H. et al. Analysis of biological effects and signaling properties of Flt-1 (VEGFR-1) and KDR (VEGFR-2). A reassessment using novel receptor-specific vascular endothelial growth factor mutants. *J Biol Chem* **276**, 3222-30 (2001).
46. Ferrara, N., Gerber, H.P. & LeCouter, J. The biology of VEGF and its receptors. *Nat Med* **9**, 669-76 (2003).
47. Papapetropoulos, A. et al. Direct actions of angiopoietin-1 on human endothelium: evidence for network stabilization, cell survival, and interaction with other angiogenic growth factors. *Lab Invest* **79**, 213-23 (1999).
48. Thurston, G. et al. Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science* **286**, 2511-4 (1999).
49. Hattori, K. et al. Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells. *J Exp Med* **193**, 1005-14 (2001).
50. Hattori, K. et al. Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. *Nat Med* **8**, 841-9 (2002).
51. Maniotis, A.J. et al. Vascular channel formation by human melanoma cells in vivo and in vitro: vasculogenic mimicry. *Am J Pathol* **155**, 739-52 (1999).
52. Folberg, R., Hendrix, M.J. & Maniotis, A.J. Vasculogenic mimicry and tumor angiogenesis. *Am J Pathol* **156**, 361-81 (2000).
53. McDonald, D.M., Munn, L. & Jain, R.K. Vasculogenic mimicry: how convincing, how novel, and how significant? *Am J Pathol* **156**, 383-8 (2000).

54. Hendrix, M.J., Seftor, E.A., Hess, A.R. & Seftor, R.E. Vasculogenic mimicry and tumour-cell plasticity: lessons from melanoma. *Nat Rev Cancer* **3**, 411-21 (2003).
55. Conejo-Garcia, J.R. et al. Tumor-infiltrating dendritic cell precursors recruited by a beta-defensin contribute to vasculogenesis under the influence of Vegf-A. *Nat Med* **10**, 950-8 (2004).
56. Conejo-Garcia, J.R. et al. Vascular leukocytes contribute to tumor vascularization. *Blood* **105**, 679-81 (2005).
57. Grunewald, M. et al. VEGF-induced adult neovascularization: recruitment, retention, and role of accessory cells. *Cell* **124**, 175-89 (2006).
58. Mantovani, A., Schioppa, T., Porta, C., Allavena, P. & Sica, A. Role of tumor-associated macrophages in tumor progression and invasion. *Cancer Metastasis Rev* (2006).
59. Schmid, M.C. & Varner, J.A. Myeloid cell trafficking and tumor angiogenesis. *Cancer Lett* (2006).
60. Dvorak, H.F. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med* **315**, 1650-9 (1986).
61. Morikawa, S. et al. Abnormalities in pericytes on blood vessels and endothelial sprouts in tumors. *Am J Pathol* **160**, 985-1000 (2002).
62. St Croix, B. et al. Genes expressed in human tumor endothelium. *Science* **289**, 1197-202 (2000).
63. Folkman, J., Browder, T. & Palmblad, J. Angiogenesis research: guidelines for translation to clinical application. *Thromb Haemost* **86**, 23-33 (2001).
64. Kerbel, R. & Folkman, J. Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer* **2**, 727-39 (2002).
65. Thorpe, P.E. Vascular targeting agents as cancer therapeutics. *Clin Cancer Res* **10**, 415-27 (2004).
66. Wright, S.D. Toll, a new piece in the puzzle of innate immunity. *J Exp Med* **189**, 605-9 (1999).
67. Janeway, C.A., Jr. The immune system evolved to discriminate infectious nonself from noninfectious self. *Immunol Today* **13**, 11-6 (1992).
68. Takeuchi, O. et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* **11**, 443-51 (1999).
69. Muzio, M., Polentarutti, N., Bosisio, D., Prahladan, M.K. & Mantovani, A. Toll-like receptors: a growing family of immune receptors that are differentially expressed and regulated by different leukocytes. *J Leukoc Biol* **67**, 450-6 (2000).
70. Janeway, C.A., Jr. & Medzhitov, R. Innate immune recognition. *Annu Rev Immunol* **20**, 197-216 (2002).
71. Ozinsky, A. et al. The repertoire for pattern recognition of pathogens by the innate immune system is defined by cooperation between toll-like receptors. *Proc Natl Acad Sci U S A* **97**, 13766-71 (2000).
72. Medzhitov, R. Toll-like receptors and innate immunity. *Nat Rev Immunol* **1**, 135-45 (2001).

73. Frantz, S., Vincent, K.A., Feron, O. & Kelly, R.A. Innate immunity and angiogenesis. *Circ Res* **96**, 15-26 (2005).
74. Dow, S.W. et al. Lipid-DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously. *J Immunol* **163**, 1552-61 (1999).
75. Whitmore, M.M., Li, S., Falo, L., Jr. & Huang, L. Systemic administration of LPD prepared with CpG oligonucleotides inhibits the growth of established pulmonary metastases by stimulating innate and acquired antitumor immune responses. *Cancer Immunol Immunother* **50**, 503-14 (2001).
76. Isaacs, A., Lindenmann, J. & Valentine, R.C. Virus interference. II. Some properties of interferon. *Proc R Soc Lond B Biol Sci* **147**, 268-73 (1957).
77. Isaacs, A. & Lindenmann, J. Virus interference. I. The interferon. *Proc R Soc Lond B Biol Sci* **147**, 258-67 (1957).
78. Pestka, S., Langer, J.A., Zoon, K.C. & Samuel, C.E. Interferons and their actions. *Annu Rev Biochem* **56**, 727-77 (1987).
79. Biron, C.A. Role of early cytokines, including alpha and beta interferons (IFN-alpha/beta), in innate and adaptive immune responses to viral infections. *Semin Immunol* **10**, 383-90 (1998).
80. Clemens, M.J. & McNurlan, M.A. Regulation of cell proliferation and differentiation by interferons. *Biochem J* **226**, 345-60 (1985).
81. De Maeyer-Guignard, J. & De Maeyer, E. Immunomodulation by interferons: recent developments. *Interferon* **6**, 69-91 (1985).
82. Sidky, Y.A. & Borden, E.C. Inhibition of angiogenesis by interferons: effects on tumor- and lymphocyte-induced vascular responses. *Cancer Res* **47**, 5155-61 (1987).
83. Pestka, S. The human interferon-alpha species and hybrid proteins. *Semin Oncol* **24**, S9-4-S9-17 (1997).
84. Pestka, S., Krause, C.D. & Walter, M.R. Interferons, interferon-like cytokines, and their receptors. *Immunol Rev* **202**, 8-32 (2004).
85. Schroder, K., Hertzog, P.J., Ravasi, T. & Hume, D.A. Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* **75**, 163-89 (2004).
86. Der, S.D., Zhou, A., Williams, B.R. & Silverman, R.H. Identification of genes differentially regulated by interferon alpha, beta, or gamma using oligonucleotide arrays. *Proc Natl Acad Sci U S A* **95**, 15623-8 (1998).
87. Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H. & Schreiber, R.D. How cells respond to interferons. *Annu Rev Biochem* **67**, 227-64 (1998).
88. Haque, S.J. & Williams, B.R. Signal transduction in the interferon system. *Semin Oncol* **25**, 14-22 (1998).
89. Platanias, L.C. Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat Rev Immunol* **5**, 375-86 (2005).
90. Wigginton, J.M. et al. IFN-gamma and Fas/FasL are required for the antitumor and antiangiogenic effects of IL-12/pulse IL-2 therapy. *J Clin Invest* **108**, 51-62 (2001).

91. Yu, D. & Thomas-Tikhonenko, A. Intratumoral delivery of an interferon gamma retrovirus-producing cells inhibits growth of a murine melanoma by a non-immune mechanism. *Cancer Lett* **173**, 145-54 (2001).
92. Bauer, J.A. et al. IFN-alpha2b and thalidomide synergistically inhibit tumor-induced angiogenesis. *J Interferon Cytokine Res* **23**, 3-10 (2003).
93. De Bouard, S. et al. Antiangiogenic therapy against experimental glioblastoma using genetically engineered cells producing interferon-alpha, angiostatin, or endostatin. *Hum Gene Ther* **14**, 883-95 (2003).
94. Piccaluga, P.P. et al. Microdose alpha-interferon shows clinical and antiangiogenic effect in extramedullary myeloid tumor: a case report. *Leukemia* **17**, 986-7 (2003).
95. Picaud, S., Bardot, B., De Maeyer, E. & Seif, I. Enhanced tumor development in mice lacking a functional type I interferon receptor. *J Interferon Cytokine Res* **22**, 457-62 (2002).
96. McCarty, M.F., Bielenberg, D., Donawho, C., Bucana, C.D. & Fidler, I.J. Evidence for the causal role of endogenous interferon-alpha/beta in the regulation of angiogenesis, tumorigenicity, and metastasis of cutaneous neoplasms. *Clin Exp Metastasis* **19**, 609-15 (2002).
97. Lee, E.R. et al. Detailed analysis of structures and formulations of cationic lipids for efficient gene transfer to the lung. *Hum Gene Ther* **7**, 1701-17 (1996).
98. Yang, Y. et al. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc Natl Acad Sci U S A* **91**, 4407-11 (1994).
99. Freimark, B.D. et al. Cationic lipids enhance cytokine and cell influx levels in the lung following administration of plasmid: cationic lipid complexes. *J Immunol* **160**, 4580-6 (1998).
100. Whitmore, M., Li, S. & Huang, L. LPD lipopolyplex initiates a potent cytokine response and inhibits tumor growth. *Gene Ther* **6**, 1867-75 (1999).
101. Blezinger, P. et al. Intravenous delivery of an endostatin gene complexed in cationic lipid inhibits systemic angiogenesis and tumor growth in murine models. *Angiogenesis* **3**, 205-10 (1999).
102. Siders, W.M., Vergillis, K., Johnson, C., Scheule, R.K. & Kaplan, J.M. Tumor treatment with complexes of cationic lipid and noncoding plasmid DNA results in the induction of cytotoxic T cells and systemic tumor elimination. *Mol Ther* **6**, 519-27 (2002).
103. Chen, Q.R., Kumar, D., Stass, S.A. & Mixson, A.J. Liposomes complexed to plasmids encoding angiostatin and endostatin inhibit breast cancer in nude mice. *Cancer Res* **59**, 3308-12 (1999).
104. Krasnici, S. et al. Effect of the surface charge of liposomes on their uptake by angiogenic tumor vessels. *Int J Cancer* **105**, 561-7 (2003).
105. Leung, D.W., Cachianes, G., Kuang, W.J., Goeddel, D.V. & Ferrara, N. Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* **246**, 1306-9 (1989).
106. Park, J.E., Chen, H.H., Winer, J., Houck, K.A. & Ferrara, N. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro

and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. *J Biol Chem* **269**, 25646-54 (1994).
107. Shalaby, F. et al. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* **376**, 62-6 (1995).
108. Carmeliet, P. et al. Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med* **7**, 575-83 (2001).
109. Claesson-Welsh, L. Signal transduction by vascular endothelial growth factor receptors. *Biochem Soc Trans* **31**, 20-4 (2003).
110. LeCouter, J. et al. Mouse endocrine gland-derived vascular endothelial growth factor: a distinct expression pattern from its human ortholog suggests different roles as a regulator of organ-specific angiogenesis. *Endocrinology* **144**, 2606-16 (2003).
111. LeCouter, J. et al. The endocrine-gland-derived VEGF homologue Bv8 promotes angiogenesis in the testis: Localization of Bv8 receptors to endothelial cells. *Proc Natl Acad Sci U S A* **100**, 2685-90 (2003).
112. Tischer, E. et al. The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *J Biol Chem* **266**, 11947-54 (1991).
113. Woolard, J. et al. VEGF165b, an inhibitory vascular endothelial growth factor splice variant: mechanism of action, in vivo effect on angiogenesis and endogenous protein expression. *Cancer Res* **64**, 7822-35 (2004).
114. Park, J.E., Keller, G.A. & Ferrara, N. The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. *Mol Biol Cell* **4**, 1317-26 (1993).
115. Meyer, M. et al. A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. *Embo J* **18**, 363-74 (1999).
116. Fuh, G., Garcia, K.C. & de Vos, A.M. The interaction of neuropilin-1 with vascular endothelial growth factor and its receptor flt-1. *J Biol Chem* **275**, 26690-5 (2000).
117. Whitaker, G.B., Limberg, B.J. & Rosenbaum, J.S. Vascular endothelial growth factor receptor-2 and neuropilin-1 form a receptor complex that is responsible for the differential signaling potency of VEGF(165) and VEGF(121). *J Biol Chem* **276**, 25520-31 (2001).
118. Kaipainen, A. et al. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci U S A* **92**, 3566-70 (1995).
119. Fan, F. et al. Expression and function of vascular endothelial growth factor receptor-1 on human colorectal cancer cells. *Oncogene* **24**, 2647-53 (2005).
120. Wey, J.S. et al. Vascular endothelial growth factor receptor-1 promotes migration and invasion in pancreatic carcinoma cell lines. *Cancer* **104**, 427-38 (2005).

121. Nor, J.E., Christensen, J., Mooney, D.J. & Polverini, P.J. Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. *Am J Pathol* **154**, 375-84 (1999).
122. Nor, J.E. & Polverini, P.J. Role of endothelial cell survival and death signals in angiogenesis. *Angiogenesis* **3**, 101-16 (1999).
123. Tran, J. et al. A role for survivin in chemoresistance of endothelial cells mediated by VEGF. *Proc Natl Acad Sci U S A* **99**, 4349-54 (2002).
124. Katoh, O. et al. Vascular endothelial growth factor inhibits apoptotic death in hematopoietic cells after exposure to chemotherapeutic drugs by inducing MCL1 acting as an antiapoptotic factor. *Cancer Res* **58**, 5565-9 (1998).
125. Bachelder, R.E. et al. Vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells. *Cancer Res* **61**, 5736-40 (2001).
126. Harmey, J.H. & Bouchier-Hayes, D. Vascular endothelial growth factor (VEGF), a survival factor for tumour cells: implications for anti-angiogenic therapy. *Bioessays* **24**, 280-3 (2002).
127. Kim, I. et al. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. *J Biol Chem* **276**, 7614-20 (2001).
128. Reinders, M.E. et al. Proinflammatory functions of vascular endothelial growth factor in alloimmunity. *J Clin Invest* **112**, 1655-65 (2003).
129. Luo, Y. et al. Targeting tumor-associated macrophages as a novel strategy against breast cancer. *J Clin Invest* (2006).
130. Gabrilovich, D. et al. Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo. *Blood* **92**, 4150-66 (1998).
131. Ohm, J.E. & Carbone, D.P. VEGF as a mediator of tumor-associated immunodeficiency. *Immunol Res* **23**, 263-72 (2001).
132. Laxmanan, S. et al. Vascular endothelial growth factor impairs the functional ability of dendritic cells through Id pathways. *Biochem Biophys Res Commun* **334**, 193-8 (2005).
133. Oyama, T. et al. Vascular endothelial growth factor affects dendritic cell maturation through the inhibition of nuclear factor-kappa B activation in hemopoietic progenitor cells. *J Immunol* **160**, 1224-32 (1998).
134. Dikov, M.M. et al. Vascular endothelial growth factor effects on nuclear factor-kappaB activation in hematopoietic progenitor cells. *Cancer Res* **61**, 2015-21 (2001).
135. Ohm, J.E. et al. VEGF inhibits T-cell development and may contribute to tumor-induced immune suppression. *Blood* **101**, 4878-86 (2003).

CHAPTER 1

LIPOSOME-DNA COMPLEXES INFUSED INTRAVENOUSLY INHIBIT TUMOR VASCULAR PRODUCTION AND ELICIT ANTITUMOR ACTIVITY IN DOGS WITH SOFT TISSUE SARCOMA

ABSTRACT

Intravenous gene delivery using liposome–DNA complexes (LDC) has previously been shown to elicit antitumor activity, but only in rodent tumor models. This study was conducted to determine whether intravenous infusions of LDC could target gene expression to cutaneous tumor tissues in a large animal spontaneous tumor model and whether repeated treatments had an effect on tumor growth or angiogenesis. A total of 13 dogs with cutaneous soft tissue sarcomas were enrolled in the study and were randomized to receive a series of 6 weekly infusions of LDC containing either canine endostatin DNA or DNA encoding an irrelevant gene (luciferase). Serial tumor biopsies were obtained to assess transgene expression, tumor microvessel density (MVD), and intratumoral leukocyte inflammatory responses. We found that intravenous infusion of LDC did not result in detectable gene expression in cutaneous tumor tissues. However, two of 13 treated dogs had objective tumor responses and

eight dogs had stable disease during the treatment period. In addition, a significant decrease in tumor MVD was noted in eight of 12 treated dogs at one or more time point over the course of treatment. These results suggest that intravenous infusions of LDC may elicit nonspecific antitumor activity and inhibit tumor angiogenesis.

BACKGROUND

Inhibition of tumor angiogenesis continues to hold great promise as an approach to controlling tumor growth [1–7]. Moreover, several recent clinical trials investigating angiogenesis inhibitors such as VEGF antagonists have shown promising results in patients [8–12]. A variety of different angiogenic inhibitors, including synthetic molecules and endogenous antiangiogenic substances, have been described, including endostatin [3,13,14]. However, early clinical trials of recombinant endostatin failed to demonstrate convincing antitumor activity, although the compound was well tolerated [15,16]. The lack of endostatin activity in the early trials was attributed in part to the inability to produce sustainable systemic levels in the bloodstream [3,5,14].

Systemic gene delivery offers an alternative to repeated administration of protein drugs. For example, use of systemic gene delivery to express endostatin or other angiogenesis inhibitors to control tumor growth has been reported previously in mouse models [17–33]. However, potential hurdles to applying systemic gene therapy in humans include achieving efficient in vivo transfection, attaining durable

in vivo gene expression, and administering the gene delivery vectors repeatedly [2,17,23]. The use of nonviral gene delivery systems, based principally on liposome–plasmid DNA-based systems, may help address some of these problems, particularly the issue of repeated gene delivery. Use of cationic liposome–DNA complexes (LDC) for inhibition of tumor angiogenesis has the added potential advantage that the complexes have been shown to preferentially target the angiogenic endothelium in tumor tissues in vivo, an effect that may be enhanced by preinjection of protamine [34,35]. Thus, it may be possible to express antiangiogenic genes directly in angiogenic blood vessels of tumors by intravenous administration of LDC. The LDC themselves have also been shown to elicit strong activation of innate immunity and release of several potent antiangiogenic cytokines, including IL-12, IFN-γ, and IFN-α [36–38]. Thus, use of LDC for delivery of angiogenesis-inhibiting genes may inhibit tumor angiogenesis by eliciting both gene-specific and gene nonspecific effects.

A key question in the gene therapy field is whether intravenous infusion of LDC could target gene delivery and expression directly to tumor tissues in extrapulmonary sites such as tumors in cutaneous tissues. If successful, such an approach would have considerable appeal for targeting of metastatic tumors not accessible to direct injection. Therefore, we designed a study in a large animal tumor model to determine whether intravenous infusions of LDC could generate transgene expression in cutaneous tumor tissues and also inhibit tumor angiogenesis. Pet dogs with large, spontaneous soft tissue sarcomas were utilized in this study, as this tumor in dogs is refractory to most conventional therapies other than surgical excision and is also

amenable to repeatable tumor biopsies [39–41]. The study was designed in part to compare tumor responses in dogs infused with canine endostatin DNA to dogs treated with control (luciferase) DNA to determine whether endostatin gene delivery was effective in inhibiting tumor angiogenesis. The control luciferase vector was included to account for the potential nonspecific antiangiogenic and antitumor effects of the LDC vector itself [36,37]. The dose of LDC delivered to dogs in this study was based on results of a prior Phase I trial of intravenous gene delivery in dogs with lung tumor metastases [42]. The study end points were gene expression in tumor tissues, the effects of LDC infusion on tumor angiogenesis, and the effects of LDC infusion on intratumoral inflammatory responses. The effects of treatment on clinical parameters and circulating levels of VEGF and basic FGF (bFGF) were also assessed.

We found that intravenous infusion of LDC did not result in detectable levels of transgene expression in any tumor tissues, even using a very sensitive detection assay for gene expression. However, we did find that intravenous infusions of LDC resulted in significant inhibition of tumor angiogenesis in more than half of the treated dogs and also elicited a strong infiltrate of CD8+ T cells in some tumors. These effects were independent of the gene delivered, indicative of a nonspecific vector effect. Our results therefore suggest that intravenous delivery using the current LDC formulations may not be effective for systemic gene delivery to tumors, but the LDC infusion may inhibit tumor angiogenesis and elicit antitumor immunity in a gene nonspecific fashion.

MATERIALS AND METHODS

Plasmid constructs and assessment of in vitro expression

The pMB517 canine endostatin expression vector was prepared by cloning the canine endostatin cDNA into a previously described pMB75.6 plasmid expression vector. The pMB75.6 expression vector utilized the CMV immediate-early promoter enhancer region, a synthetic intron (pGL3) immediately upstream of the start site, an SV40 early poly(A) site, and the kanamycin resistance gene, as described previously.43 The cDNA for canine endostatin was synthesized (Operon Inc., Huntsville, AL) based on published sequence data and sequence information provided by Entremed Inc. (Rockville, MD). The canine endostatin cDNA was also linked to the human Ig secretory signal to facilitate secretion of the endostatin gene product. The luciferase cDNA was kindly provided by R Debs (California Pacific Research Institute) and was also cloned into the pMB75.6 expression vector to produce the pMB408 vector. Expression of the canine endostatin gene construct was assessed by transfecting a canine melanoma cell line with the plasmid pMB517 construct or with an irrelevant plasmid (pMB408), using lipofection. Immunohistochemistry was performed using a polyclonal rabbit antiserum raised against canine endostatin (Cytimmune Sciences, Rockville, MD) to demonstrate endostatin expression in the canine cell line. Briefly, fixed cells were incubated with appropriately diluted primary antibody for 20 min at room temperature, washed, incubated with biotinylated anti-

rabbit antisera (Jackson ImmunoResearch, West Grove, PA), then washed and incubated with HRP-conjugated streptavidin (Jackson ImmunoResearch), then washed and incubated with diaminobenzidine (DAB) substrate (Sigma-Aldrich, StLouis, MO), then briefly counterstained with hematoxylin and coverslipped. Controls included use of nonimmune rabbit Ig and immunostaining of nontransfected cells and cells transfected with irrelevant plasmid DNA. Immunostained cells were photographed using an Olympus microscope.

Secretion of endostatin was assessed by commercial ELISA assay (Cytimmune). Briefly, cells in six-well plates were transfected by lipofection, then rinsed and cultured for an additional 24h. Supernatants were collected and assayed for endostatin production using a commercial canine endostatin-specific ELISA (Cytimmune), according to the manufacturer's directions. Supernatants from mock-transfected cells were used as controls. Endostatin concentrations were determined by comparison to a standard curve generated with recombinant canine endostatin (Cytimmune). The biological activity of the secreted canine endostatin construct was also assessed using an endothelial cell migration inhibition assay. Briefly, nontransformed murine capillary endothelial cells (CD3 cells, kindly provided by Clement Diglio, WayneState University) were grown to confluence in 150mm tissue culture dishes (Corning). Then, using a sharp blade, a scratch was made through the center of the culture to produce a sharply demarcated zone denuded of viable cells. The plates were washed to remove detached cells, then supernatants from transiently transfected canine melanoma cells were added at a 1:1 dilution to the CD3 cells, which were then cultured for an additional 24h. Then, the cells were fixed in methanol and

counterstained with hematoxylin. Using a dissecting microscope, the number of endothelial cells that had migrated into the denuded zone of the plate were counted in 10–20 high-power fields per plate to determine the mean number of migrating cells. Controls included cells cultured with nontransfected CHO supernatants and with supernatants from CHO cells transfected with irrelevant plasmid DNA.

DNA and liposome preparation

Plasmid DNA was propagated in Escherichia coli and isolated by alkaline lysis, followed by column chromatography, as reported previously [44]. The endotoxin content of the DNA was determined by LAL assay (Biowhittaker,Walkersville,MD) and was found to be less than 0.25EU/mgDNA. The stock DNA solution (prepared in a 10mM solution of Tris-HCl (2-amino-2-hydroxymethyl-1,3-propanediolhydro-chloride), pH 8.0 at 5.0 mg/ml, was diluted to 0.625 mg/ml with 5% w/v dextrose (D5W). Liposomes were prepared by dissolving cholesterol (Sigma ChemicalCo., St Louis, MO) and the cationic lipid DOTIM (octadecenolyoxy{ethyl-2-heptadecenyl-3 hydroxyethyl}imidazolinium chloride) in chloroform (EM Sciences, Gibbstown, NJ) at equimolar concentrations in a 3-l round-bottom flask. The clear solution was rotated on a Buchi rotovapor R-134 at 301C for 30 min and hydrated in 5% w/v dextrose (McGraw, Irvine, CA) to produce a nominal concentration of 40 mM. The liposome dispersion was extruded through successive filters (0.4, 0.2, 0.1, and 0.05 mm) to obtain a vesicle size of approximately 100nm as measured by a Nicomp C-370 particle sizer. Immediately prior to infusion, CLDC were prepared by first dissolving liposomes in 5% dextrose in water at a concentration of 100μl liposomes

per 1ml of 5% dextrose solution, as described previously [45]. Next, the plasmid DNA was added to the liposome solution at a final concentration of 100μg DNA/ml solution and complexes were formed by gentle pipetting. The LDC were infused intravenously within 15 min of preparation.

Preparation of BODIPY-labeled LDC

Fluorescently labeled cationic liposomes were prepared by addition of BODIPY-labeled cholesteryl (MolecularProbes, Eugene, OR) to the unlabeled cholesterol and DOTIM at the time the liposomes were prepared. The BODIPY-labeled cholesterol was added to unlabeled cholesterol at a 1:5M ratio and the labeled and unlabeled cholesterol was added at a 1:1M ratio with DOTIM, then dried down. The labeled liposomes were rehydrated as described above, then used to prepare LDC, which were infused intravenously as for unlabeled LDC.

Trial design

Pet dogs with biopsy-confirmed cutaneous soft tissue sarcomas were eligible for entry into the study. These studies were approved by the Institutional Animal Care and Use Committees at the National Jewish Medical and Research Center and at Colorado State University. Dogs with concurrent medical diseases or those receiving concurrent immunosuppressive therapy or prior radiation therapy to the tumor site were excluded from entry. Dogs were randomized by block randomization scheme

(Research Randomizer) to receive treatment with either canine endostatin DNA or with irrelevant (luciferase) DNA. Investigators administering the treatment were blinded as to treatment groups. Dogs were treated once weekly with an intravenous infusion of LDC prepared with either endostatin or luciferase plasmid DNA. A total of six treatments were administered over the course of 6 weeks. The tumor was then surgically removed 3 days after the last treatment and analyzed by routine histology and immunohistochemistry.

Treatment and monitoring

Prior to treatment, all dogs had an indwelling intravenous catheter placed in either the cephalic vein or the medial saphenous vein, without sedation. Using a syringe pump (Harvard Apparatus, Holliston, MA), the LDC solution was infused as a continuous rate infusion over a 90-min period. Each dose of LDC consisted of 20μg/kg body weight of plasmid DNA. This dose of LDC and rate of infusion was selected based on preclinical studies of intravenous LDC infusion and gene expression in rabbits (D Liggitt, unpublished data) and on the results of a phase I study completed previously in pet dogs with lung tumor metastases [42]. Thus, for a typical 25 kg dog receiving a 20μg/kg dose of plasmid DNA, the total DNA dose delivered was 500 μg and the infusion rate was 55μl/min. For the first 24h after the first infusion, dogs were kept under observation in the hospital and body temperature and respirations were monitored every 2h. For subsequent treatments, dogs were monitored for the first 4–6 h post-infusion, then returned home with their owners.

Routine complete blood counts and serum biochemistry analysis were performed before treatment, 24h after the first treatment, and again on weeks 1, 4, and 6 of the study. Tumor measurements in three dimensions were obtained using calipers at each treatment and the tumor volume was calculated. Tumor responses were classified as partial response if tumor volume decreased by >50% from pretreatment measurements and complete response if the tumor regressed completely and was no longer detectable. Stable disease was defined as tumor volume that did not increase or decrease by >50% from starting values, whereas progressive disease was defined as tumor volume that increased >50% from starting values.

Tumor biopsy and assessment of tumor microvessel density and inflammation scores

Tumor biopsies were obtained under local anesthesia by wedge biopsy immediately prior to the first treatment, at 24h after the first treatment, at 7 days after the first treatment, and on week 6 of the study. Portions of the biopsy were flash-frozen for RNA analysis (see below) while the remainder of tumor tissue was embedded in Tissue-Tek OCT compound (TedPella Inc., Redding, CA) and snap-frozen in isopentane. Tissues were cryosectioned to a thickness of 4μm and adhered to positively charged glass slides. For identification of tumor microvessels and angiogenic endothelium, a mAb specific for human CD146 (clone P1H12, Chemicon, Temecula, CA) that cross reacts with dog endothelium was utilized [46]. After fixation in acetone and blocking nonspecific binding, tissues were incubated first with

the primary antibody, followed by donkey anti-mouse IgG antiserum (Jackson ImmunoResearch), then with streptavidin–HRP and AEC substrate (Vector Laboratories, Burlingame, CA), followed by hemtoxylin counterstain. Negative controls included incubation with irrelevant mAb and omission of the primary antibodies. Staining of normal canine splenic tissue was utilized for positive control for microvessel density (MVD) and inflammation assays.

For quantitation of tumor MVD, four photomicrographs at 10X magnification were obtained by digital camera (Leica Microscope equipped with digital camera with SPOT Advanced Imaging software). Utilizing Adobe Photoshop and Reindeer Graphics Quantitative Analysis Plug-ins (Asheville, NC), the photomicrographs were converted to binary images and the number of pixels per section was determined digitally. MVD values for each tumor biopsy were calculated based on the average number of vessels for the four 10X fields.

Pre- and post-treatment tumor biopsies were also immunostained for detection of tumor-infiltrating leukocytes (TIL). Monoclonal antibodies specific for canine CD4 (clone YKIX 302.9, Serotec, Oxford, UK), canine CD8 (clone YCATE 55.9, Serotec), and canine CD11b (clone CA16.3E10, Serotec) were utilized for these studies. Appropriately diluted antibodies were incubated with acetone fixed sections as described above, along with appropriate negative controls. The degree of leukocyte infiltration was assessed by examining at least 10 random high-power (40X) fields per slide. Semi-quantitative scores of the degree of leukocyte infiltration were

assigned based on the following system: 0 = no leukocytes found; 1 = 1–5 cells/high-power field (hpf); 2 = 6–10 cells/hpf, 3 = 11–15 cells/hpf; and 4≥15 cells/hpf.

Quantitative RT-PCR assay for transgene and cytokine gene expression in tumor tissues*

Quantitative RT-PCR was utilized to detect transgene expression in tumor tissues, as previously described [43]. Tumor biopsies were obtained pretreatment and again 24h after the first LDC infusion and were then snapfrozen in isopentane in dry ice and stored at -80^{0}C prior to analysis. After thawing, tumor biopsies were immersed in RNA storage solution (RNA Later, Invitrogen,Carlsbad, CA) and then pulverized using disposable tissue homogenizers. The RNA was then extracted, reverse transcribed to cDNA, treated with DNAse, and amplified using a set of primers specific for a synthetic intron (pgl) incorporated between the promoter and the start site of the gene expression vector pMB75.6 used in these experiments [43]. The primer and probe combinations were those described previously and spanned the 5′UTR region of the expression vector. This strategy allowed amplification of the synthetic intron sequence from vector-encoded mRNA and avoided contamination with endogenous transcripts. This assay was found to be sensitive to one copy of template DNA when template DNA was added to control tissue homogenates (data not shown). Quantitation of mRNA transcripts was performed on an ABI Prism 7000 cycle sequencer and the level of mRNA expression was indexed to mRNA levels of a housekeeping gene (GAPDH). *This work was performed by research associate Lisa Coro.

Measurement of plasma VEGF and bFGF concentrations

Plasma was collected from each dog prior to treatment, at 8, 12, and 24h after the first treatment, and then again at weeks 1, 2, and 6 of treatment. Plasma was stored frozen at -80^{0}C prior to analysis. Plasma VEGF and bFGF concentrations were determined utilizing commercial ELISA assays (R&D Systems, Minneapolis, MN), according to the manufacturer's directions. Both of these assays have been previously validated for use in detecting canine VEGF and canine bFGF [47–49].

Endothelial cell toxicity assay

Nontransformed mouse capillary endothelial cells (a gift of C. Diglio, Wayne State University) were used to assess endothelial toxicity of LDC. Endothelial cells were plated at a density of $5x10^4$ cells/well in 96-well plates in complete medium (minimum essential medium {InvitrogenCorp., Grand Island, NY}), supplemented with 10% fetal bovine serum (Sigma, St Louis, MO), penicillin streptomycin,L-glutamine, essential amino acids, and nonessential amino acids (Gibco, Grand Island, NY)). Cells in quadruplicate wells were incubated with serial dilutions of LDC prepared with noncoding plasmid DNA, at the same concentration as used for in vivo infusions. After addition of LDC, the cells were incubated for 24h and then cell viability was determined by MTT assay. Briefly, cells were incubated with MTT solution (Sigma) for 2h, lysed with acidified isopropanol, then the optical density

determined by optical scanner (MultiscanAscent; Thermo Labsystems, Franklin, MA). Cell survival was calculated by determining the mean OD 570nm for quadruplicate wells.

Tumor inhibition assay

The ability of LDC to elicit cytokines capable of inhibiting fibrosarcoma tumor cell growth in vitro was assessed using spleen supernatants from mice injected intravenously with LDC prepared with noncoding DNA. Briefly, supernatants were prepared by overnight culture of spleen cells obtained from LDC-injected mice (four mice per group) 3h after intravenous injection of LDC, at a dose of 10μg DNA per mouse or untreated control mice. Supernatants were collected and stored frozen at -80^{0}C prior to assay. Murine fibrosarcoma cells (MCA 2.1; derived in our laboratory by methylcholanthrene induced carcinogenesis in the skin of mice) were cultured in 96-well plates at a density of 5x10^3/well and incubated with serial dilutions of supernatants from spleen cells of control or LDC-injected mice to assess their effects on tumor cell viability and proliferation. After 24h of incubation of quadruplicate wells with serial dilutions of spleen cell supernatants, the number of viable cells in each well was determined by MTT assay as described above.

Statistical analysis

Statistical differences in tumor microvessel densities were assessed using one way analysis of variance (ANOVA) to compare pre-treatment tumor MVD to tumor MVD

at other time points. Differences in inflammatory pre- and post-treatment inflammatory infiltrates in tumor tissues were assessed by unpaired t-test. Comparison of differences in tumor cell viability and endothelial cell viability following treatment with spleen cell supernatants or LDC, respectively, was made by paired t-test.

RESULTS

*In vitro expression of canine endostatin cDNA**

To assess expression of canine endostatin by the pMB5176 vector, cell lines were transfected with plasmid DNA and expression was quantitated by canine endostatin ELISA and by immunohistochemistry. Production of secreted endostatin was detected in supernatants of cells transfected with the canine endostatin construct, but not in supernatants from cells transfected with irrelevant DNA (Figure 1a). Canine endostatin activity was also detected by immunohistochemistry in transfected canine melanoma cells (data not shown). Production of biologically active endostatin by transfected cells was demonstrated by inhibition of endothelial cell migration (Figure 1b). Therefore, the pMB517 construct produced and secreted biologically active canine endostatin following transfection of canine cell lines. *This data was acquired by the primary investigator Dr. Steve Dow.

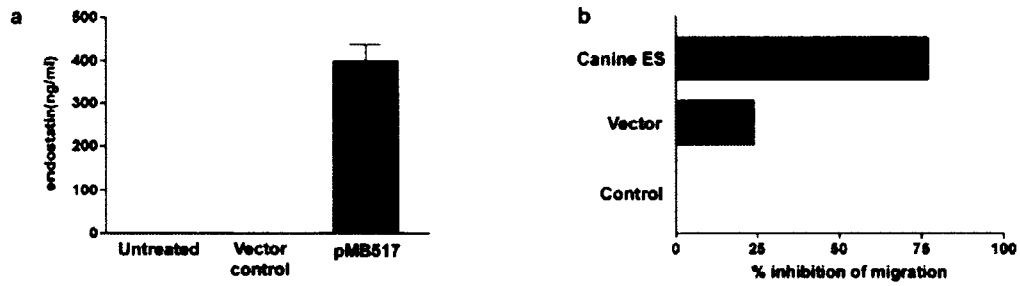


Figure 1. Secretion of canine endostatin by transfected cells and demonstration of biological activity. (a) To assess the ability of the pMB571 vector to produce and secrete canine endostatin, canine melanoma cells in triplicate wells were transfected in vitro using lipofection. Controls included nontransfected cells and cells transfected with a luciferase plasmid. At 24h after transfection, supernatants were collected and assayed for the presence of canine endostatin protein by specific ELISA (Cytimmune). Cells transfected with pMB517 vector produced substantial amounts of endostatin. **(b)** The biological activity of the secreted endostatin was assessed using an endothelial migration assay as described in materials and methods. Supernatants from canine melanoma cells transfected with the canine endostatin construct (pMB571) or a luciferase control plasmid by lipofection were collected 24 h post-transfection and diluted 1:1 with medium and the effects on endothelial migration were assessed. Incubation with supernatants from pMB517-transfected cells resulted in significant ($P<0.05$) inhibition of endothelialcell migration, compared to control supernatants. Similar results were obtained in one additional experiment.

Treatment responses and tumor responses following treatment with LDC encoding endostatin or luciferase DNA

Dogs enrolled in this study all had cutaneous soft tissue sarcomas where they were accessible for measurement and biopsy. A total of 13 dogs were enrolled in the study and received at least one infusion of LDC (Table 1). One dog (number 13; Table 1) was withdrawn shortly after entering the study due to progressive tumor growth, while the rest of the dogs received the full course of six treatments. Seven dogs were treated with LDC containing the endostatin gene and six received infusions

Of LDC containing the luciferase gene. All dogs tolerated the LDC infusions well. Most dogs developed fever within 6h of beginning the infusion, and most dogs also

developed transient lymphopenia for the first 24h following treatment, consistent with what has been reported previously (data not shown) [42]. Significant changes in other hematologic or biochemical parameters were not observed during the course of the study in any treated dogs (data not shown). For the 13 dogs treated in the study, objective tumor responses were observed in two animals: one dog experienced complete tumor regression, one dog had partial tumor regression, while eight dogs had stable disease and three dogs experienced progressive tumor growth during the course of the 6-week study.

Table 1. Clinically observed tumor responses and effects on microvessel density in dogs with soft tissue sarcoma treated with intravenous infusion of LDC

Dog	Treatment	Clinical Response	MVD
1	ES	SD	Decreased
2	ES	SD	Decreased
3	ES	SD	Decreased
4	ES	SD	Decreased
5	ES	PD	Decreased
6	ES	SD	Unchanged
7	ES	SD	Increased
8	Lucif	CR	Decreased
9	Lucif	PD	Decreased
10	Lucif	SD	Unchanged
11	Lucif	SD	Decreased
12	Lucif	PR	Unchanged
13	Lucif	PD	NA

Clinical data for 13 dogs with soft tissue sarcoma enrolled in a clinical study of intravenous LDC gene delivery. Of the 13 dogs enrolled, seven dogs were treated with LDC containing canine endostatin plasmid DNA (ES) and six dogs were treated with LDC containing luciferase DNA (lucif). Tumor responses were classified as CR (complete regression of all visible tumor), PR (partial regression; >50% decrease in overall tumor volume), SD (stable disease, no increase or decrease in tumor volume >50%), and PD (progressive disease, >50% increase in tumor volume). Tumor microvessel density (MVD) was considered decreased if the MVD was decreased significantly ($P<0.05$) at one or more time points over the course of treatment compared to pretreatment MVD, or increased if the MVD increased significantly over the course of treatment compared to pretreatment MVD. The tumor MVD was considered unchanged if no significant differences in MVD were noted. NA = not available for analysis.

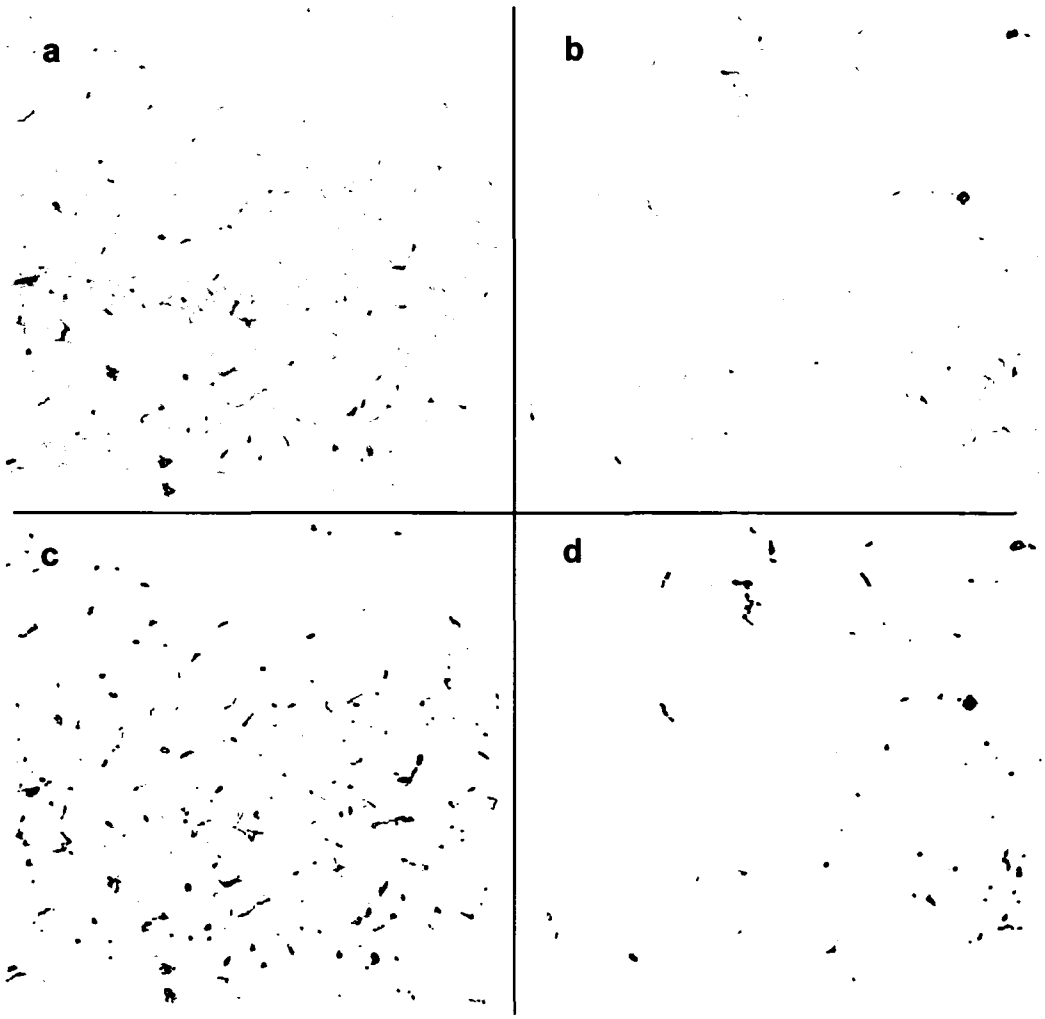
Effects on tumor MVD following LDC infusion

Tumor biopsies were processed by immunohistochemistry for assessment of tumor MVD, using the P1H12 antibody. An example of tumor MVD changes in pre- and post-treatment tumor biopsies is illustrated in Figure 2, as is the conversion of the initial digital images into binary images for quantitation of MVD. The values for tumor MVD were determined from pre-treatment biopsies and from biopsies taken 7 days after the first infusion of LDC and again at day 42 of the study (Figure 3 and Table 1). It should be noted that although there was a moderate range of starting MVD values between tumors in different dogs, within a given dog the tumor MVD values were relatively consistent from biopsy to biopsy, suggesting that sample biopsy site variability was not a major factor in the MVD analysis. Some biopsy samples however were inadequate, or not available, for MVD analysis due to tissue necrosis, increased presence of surrounding tissue with lack of sufficient tumor tissue, or due to complete remission (dog 8). MVD at these time points was thus not represented as reflected in Figure 3.

When MVD values for pretreatment biopsies were compared to those during and at the end of treatment, we found that tumor MVD decreased significantly at one or more time points in eight of the 12 treated dogs. Of these eight dogs, two dogs initially experienced a significant decrease in tumor MVD at day 7 of the study, but by day 42 the tumor MVD had increased again to values that were not statistically different from pretreatment values. Additionally, two of these dogs experienced a significant decrease in tumor MVD at day 7 however either lacked a sufficient biopsy

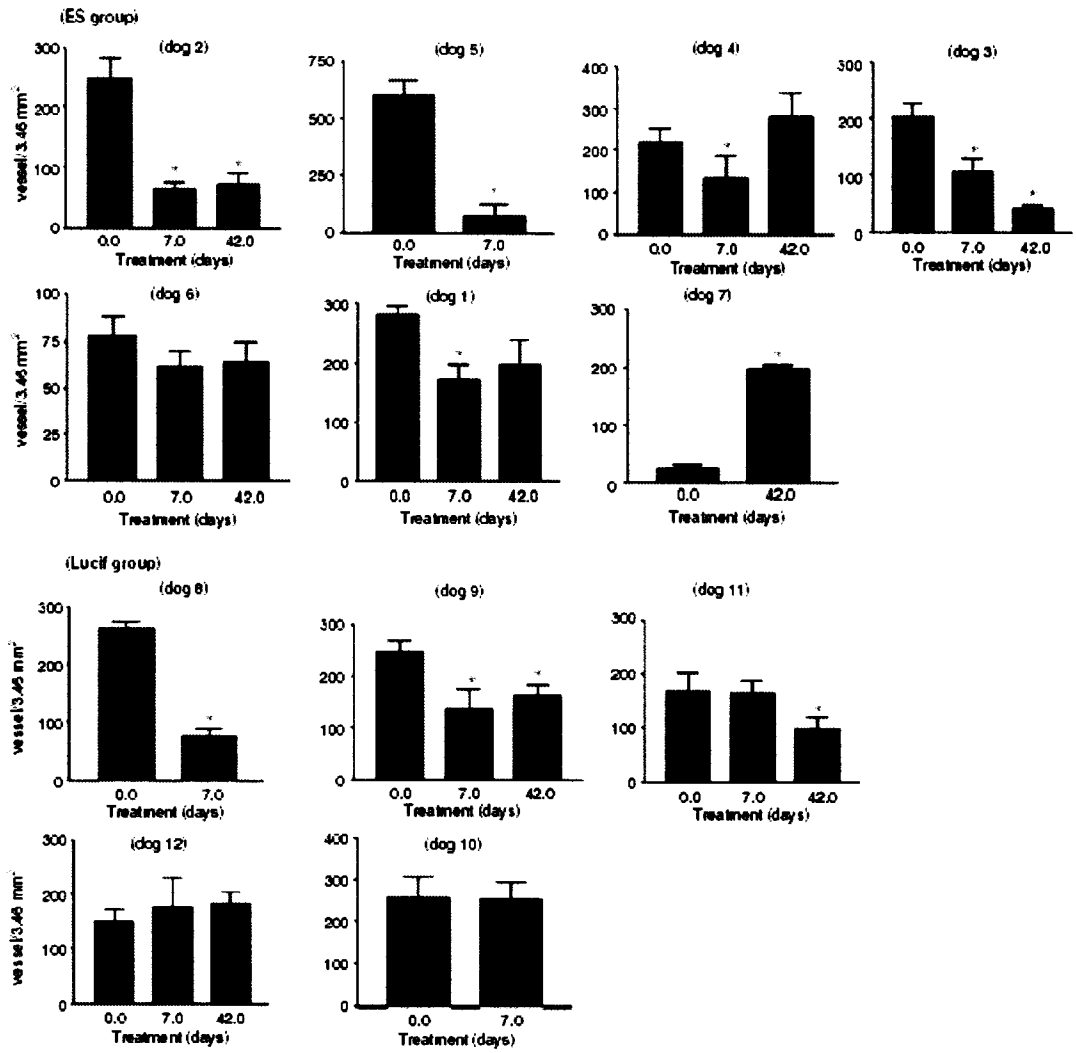
sample to determine MVD at day 42 (dog 5) or had no tumor tissue to biopsy due to complete remission (dog 8). Overall, three dogs had no significant change in tumor MVD over the course of treatment, while one dog developed a significant increase in tumor MVD at the end of the study. When tumor MVD responses were compared between treatment groups, there was no statistically significant difference between response rates in dogs treated with endostatin DNA and dogs treated with luciferase DNA.

Figure 2. Assessment of tumor MVD by immunohistochemistry and digital analysis



Tumor biopsies were obtained from a dog prior to treatment and again after six infusions of LDC encoding the canine endostatin gene. Cryosectioned tissues were then immunostained with the P1H12 antibody for quantitation of MVD, as described in Materials and methods. Multiple low-power fields from each biopsy were digitally photographed and the images converted to binary images (Reindeer Graphics) for quantitation of MVD. Pre-treatment tumor biopsies demonstrating tumor microvessels by light microscopy (vessels are red, panel a) and again after the image was converted to a binary image for quantitation (vessels are black; panel c) as shown. In panel b (and binary image in panel d), post-treatment biopsies demonstrated a significant reduction in tumor MVD.

Figure 3. Tumor angiogenic responses to intravenous infusions of endostatin or luciferease LDC



Serial tumor biopsies were obtained from 12dogs that each received a series of six infusions of LDC containing either canine endostatin plasmid DNA (ES group) or luciferase plasmid DNA (Lucif group). Tumor biopsies were obtained before treatment, at day 7 after the first treatment, and on day 42 after six treatments had been administered. The tumor biopsies were cryosectioned and immunostained for identification of tumor microvessels by immunohistochemistry and tumor MVD was quantitated for each biopsy as described in materials and methods, and the mean (± s.e.) MVD for each biopsy was plotted. (ES group) Assessment of tumor MVD in seven dogs treated with six infusions of LDC encoding the canine endostatin cDNA. (Lucif group) Assessment of tumor MVD in five dogs treated with six infusions of LDC encoding luciferase DNA (*P< 0.05, for mean MVD value, compared to pretreatment MVD). In some dogs not all biopsy samples were adequate to appropriately assess MVD, thus those time points are not represented above (dogs 5, 7, 8, 10).

Assessment of transgene expression in tumor tissues following in vivo gene delivery

Transgene expression was assessed in pretreatment and 24h post-treatment tumor biopsies from study dogs. For this analysis, a sensitive RT-PCR assay was utilized, based on the unique design of the expression plasmid. In vitro plasmid add-back studies revealed that the assay used was sensitive down to the level of approximately 10 gene copies (data not shown). When 24h post-treatment tumor biopsies were analyzed from the treated dogs, transgene expression was not detected in any of the samples analyzed. The failure to detect transgene expression was not, however, due to RNA degradation, as GAPDH mRNA was detectable in all the samples. Serum from several dogs obtained at 8 or 24 h after their first LDC infusion was also evaluated by endostatin ELISA for the presence of circulating canine endostatin activity, but in no case was endostatin detected (data not shown). Based on these results, it appeared therefore that intravenous infusion of LDC was not an effective means of generating transgene expression in tumor tissues of dogs with established tumors. It is still possible, however, that low levels of transgene expression may have occurred in normal tissues such as the lung. However, given that tumor inhibition by endostatin generally requires exposure to high doses in vivo, the amount theoretically produced in the lungs and released into circulation is unlikely to have been biologically relevant [14,15]. *This data was acquired by research associate Lisa Coro.

Localization of labeled LDC in tumor tissues

To determine whether the LDC were in fact delivered to the tumors, LDC labeled with the fluorescent dye BODIPY were infused in one dog and tumor biopsies were obtained pretreatment and again 120 min post-infusion. When the tumor tissues were examined by fluorescence microscopy, we observed numerous cells containing BODIPY +LDC within the tumor tissues (Figure 4). The location of the labeled LDC suggested that they were present in or adjacent to blood vessels, although this could not be confirmed by dual labeling studies. Thus, intravenous infusion of LDC resulted in delivery of the complexes to tumor tissues. *This data was acquired by the primary investigator Dr. Steve Dow.

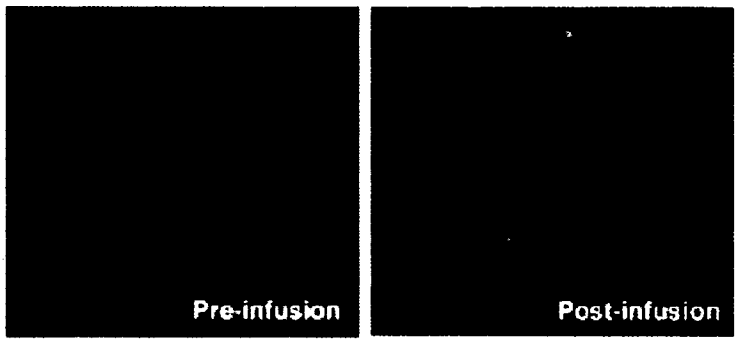
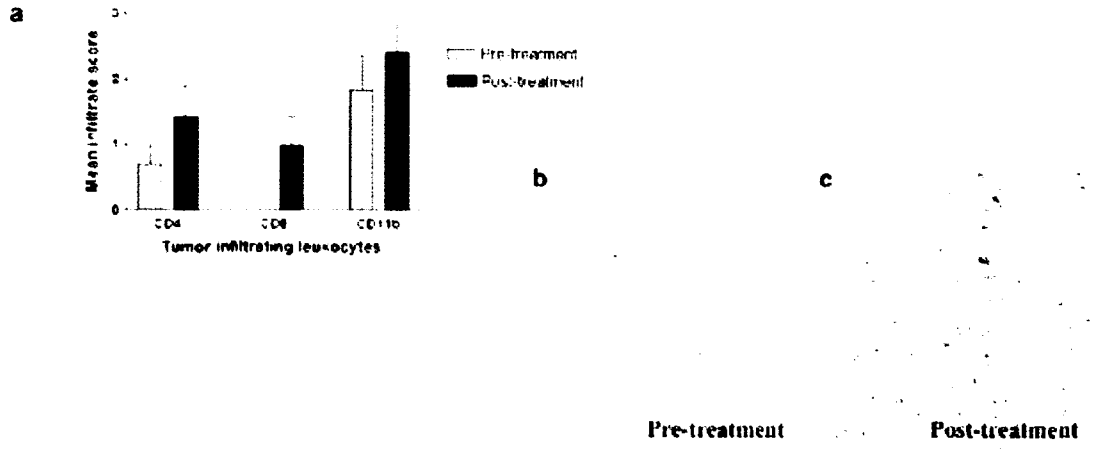


Figure 4 Localization of fluorescently labeled LDC in tumor tissues. To determine whether LDC were taken up by tumor tissues following intravenous infusion, LDC were prepared using BODIPY-labeled liposomes and infused intravenously into a dog with soft tissue sarcoma, as described in materials and methods. A pretreatment tumor biopsy was obtained, the labeled LDC were then infused over 90 and 60 min later, a second tumor biopsy was obtained. Tissues were frozen, then cryosectioned and examined by fluorescence microscopy. Several cells with BODIPY+ material are visualized in the post-infusion biopsy sample.

Infiltration of leukocytes in tumor tissues following LDC infusion

To assess the response of TIL to LDC infusion, pretreatment and day 42 tumor biopsies were immunostained for quantitation of numbers of CD4+ and CD8+ T cells and CD11b+ macrophages in tumor tissues (Figure 5a). The numbers of CD4+ T cells and CD11b+ macrophages were not significantly different in tumor tissues following LDC treatment when compared to pretreatment values. However, four dogs developed an increase in CD8+ T cells in tumor tissues following treatment, whereas CD8+ T cells were not observed in any tumor biopsies obtained prior to the start of treatment. In one treated dog, there was a dramatic increase in infiltrating CD8+ T cells following treatment (Figure 5b and c). Thus, infusion of LDC appeared to trigger an influx of CD8+ T cells into tumor tissues in some dogs, although the functional status of T cells was not assessed in this study.

Figure 5. Leukocyte inflammatory responses in tumors before and after LDC infusion



Pre-treatment and post-treatment (day 42) biopsy samples were available from 9 of the 12 dogs with soft tissue sarcoma after six treatments with LDC. Specimens were cryosectioned and immunostained with mAbs specific for canine CD4, CD8, or CD11b, as described in materials and methods. A total of 10 random high-power (40X) fields of each slide were then examined microscopically to determine the degree of leukocyte infiltration. Tumor infiltrate scores were assigned based on a scale of 0–4, where a score of 0 was equivalent to 0 infiltrating leukocytes, 1 was equivalent to 1–5 infiltrating cells, 2 was equivalent to 6–10 infiltrating cells, 3 was equivalent to 11–15 infiltrating cells, and 4 was equivalent to $\geq$15 infiltrating cells, as described in materials and methods. (a) Mean ($\pm$ s.e.) infiltrate scores for pre- and post-treatment tumor biopsies were plotted for CD4+ T cells, CD8+ T cells, and macrophages. Statistical analysis of infiltrate scores did not reveal significant differences between pre- and post-treatment biopsies, although the difference in CD8+ T cell infiltrates approached significance ($P = 0.08$). (b, c) Marked infiltration of CD8+ T cells (red staining; 20X) into tumor tissues (c) was observed in one dog following six infusions of LDC encoding the endostatin gene, whereas pretreatment tumor biopsies (b) did not contain CD8+ T cells. Inset shows higher power view (40X) of the T-cell infiltrate.

LDC elicit endothelial cell cytotoxicity

The preceding results suggested that most of the effects observed in the study (tumor regression or tumor stasis, decrease in MVD, infiltration of CD8 T cells) are likely to have been elicited by the vector itself. These nonspecific vector-induced effects could

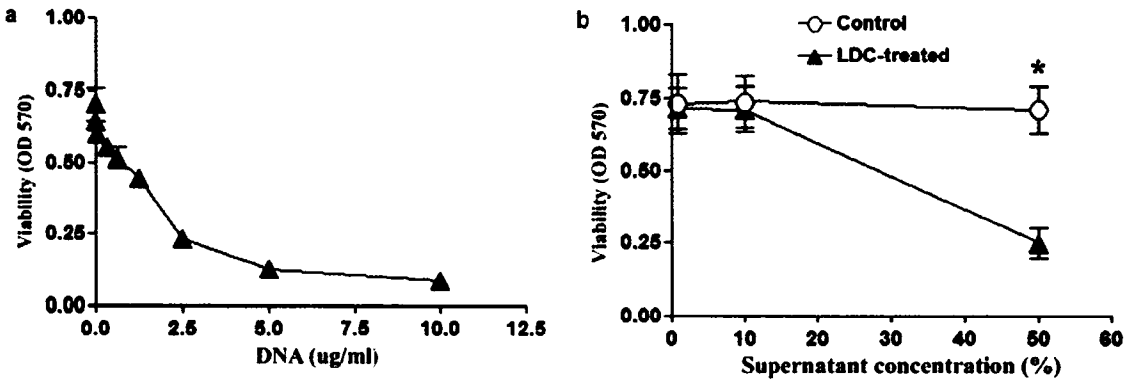
have included direct endothelial cell toxicity by LDC or inhibition of tumor growth by cytokines elicited by the immune stimulatory effects of LDC [36,50]. To assess direct effects of LDC on endothelial cells, primary cultures of mouse endothelial cells were used, as normal canine endothelial cell lines were not available. LDC are known to be toxic to other cells in vitro, but their effects on endothelial cells have not been reported previously. We found that endothelial cells were in fact quite sensitive to killing by LDC. For example, incubation of endothelial cells with doses of LDC as low as 0.25μg/ml elicited substantial killing (Figure 6a). Thus, it is possible that inhibition of tumor angiogenesis by LDC infusions may have been mediated in part by direct endothelial cell killing, particularly in dogs where significant inhibition of angiogenesis was observed as early as 7 days following the first LDC infusion (Figure 3). In support of this theory, LDC could be localized in tumor tissues within 2 h of infusion (Figure 4).

Infusion of LDC triggers release of cytokines with tumor inhibitory activity

Two dogs in this study experienced significant tumor regression and eight dogs experienced lack of tumor growth over the 6-week treatment period. Although angiogenesis inhibition might account for some of this effect, we also observed that tumor responses did not always correlate with changes in MVD. For example, one dog with a partial tumor response and one dog with stable disease at the end of the study had no significant change in MVD. Thus, it was possible that immunological mechanisms might also account in part for the antitumor activity observed. For

example, previous studies in mice have shown that LDC injected intravenously trigger potent release of cytokines with antitumor activity, including IL-12, IFN-γ, IFN-α, and TNF [36,37,50]. Studies in mice have also demonstrated that a large portion of the infused complexes localize to the spleen, where they are taken up by macrophages and induce immune activation [34]. Thus, cytokine production by the spleen in response to LDC infusion could trigger immune responses capable of suppressing tumor growth at distant sites. To address this question, we determined whether cytokines released from spleen tissues in mice following intravenous injection of LDC could also inhibit tumor growth in vitro. For these studies, a murine fibrosarcoma cell line (similar to the tumor type studied in dogs in this study) was used to assess antitumor responses. Addition of supernatants from spleens of LDC-injected mice, but not supernatants from control spleens, inhibited fibrosarcoma cell growth in vitro (Figure 6b). These results therefore suggest that LDC-triggered systemic release of cytokines with antitumor activity would be capable of inhibiting tumor cell growth in vivo. In fact, such antitumor activity of LDC has been demonstrated previously in several different tumor models in mice [36, 50].

Figure 6. Effects of LDC on endothelial cell survival and on release of tumor inhibitory cytokines by spleen cells



Studies were performed to assess gene nonspecific effects of LDC on endothelial cell survival and on release of tumor inhibitory cytokines. (a) Normal mouse capillary endothelial cells were plated in quadruplicate wells and were incubated with serially diluted LDC (formulated with noncoding plasmid DNA) in complete medium for 24 h, and then cell viability was determined by MTT assay, as described in materials and methods. The mean (± s.d.) optical density reading for each dilution of LDC (expressed as DNA content) was plotted. Similar results were obtained in two additional experiments. (b) Mice (n = 5 per group) were injected intravenously with LDC (equivalent to 10 μg DNA per mouse), then the spleens were collected 6 h later and cultured for 18 h in complete medium and supernatants collected and frozen. Supernatants from spleens from untreated control mice were processed similarly. The supernatants were then diluted 1:1 and incubated with murine fibrosarcoma cells (MCA 2.1) in quadruplicate wells for 24 h, and then viability determined by MTT assay as described in materials and methods. The mean optical density (±s.d.) for tumor cells treated with LDC-elicited or control spleen supernatants were then plotted. Similar results was obtained in one additional experiment (*P< 0.01).

Response of plasma VEGF and bFGF concentrations to LDC infusion

To determine whether infusions of LDC might have inhibited tumor angiogenesis by decreasing circulating concentrations of key proangiogenic cytokines, VEGF and bFGF concentrations in plasma were quantitated before, during, and after LDC infusions, using cross-reactive ELISA assays (see materials and methods). Significant changes in the concentrations of VEGF or bFGF were not observed in plasma

samples collected during treatment, as compared to pretreatment plasma VEGF or bFGF concentrations (data not shown). Thus, any effects of LDC infusion on tumor angiogenesis were most likely not due to suppression of circulating concentration of key proangiogenic cytokines.

DISCUSSION

This study was designed primarily to address two questions. Firstly, could intravenous infusions of LDC in a large animal spontaneous tumor model lead to transfection of cutaneous tumors. And secondly, could infusion of LDC inhibit tumor angiogenesis in large, well established tumors. The answer to the first question appears to be no, in that we were unable to detect transgene expression in cutaneous tumor tissues following intravenous infusion of LDC. However, several caveats may apply. First, it may be that soft tissue sarcomas are more difficult to transfect than other tumor types, although it is likely that the major target for transfection by this route is probably the endothelium and not the tumor itself. Also, the LDC used for this study were optimized for pulmonary delivery of genes, and it may be that additional modifications of the LDC (e.g. addition of PEG groups to the liposome surface) might improve gene delivery to tumor tissues following intravenous delivery. More efficient tumor transfection might also be achieved if higher doses of the LDC were administered. However, doses much above the dose used in the current study

(20μg DNA per kg body weight) would be likely to elicit toxicity, as was found in a previous study in dogs [42].

Intravenous delivery of LDC appeared to inhibit tumor angiogenesis in some of the dogs in this study. The effect was, however, independent of the transgene delivered, inasmuch as there were no significant differences between dogs treated with endostatin or luciferase DNA. Admittedly, with the small sample size evaluated in this study, it is impossible to exclude the possibility that endostatin gene delivery may have actually been more effective than infusion of an irrelevant plasmid. Additionally arguing against an endostatin specific effect was the fact that transgene expression in the tumors was not observed and that increased circulating concentrations of endostatin were not detected.

The antitumor effects observed in this study, therefore, appeared to be largely transgene independent and were most likely induced by the LDC themselves. There is ample precedent for noncoding LDC (empty vector) eliciting antitumor activity [36, 38, 50]. In addition, the ability of noncoding LDC to inhibit angiogenesis has also been demonstrated previously in mouse tumor models [21, 24, 51]. However, in the studies conducted in mice, the doses of LDC that were administered were much higher (up to 200 times more DNA per kg body weight) than the doses used in dogs in this study. Therefore, it is noteworthy that the effects observed in this study occurred at a very low dose of LDC, and in large animals with large, established spontaneous tumors. The slow infusion of LDC used for these studies was also an

important difference between this study and studies typically conducted in mice. Large animals did not tolerate bolus infusions of LDC, and slow infusions also resulted in much more efficient gene delivery systemically (D Liggitt, unpublished data).

Although a placebo-treated control group was not included in this phase I study, clinically it is recognized that spontaneous regressions of soft tissue sarcomas in dogs are extremely rare [39]. Thus, the fact that eight animals experienced stabilization of their tumor growth and two had tumor regression suggests that a treatment effect was induced by repeated infusions of the LDC. The effects of LDC infusion on tumor growth and tumor angiogenesis could have been mediated by a combination of factors, including direct endothelial cell injury by LDC, release of cytokines with antiangiogenic and antitumor properties, and recruitment of inflammatory cells into tumor tissues. For example, we observed that changes in tumor angiogenesis did not always correlate with tumor responses, as four of 9 dogs with stable disease or decreased tumor size had a decrease in MVD at the end of treatment, whereas MVD was unchanged or increased in the other five dogs. This finding suggested that a combination of antiangiogenic and direct antitumor effects was elicited by infusion of the LDC. For example, infiltration of CD8+ T cells was observed in tumor tissues of some dogs in this study following infusion of LDC. Thus, deposition of LDC in the tumor may have recruited CD8+ T cells into the tumor tissues, or triggered activation of local dendritic cells with subsequent stimulation of cytotoxic T cells, as has been reported previously [38].

Results from this study additionally suggest that LDC infusion did not have an effect on circulating VEGF and bFGF concentrations as no significant changes in these parameters were observed over the course of treatment when compared to pre-treatment levels. These results are drawn on the foundation that the levels obtained prior to treatment represent a control or untreated group yet it is recognized that these levels are obtained only at a single time point. It is additionally recognized that these conclusions could be strengthened with the inclusion of a placebo-treated group assuming the placebo-treated group also maintained constant VEGF and bFGF levels throughout treatment.

In summary, the studies reported here indicate that infusion of LDC appears to elicit substantial antitumor and antiangiogenic activity, probably mediated by the immune stimulatory properties of the LDC. Further investigations of intravenous LDC infusion, alone or combined with radiation therapy or chemo therapy for tumor treatment, are warranted. For example, our preliminary observations suggest enhancement of tumor responses when dogs with advanced lung metastases were treated with LDC plus chemotherapy (R Elmslie, unpublished data). In addition, recent reports indicate that activation of innate immunity by administration of CpG oligonucleotides, which elicit immune responses very similar to those elicited by LDC, markedly enhances tumor sensitivity to radiation therapy [52, 54].

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REFERENCES

1 Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* 2003; 3: 401–410.

2 Folkman J. Angiogenesis inhibitors: a new class of drugs. *Cancer Biol Ther* 2003; 2: S127–S133.

3 Folkman J. Endogenous angiogenesis inhibitors. *Apmis* 2004; 112: 496–507.

4 Kerbel RS. Tumor angiogenesis: past, present and the near future. *Carcinogenesis* 2000; 21: 505–515.

5 Kerbel R, Folkman J. Clinical translation of angiogenesis inhibitors. *Nat Rev Cancer* 2002; 2: 727–739.

6 Kerbel RS, Kamen BA. The anti-angiogenic basis of metronomic chemotherapy. *Nat Rev Cancer* 2004; 4: 423–436.

7 Ziche M, Donnini S, Morbidelli L. Development of new drugs in angiogenesis. *Curr Drug Targets* 2004; 5: 485–493.

8 Glade-Bender J, Kandel JJ, Yamashiro DJ. VEGF blocking therapy in the treatment o cancer. *Expert Opin Biol Ther* 2003; 3: 263–276.

9 Heymach JV, Desai J, Manola J, Davis D, McConkey J,Harmon D et al. Phase II study o the antiangiogenic agent SU5416 in patients with advanced soft tissue sarcomas. *ClinCancer Res* 2004; 10: 5732–5740.

10 Iqbal S, Lenz HJ. Angiogenesis inhibitors in the treatment of colorectal cancer. *Semin Oncol* 2004; 31: 10–16.

11 Zangari M, Anaissie E, Stopeck A, Morimoto A, Tan N,Lancet J et al. Phase II study of SU5416, a small molecule vascular endothelial growth factor tyrosine kinase receptor inhibitor, in patients with refractory multiple myeloma. *ClinCancer Res* 2004; 10: 88–95.

12 Zondor SD, Medina PJ. Bevacizumab: an angiogenesisinhibitor with efficacy in colorectal and other malignancies.*Ann Pharmacother* 2004; 38: 1258–1264.

13 O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, LaneWS et al. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 1997; 88: 277–285.

14 Kisker O, Becker CM, Prox D, Fannon M, D'Amato R, Flynn E et al. Continuous administration of endostatin by intraperitoneally implanted osmotic pump improves the efficacy and potency of therapy in a mouse xenograft tumor model. *Cancer Res* 2001; 61: 7669–7674.

15 Eder Jr JP, Supko JG, Clark JW, Puchalski TA, Garcia-Carbonero R, Ryan DP et al. Phase I clinical trial of recombinant human endostatin administered as a short intravenous infusion repeated daily. *J Clin Oncol* 2002; 20:3772–3784.

16 Jouanneau E, Alberti L, Nejjari M, Treilleux I, Vilgrain I,Duc A et al. Lack of antitumor activity of recombinant endostatin in a human neuroblastoma xenograft model.*J Neurooncol* 2001; 51: 11–18.

17 Folkman J. Antiangiogenic gene therapy. *Proc Natl Acad Sci USA* 1998; 95: 9064–9066.

18 Jin X, Bookstein R, Wills K, Avanzini J, Tsai V, LaFace Det al. Evaluation of endostatin antiangiogenesis gene therapy in vitro and in vivo. *Cancer Gene Ther* 2001; 8: 982–989.

19 Li PY, Lin JS, Feng ZH, He YF, Zhou HJ, Ma X et al.Combined gene therapy of endostatin and interleukin 12 with polyvinylpyrrolidone induces a potent antitumor effect on hepatoma. *World J Gastroenterol* 2004; 10:2195–2200.

20 Nakashima Y, Yano M, Kobayashi Y, Moriyama S, SasakiH, Toyama T et al. Endostatin gene therapy on murine lung metastases model utilizing cationic vector-mediated intravenous gene delivery. *Gene Therapy* 2003; 10: 123–130.

21 Blezinger P, Yin G, Xie L, Wang J, Matar M, Bishop JS et al. Intravenous delivery of an endostatin gene complexed in cationic lipid inhibits systemic angiogenesis and tumor growth in murine models. *Angiogenesis* 1999; 3: 205–210.

22 Chen QR, Kumar D, Stass SA, Mixson AJ. Liposomes complexed to plasmids encoding angiostatin and endostatin inhibit breast cancer in nude mice. *Cancer Res* 1999; 59:3308–3312.

23 Davidoff AM, Nathwani AC. Antiangiogenic gene therapy for cancer treatment.*Curr Hematol Rep* 2004; 3: 267–273.

24 Dutour A, Monteil J, Paraf F, Charissoux JL, Kaletta C, Sauer B et al. Endostatin cDNA/Cationic liposome complexes as a promising therapy to prevent lung metastases in osteosarcoma: study in a human-like rat orthotopic tumor.*Mol Ther* 2005; 11: 311–319.

25 Hong SY, Lee MH, Kim KS, Jung HC, Roh JK, Hyung WJ et al. Adeno-associated virus mediated endostatin gene therapy in combination with topoisomerase inhibitor effectively controls liver tumor in mouse model. *World J Gastroenterol* 2004; 10: 1191–1197.

26 Indraccolo S. Undermining tumor angiogenesis by gene therapy: an emerging field. *Curr Gene Ther* 2004; 4: 297–308.

27 Regulier E, Paul S, Marigliano M, Kintz J, Poitevin Y,Ledoux C et al. Adenovirus-mediated delivery of antiangiogenic genes as an antitumor approach. *Cancer Gene Ther* 2001; 8: 45–54.

28 Shi W, Teschendorf C, Muzyczka N, Siemann DW. Gene therapy delivery of endostatin enhances the treatment efficacy of radiation. *Radiother Oncol* 2003; 66: 1–9.
29 Subramanian IV, Ghebre R, Ramakrishnan S. Adeno-associated virus-mediated delivery of a mutant endostatin suppresses ovarian carcinoma growth in mice. *Gene Therpy* 2005; 12: 30–38.
30 Sun X, Qiao H, Jiang H, Zhi X, Liu F, Wang J et al. Intramuscular delivery of antiangiogenic genes suppresses secondary metastases after removal of primary tumors. *Cancer Gene Ther* 2005; 12: 35–45.
31 Szala S, Szary J, Cichon T, Sochanik A. Antiangiogenic gene therapy in inhibition of metastasis. *Acta Biochim Pol* 2002;49: 313–321.
32 Wang L, Schmitz V, Perez-Mediavilla A, Izal I, Prieto J,Qian C. Suppression of angiogenesis and tumor growth by adenoviral-mediated gene transfer of pigment epithelium derived factor. *Mol Ther* 2003; 8: 72–79.
33 Yano M, Nakashima Y, Kobayashi Y, Mizuno K, KonishiA, Sasaki H et al. Endostatin gene transfection using a cationic lipid: advantages of transfection before tumor cell inoculation and repeated transfection. *Cancer Gene Ther* 2004; 11: 354–362.
34 Thurston G, McLean JW, Rizen M, Baluk P, Haskell A,Murphy TJ et al. Cationic liposomes target angiogenic endothelial cells in tumors and chronic inflammation in mice. *J Clin Invest* 1998; 101: 1401–1413.
35 Eichhorn ME, Strieth S, Krasnici S, Sauer B, Teifel M,Michaelis U etal. Protamine enhances uptake of cationic liposomes in angiogenic microvessels. *Angiogenesis* 2004; 7:133–141.
36 Dow SW, Fradkin LG, Liggitt DH, Willson AP, Heath TD, Potter TA. Lipid–DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously. *J Immunol* 1999; 163: 1552–1561.
37 Whitmore MM, Li S, Falo Jr L, Huang L. Systemic administration of LPD prepared with CpG oligonucleotides inhibits the growth of established pulmonary metastases by stimulating innate and acquired antitumor immuneresponses. *Cancer Immunol Immunother* 2001; 50: 503–514.
38 Siders WM, Vergillis K, Johnson C, Scheule RK, Kaplan JM. Tumor treatment with complexes of cationic lipid and noncoding plasmid DNA results in the induction of cytotoxic T cells and systemic tumor elimination. *Mol Ther* 2002; 6:519–527.
39 Withrow SJ. Soft tissue sarcomas. *Vet Q* 1998; 20(Suppl 1):S16–S17.
40 Rassnick KM. Medical management of soft tissue sarcomas.*Vet Clin N Am Small Anim Pract* 2003; 33: 517–531.
41 Ettinger SN. Principles of treatment for soft-tissue sarcomas in the dog. *Clin Tech Small Anim Pract* 2003; 18: 118–122.
42 Dow S, Elmslie R, Kurzman I, Macewen G, Pericle F, Liggitt D. Phase I study of liposome-DNA complexes encoding the interleukin-2 gene in dogs with osteosarcoma lung metastases. *Hum Gene Ther* 2005; 16(8): 937–946.

43 Fairman J, Roche L, Pieslak I, Lay M, Corson S, Fox E et al. Quantitative RT-PCR to evaluate in vivo expression of multiple transgenes using a common intron. *Biotechniques*1999; 27: 566–570, 572–564.
44 Liu Y, Mounkes LC, Liggitt HD, Brown CS, Solodin I, Heath TD et al. Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery. *Nat Biotechnol* 1997; 15: 167–173.
45 Dow SW, Elmslie RE, Fradkin LG, Liggitt DH, Heath TD, Willson AP et al. Intravenous cytokine gene delivery by lipid-DNA complexes controls the growth ofestablishedlung metastases. *Hum Gene Ther* 1999; 10: 2961–2972.
46 St Croix B, Rago C, Velculescu V, Traverso G, Romans KE,Montgomery E et al. Genes expressed in human tumor endothelium. *Science* 2000; 289: 1197–1202.
47 Scheidegger P, Weiglhofer W, Suarez S, Kaser-Hotz B, Steiner R, Ballmer Hofer K et al. Vascular endothelial growth factor (VEGF) and its receptors in tumor-bearing dogs. *Biol Chem* 1999; 380: 1449–1454.
48 Clifford CA, Hughes D, Beal MW, Henry CJ, Drobatz KJ,Sorenmo KU. Vascular endothelial growth factor concentrations in body cavity effusions in dogs. *J Vet Intern Med* 2002; 16: 164–168.
49 Allen DK, Waters DJ, Knapp DW, Kuczek T. High urine concentrations of basic fibroblast growth factor in dogs with bladder cancer. *J Vet Intern Med* 1996; 10: 231–234.
50 Whitmore M, Li S, Huang L. LPD lipopolyplex initiates a potent cytokine response and inhibits tumor growth. *GeneTherapy* 1999; 6: 1867–1875.
51 Lesoon-Wood LA, Kim WH, Kleinman HK, Weintraub BD, Mixson AJ. Systemic gene therapy with p53 reduces growth and metastases of a malignant human breast cancer in nude mice. *Hum Gene Ther* 1995; 6: 395–405.
52 Koski GK, Czerniecki BJ. Combining innate immunity with radiation therapy for cancer treatment. *Clin Cancer Res* 2005; 11: 7–11.
53 Mason KA, Ariga H, Neal R, Valdecanas D, Hunter N, Krieg AM et al. Targeting toll-like receptor 9 with CpG oligodeoxynucleotides enhances tumor response to fractionated radiotherapy. *Clin Cancer Res* 2005; 11: 361–369.
54 Milas L, Mason KA, Ariga H, Hunter N, Neal R, Valdecanas D et al. CpG oligodeoxynucleotide enhances tumor response to radiation. Cancer Res 2004; 64: 5074–5077.

CHAPTER 2

REGULATION OF TUMOR-ASSOCIATED VASCULAR PRODUCTION BY ENDOGENOUS TYPE I AND II INTERFERONS

ABSTRACT

The objective of this work was to explore the effects of endogenous type I and II interferons (IFN) on tumor angiogenesis under physiological tumor-bearing conditions. We hypothesized that type I and II IFNs function in tumor surveillance as endogenous anti-angiogenic factors inhibiting tumor-associated vascular production and hence tumor growth. Interferon receptor knock out (IFNR-/-) mice were inoculated intradermally with syngeneic methylcholanthrene-induced sarcoma cells for tumor induction. Post-inoculation, tumor growth rates were monitored clinically and serial tail bleeds were performed to assess the presence of circulating endothelial progenitor cells (CEPs) by fluorescence activated cell sorting (FACS). At sacrifice, tumor microvessel density (MVD) and tumor infiltrating leukocytes (TIL) were determined via immunohistochemistry (IHC) and FACS respectively while blood collection was pursued to evaluate circulating VEGF levels by enzyme linked immunosorbent assay (ELISA). Tumor growth rates as well as tumor MVD in both

type I and II IFNR-/- mice exceeded that of wild-type (wt) mice supporting the role of endogenous type I and II IFN in tumor immunity, at least in part, by inhibiting tumor-associated vascularization. Decreasing numbers of CEPs were noted in IFNR-/- mice as compared to wt mice suggesting a more specific role for these cytokines in the inhibition of vasculogenesis by impeding recruitment of CEPs to the tumor microenvironment. While no significant differences in numbers of tumor infiltrating macrophages, neutrophils, or NK cells were noted, an increased number of infiltrative dendritic cells (DCs) was present in the tumors of IFNR-/- mice suggesting an additional role of these cytokines in suppression of DC recruitment and or differentiation. No difference in circulating VEGF levels was identified.

INTRODUCTION

Progressive growth and metastasis of solid tumors is dependent on the development of a tumor-associated blood supply.[1,2] This blood supply can occur by angiogenesis, the sprouting of new capillaries from pre-existing vessels, or vasculogenesis, the recruitment of endothelial progenitor cells from the bone marrow, or likely a combination of both.[3-8] Regardless of the method, the induction of this vascular supply is dependent on the initiation of the angiogenic switch which itself depends on the shift in the balance of angiogenic regulatory molecules toward angiogenic activators.[9-12] Tumors in the avascular state, aggregates of neoplastic cells measuring

no more than 2mm in diameter (microtumors), remain dormant and thus do not induce disease. While the presence of these microtumors frequently exist in the antemortem state, they go undetected until postmortem examination as they are subclinical.[13,14] The questions behind the mechanisms which allow some tumors to remain in an avascular, dormant, non-lethal state, while other become vascularized and thus induce disease remain to be answered. It is well accepted that the induction of this vascular network is dependent on the balance between pro- and anti-angiogenic factors in the tumor micro-environment. Additionally, it has been recognized that neoplastic cells can and do secrete pro-angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), interleukin-8 (IL-8), and platelet derived growth factor (PDGF).[10,15] What perhaps is less clear, are the natural host defenses which exist in the tumor-bearing state and, more specifically as it pertains to this work, what natural endogenous anti-angiogenic armory does the host posses to combat the tumor-associated angiogenic activators thus preventing induction of tumor vascularization.

Previous studies in our lab and by others have demonstrated a role of innate immunity in antitumor and antiangiogenic activity.[16-18] Additionally, antitumor effects have been noted following CLDC administration, as demonstrated in chapter one, and CLDCs have been shown to induce potent innate immune responses thus further supporting the association of innate immunity and antitumor activity.[16,19-23] Moreover, cytokines associated with innate immunity such as IL-12, type I IFNs (IFNα/β) and type II IFN (IFNγ) have been implicated and have been shown to elicit

antitumor effects and to inhibit angiogenesis via many mechanisms including downregulation of proangiogenic molecules such as VEGF, bFGF, matrix metalloproteinase-9 (MMP-9), and IL-8.[16,17,24-31]

Thus, type I and II interferons are known inhibitors of angiogenesis but have been recognized as such either *in vitro* or at supraphysiological levels *in vivo*. The study within sought to assess the potential role of endogenous interferons (that is at normal physiological levels without the presence of IFN inducers or exogenous interferon administration) in tumor surveillance in an *in vivo* model. Evaluation of this proposed biological activity was achieved by comparing tumor growth in IFNR-/- mice (which are unable to respond to endogenous interferons) to tumor growth in wild-type mice after intradermal inoculation with syngeneic methylcholanthrene-induced neoplastic mesynchymal cells. As these interferons are known angiogenic inhibitors, we hypothesized that they would elicit antitumor effects by inhibiting tumor-associated vascularization under physiological conditions. To further elucidate the mechanism by which these effects might occur, we additionally evaluated the presence of circulating endothelial progenitor cells, tumor infiltrating leukocytes, and circulating levels of VEGF.

Results from these studies demonstrate that, when compared to wt mice, IFNR-/- mice have an increased tumor growth rate, an increased tumor MVD, decreased circulating endothelial progenitor cells, and an increased infiltrate of tumor-associated dendritic cells. These findings support the role of endogenous type I and II

interferons in natural tumor surveillance by inhibiting tumor-associated vascular production which, more specifically may be, at least in part, by inhibiting the recruitment of circulating endothelial progenitor cells to the tumor microenvironment and by inhibiting recruitment and / or differentiation of dendritic cells.

MATERIALS AND METHODS

IFNR -/- mice

The original knock out strain (A129 mice) lacking a functional interferon receptor (homozygous for receptor mutations interfering with and inhibiting signal transduction) originated in the lab of Michael Aguet at the Swiss Institute for Experimental Cancer Research.[32] For the experiments within, wild-type interferon receptor (129S6/SvEv) mice were obtained from Taconic (Germantown, NY), IFNγR-/- mice derived on a pure 129 SvEv genetic background (129-Ifngr^{tm1Agt}) were obtained from The Jackson Laboratory (Bar Harbor, Maine), and breeding pairs of IFNα/βR-/- mice were obtained from the National Jewish Medical and Research Center and the University of Colorado Denver Health Science Center, Denver CO. All mice were free of specific pathogens, housed according to ACUC requirements at a maximum of 5 mice per cage and fed autoclaved pellets and water *ad libitum*. For all experiments, mice were age matched ranging from 8 to 12 weeks old, and

consisted of 5 mice from each respective genetic variant per group. All procedures were approved by the Animal Care and Use Committee at Colorado State University.

Tumor cell line and in vitro conditions

The MCA 2.1.1 cell line utilized in these experiments was originally established by chemotoxic intradermal injection of the carcinogen 3- methylcholanthrine (MCA) diluted in peanut oil into interferon receptor wild-type 129 mice. MCA induced tumors were then harvested from these mice, neoplastic cells were purified, cultured, harvested, and stored in liquid nitrogen. For use in the following experiments, frozen cells were retrieved by quick thaw in a 37^{0} water bath, followed by routine culturing techniques including an initial wash in 15mls of minimum essential medium (MEM), centrifugation, and resuspension, two times followed by plating initially in a T-25 Falcon flask (BD Bioscience, San Jose CA) with complete medium (MEM (Invitrogen Corp., Grand Island, NY) supplemented with 10% fetal bovine serum (FBS, Sigma, St Louis, MO), penicillin-streptomycin, L-glutamine, essential amino acids, and nonessential amino acids (Gibco, Grand Island, NY) and incubated in 6% CO2 at 37^{o}C. Cells were maintained in a monolayer, split appropriately as needed, and ultimately transferred to T-75 flasks.

Tumor induction

For *in vivo* injections, tumor cells in exponential growth phase were washed in 1x PBS followed by a brief treatment with .2% trypsin and harvested by routine culture techniques. Cell counts for appropriate suspension concentration and viability were obtained via hematocytometer and trypan blue. Cells were suspended at $1X10^6$/100ul of sterile 1x PBS and inoculated at a volume of 100ul ($1X10^6$cells) intradermally in a clipped area over the right iliac crest region of anesthetized mice. Mice were anesthetized with an intraperitoneal injection of 200ul of 2.5% Avertin in sterile 1x PBS.

Monitoring

Following inoculation of MCA 2.1.1 cells, inoculation sites were monitored every two to three days for tumor growth. Upon initial detection of a tumor mass, size was measured in three dimensions with calipers every two to three days and mice were sacrificed when tumor size measured ~1.5cm in diameter.

Blood collection

Prior to inoculation and over the course of tumor development, whole blood (6-8ggt / mouse) was collected via tail vein once weekly for analysis of CEPs. Due to minimal sample size, blood for each genetic variant of mouse was pooled allowing for an increased volume and resulting in one test sample for each group of mice (wt, tumor-

bearing wt, IFNα/βR-/-, and IFNγR-/- respectively). At sacrifice, plasma was collected via cardiac stick to assess circulating VEGF levels. Following collection, samples for CEPs were processed immediately while plasma was stored at -80°C.

Assessment of MVD

At sacrifice, tumors were harvested *en toto* and divided into thirds for MVD analysis, TIL analysis, and storage in -80 °C. For MVD analysis, sections were embedded in Tissue-Tek OCT compound (TedPella Inc., Redding, CA) and snap frozen in either isopentane or liquid nitrogen. Tissues were cryosectioned to a thickness of 4μm and adhered to positively charged glass slides. For identification of endothelial cells associated with tumor microvasculature, a monoclonal antibody specific for murine CD31/Platelet Endothelial Cell Adhesion Molecule (PECAM) (BD PharMingen, SanDiego, CA) was utilized. After fixation in acetone and blocking nonspecific binding, tissues were incubated first with the primary antibody, followed by biotinylated donkey anti-rat IgG antiserum (Jackson ImmunoResearch, West Grove, PA), then with streptavidin–HRP and AEC substrate (Vector Laboratories, Burlingame, CA), followed by hematoxylin counterstain. Negative controls included incubation with irrelevant mAb and omission of the primary antibodies. Staining of normal murine splenic tissue was utilized for positive control.

For quantitation of tumor MVD, five photomicrographs at 20X magnification were obtained by digital camera (Leica Microscope equipped with digital camera with

SPOT Advanced Imaging software). Utilizing Adobe Photoshop and Reindeer Graphics Quantitative Analysis Plug-ins (Asheville, NC), the photomicrographs were converted to binary images and the number of microvessels per section was determined digitally. MVD values for each tumor biopsy were calculated based on the average number of vessels (quantitated as pixels) for the five 20X fields identified for each individual mouse.

Assessment of tumor infiltrating leukocytes

At sacrifice, 1/3 of the tumor was harvested for tissue digestion to characterize and quantitate tumor infiltrating leukocytes by FACS analysis. First, tissue was minced in a Petri dish containing a small volume of collagenase solution after which it was transferred to a 15ml conical tube containing 3-4ml of collagenase solution. Conical tubes were incubated in a 37°C waterbath for ~60minutes and mixed vigorously every 15minutes during incubation. Specimens were next trituated 3-4 times using an 18guage needle and 3cc syringe in the 15ml conical which was then filled with 1xPBS and centrifuged in the Thermo IEC centrifuge for 5 minutes at 1200 RPM. Supernatant was discarded and the sample was resuspended in 1ml of 1xPBS followed by the addition of 10ml NH_4Cl for 5 minutes. Samples were then serially filtered through a 70um and 40um strainer into a 50ml conical and a final wash performed with 1xPBS. Supernatant was discarded and cells resuspended in 500ul of complete tumor medium with 10% FBS and kept on ice.

Following preparation, samples were appropriately distributed to respective wells in a 96-well round bottom plate along with individual cell suspensions of splenocytes to determine compensation values and unstained cell samples to determine forward and side scatter. Following centrifugation, 10ul of FACS Block was added to each well. Following blocking, samples were incubated for 25 minutes at 4°C with 50ul of respective primary antibody at 1:200 which were purchased from either BD PharMingen (San Diego, CA) or eBiosciences (San Diego, CA). Antibodies utilized included, in 5-color panels: anti-CD4 (flouresceine isothicynate (FITC)), anti-CD8 (allophycocyanin (APC)), anti-B220 (APC-Cy7), anti-CD25 (PE), anti-CD62c (PeCy5), and, anti-CD11b (PeCy5), anti-CD11c (APC), anti-GR1 (PeCy7), anti-MHCII (PE), and biotinylated NKG2D. All splenocytes were treated with biotinylated CD8 at 1:400 followed by respective streptavidin conjugated fluorescent antibodies at 1:400 including Fitc, PE, APC, PeCy5, PeCy7, and APC-Cy7. For the majority of experiments, following the final wash cells were fixed in 1% paraformaldyhyde for 30 minutes at 4°C, washed once, resuspended in FACS buffer, and stored at 4°C until analyzed. Cells were analyzed via flow cytometry using a Cyan MLE cytometer (Dako-cytomation, Fr Collins, CO) for multi-color channel analysis. Macrophages were defined as $CD11b^{+}/MHCII^{+}$ cells, DCs as $CD11b^{+}/CD11c^{+}$ cells, granulocytes by $CD11b^{+}/GR\text{-}1^{+}/MHCII^{-}$ cells and natural killer cell (NK) as $CD11b^{+}/CD11c^{+}$. Gates were set on viable unstained splenocytes designed to include all viable cell populations. $1x10^{6}$ events were collected per sample and the percentage of each respective cell population determined. Experimental and control groups consisted of five animas each.

Assessment of circulating endothelial progenitor cells

Circulating endothelial progenitor cells (CEPs) were assessed by immunostaining and flow cytometry. Following blood collection as previously described, samples were centrifuged in 14mls of heparinized hanks balanced salt solution (hep-HBSS). Supernatant was aspirated off and the pellet resuspended in 1ml of HBSS and 14mls of NH_4CL lysing solution and incubated for 10 minutes. Samples were then recentrifuged, supernatant discarded, and washed again in HBSS followed by centrifugation and resuspension in appropriate volume of tissue culture media.

Following preparation, samples were appropriately distributed to respective wells in a 96-well round bottom plate along with individual cell suspensions of cultured murine endothelial cells and murine splenocytes to determine forward and side scatter and compensation values respectively. Cultured murine endothelial cells were also utilized as positive controls for each panel and for isotype control antibodies. Isotype controls were run at concentrations consistent with the primary antibody for which it was being run and consisted of antibodies purchased from BD PharMingen or eBiosciences including IgG2a,κ (PE or FITC), IgG1,κ (PE, FITC, or biotinylated), and IgG2a (biotinylated). Primary antibodies used for flow cytometric analysis were also purchased from either BD PharMingen or eBiosciences and included, in various combinations: anti-CD34 (fluorescence isothicynate (FITC); clone RAM34), anti-Flk-1 (PE; clone Avas12α1), anti-AC133 (biotin; clone 13A-4) anti-CD144 (biotin), anti-

CD11b (PeCy5; clone M1), anti-CD31 (FITC; clone Mec 13.3), anti-CD146 (PE; clone P1H12), and anti-Tie2 (biot; clone TEK4). The immunostaining protocol was pursued as described above and again, splenocytes were treated with biotinylated-CD8 at 1:400 followed by respective streptavidin conjugated fluorescent antibodies at 1:500 including Fitc, PE, Alexa647, PeCy5, and PeCy7. Circulating endothelial progenitor cells were defined as CD133 $(AC133)^{+}$/ VEGFR-2 $(Flk\text{-}1)^{+}$ cells.[33-35] Gates were set on unstained cultured murine endothelial cells to include all viable cell populations and $1X10^{5}$ events were collected per sample and the percentage of each respective cell population determined. As samples were pooled, one value for each treatment group (genetic variant of mouse) per respective time point was obtained.

Assessment of circulating VEGF

Plasma was collected from mice via cardiac stick at sacrifice and stored frozen at -80°C prior to analysis. All samples were then run simultaneously utilizing a commercially available VEGF ELISA kit (R&D Systems, Minneapolis, MN) according to manufacturer's directions.

Statistical analysis

Statistical differences between more than two treatment groups were performed using one way analysis of variance (ANOVA), followed by Tukey's multiple means comparison test. Comparisons between two groups were determined by unpaired

nonparametric analysis utilizing the Mann Whitney test. Analysis was carried out using GraphPad Prism software (SanDiego, CA). For all analysis, p values < 0.05 were considered statistically significant.

RESULTS

Tumor progression occurs more rapidly in IFNR-/- mice

As shown in Figure 1, tumor development occurred more rapidly in the INFR-/- mice where tumor growth in the IFNα/βR-/- mice exceeded that of both IFNγR-/- and wt mice. Palpable tumors were detected at approximately 10 days post-inoculation in all groups while notable differences in tumor size between groups were identified by day 20 to 23. Error bars represent standard error of the mean (SEM) for tumor size within each group of mice for the respective time point. Survival curves were not generated as mice were sacrificed when tumor size attained a diameter of ~1.5 cm. All mice at time of sacrifice appeared clinically healthy, were ambulatory, and in good body condition. No evidence of metastatic disease was identified grossly in any mice.

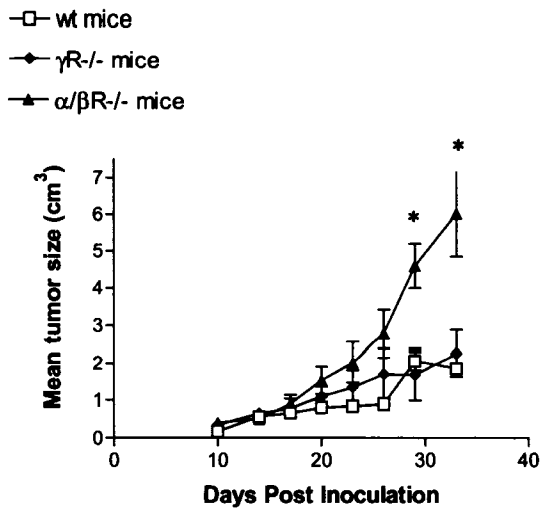

Figure 1. Tumor growth rate in IFNR-/- and wt mice following intradermal inoculation with $1X10^6$ MCA2.1.1 cells. Enhance tumor development was noted in IFNR-/- mice as compared to wt mice. The most dramatic difference was noted in the rate of tumor growth in IFNα/βR-/- mice which also exceeded that of γR-/- mice. * denotes a significant ($p < 0.05$) difference when compared to wt mice as determined by ANOVA. This experiment was repeated two additional times, each yielding similar results.

Increased tumor MVD in IFNR-/- mice

As demonstrated in Figure 2, a higher MVD was identified in tumors which developed in the IFNR-/- mice as compared to tumors in the wild type mice, while, similar to growth rate, MVD in the IFNα/βR-/- mice exceeded that in both the IFNγR-/- and wt mice. Error bars represent the SEM between each of the 5 mice per group where the MVD value for each individual mouse was obtain by calculating the average number of vessels from five random 20x fields as described in materials and methods. Figure 3 demonstrates immunoreactivity of tumor microvasculature to CD31/PECAM in each of the respective groups of mice. Although calculated MVD values were higher in tumors of the IFNR-/- mice, differences between the tumors in

IFNR-/- mice and wt mice were not statistically significant with ANOVA analysis. The lack of statistical significance may, in part, be due to the low number of subjects per group.

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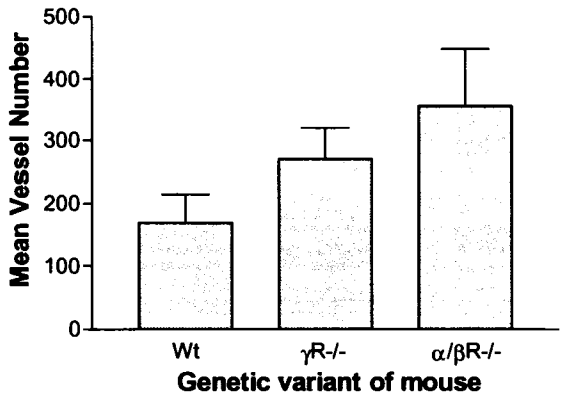


Figure. 2 Tumor microvessel density analysis between genetic variants of mice. Tumor MVD was determined by analysis of immunohistochemically stained cryosections with anti-CD31/PECAM monoclonal antibody (see Fig 3). Error bars represent the SEM between each of the 5 mice per group where the MVD value for each individual mouse was obtain by calculating the average number of vessels from five random 20x fields. MVD in tumors which developed in the IFNR-/- mice exceeded MVD in the tumors from wt mice where the highest tumor MVD was noted in the IFNα/βR-/- mice. Statistically significant differences were not present by one-way analysis of variance (ANOVA) with parametric multiple comparison (Tukey) post-test. This lack of statistical significance may be due, in part, to the low numbers of subjects per group. MVD was determined in the two additional repeated experiments and similar results were obtained.

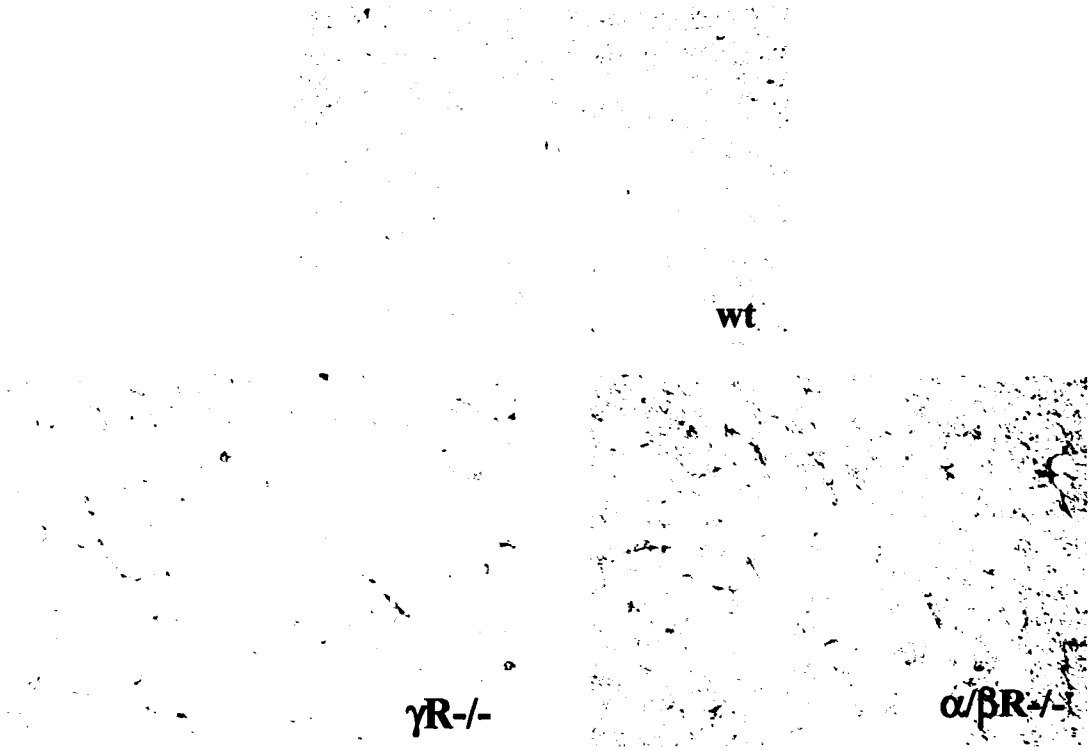


Figure. 3 Three photomicrographs at 20x magnification representative of CD31 immunoreactivity in tumor cryosections from wt, IFNγR-/-, and IFNα/βR-/- mice inoculated with MCA2.1.1 sarcoma cell line for MVD analysis. As is reflected in the calculated data presented in figure 2, tumors from IFNR-/- mice demonstrate an increased number of vessels when compared to the tumor from the wt mouse while vessel density in the α/βR-/- mouse also appears to be slightly increased as compared to the tumor from and IFNγR-/- mouse. Again, while calculated values were different, they were not considered statistically significant.

Tumor infiltrating leukocytes

Assessment of tumor infiltrating leukocytes (TIL) including macrophages, neutrophils, natural killer (NK) cells and dendritic cells (DCs) was performed by flow cytometry as described in materials and methods. $CD11b^+$ cells (expressed on macrophages, granulocytes, as well as subsets of NK cells, and DCs) represented the largest percentage of infiltrating leukocytes in tumors from all groups. No significant

differences in infiltrating macrophages ($CD11b^+/MHCII^+$), neutrophils ($CD11b^+/GR1^+$), or NK cells ($NKG2D^+$) were identified between groups (Figure 4a). When comparing tumor infiltrating DCs ($CD11b^+/CD11c^+$) from each of the respective IFNR-/- mice to tumors of wt mice, a statistically significant increase was noted in tumors from IFNR-/- mice (Figure 4b). The overall percentage (y-axis) of infiltrative NK cells ($NKG2D^+$), and DCs ($CD11b^+/CD11c^+$) is remarkably less than that of infiltrating macrophages and neutrophils in tumors from all mice.

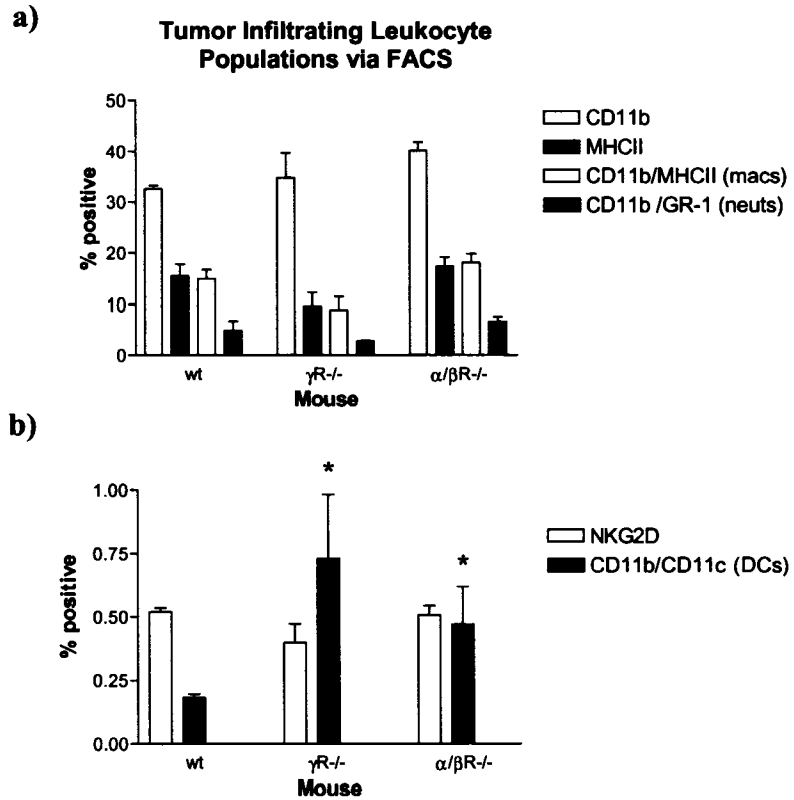
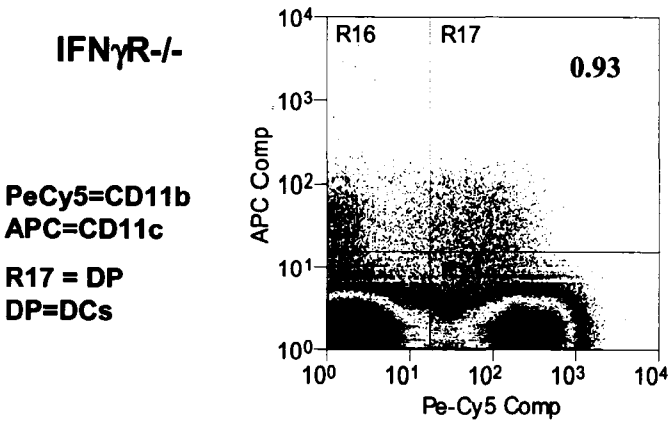


Figure 4. Tumor Infiltrating Leukocytes: No significant differences in infiltrating macrophages or granulocytes were present between tumors of IFNR-/- and wt mice **(a)**. Dendritic cell (DC) infiltrates in tumors of IFNR-/- mice however were significantly greater than that of tumors in wt mice as statistically determined with the one-tailed Mann-Whitney test **(b)**. * denotes $p < 0.05$. This experiment was performed successfully and to completion one time.

As another means to demonstrate the difference in the percentage of tumor infiltrating DCs between the IFNR-/- mice and wt mice, figure 5 provides two individual scatterplots from flow cytometric analysis. Immunostaining for CD11b and CD11c expression is represented on the x and y axes respectively. Double positive cells in the upper right hand quadrant represent DCs and the percentage of positive cells is indicated. "A" represents tumor-associated DC infiltrates from an IFNγR-/- mouse while "B" reflects tumor-associated DC infiltrates from a wt mouse.

a)



b)

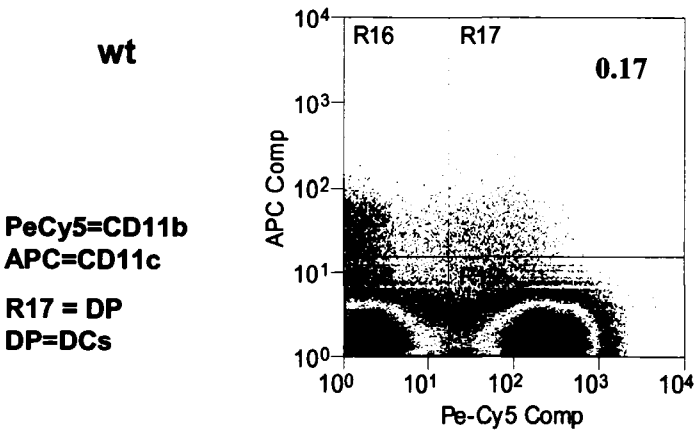


Figure 5. Flow cytometric data of TILs: The two scatter plots demonstrate the intratumoral population of DCs, represented as double positive (DP) cells ($CD11b^{+}/CD11c^{+}$) in the upper right hand quadrant (R17), from an IFNγR-/- mouse **(a)** and a wt mouse **(b)** respectively. The percentage of positive cells, as determined by flow cytometery, is indicated in quadrant R17. Anti-CD11b (Pe-Cy5) immunoreactivity is represented on the x-axis while anti-CD11c (APC) immunoreacctivity is represented on the y-axis.

Circulating endothelial progenitor cells (CEPs)

Assessment of CEPs was performed via immunostaining and flow cytometery as described in materials and methods. Again, blood was collected via tail vein prior to inoculation and once weekly during tumor development. Due to limited blood volumes physiologically attainable per individual mouse, blood samples from mice within each group were pooled resulting in one value for each genetic variant of mouse (wt, IFNγR-/-, IFNα/βR-/-) per respective time point. Evaluation of CEPs from non-tumor bearing wild type mice as an additional control were also included in these experiments. CEPs were identified as cells which co-expressed VEGFR-2 (Flk-1) and CD133 (AC133). CEPs prior to inoculation (baseline levels) were slightly higher in IFNR-/- mice however, over the course of tumor development, circulating endothelial progenitor cells progressively decreased in the IFNR-/- mice as compared to tumor-bearing wt mice and were ultimately lower than wt mice at days 14 and 21 post-inoculation (Fig. 6). The percentage of CEPs as determined by flow cytometry and indicated on the y-axis ranged from ~0.025 - 0.225%. The normal percentage of circulating endothelial progenitor cells in humans is reported to be ~.01%.[35-38]

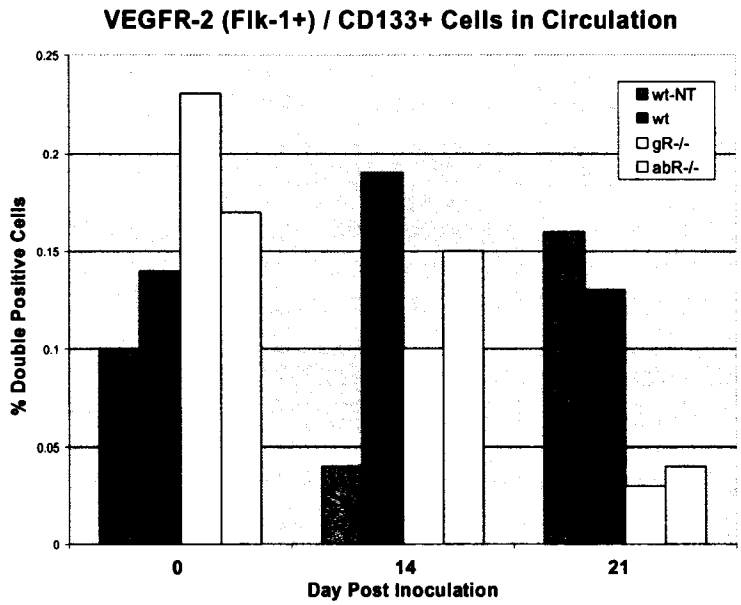


Figure 6. Circulating endothelial progenitor cells (CEPs) pre-inoculation and during tumor development: CEPs were identified as VEGFR-2^{+}/CD133+ cells by flow cytometery and percentages determined for $1x10^{5}$ collected events. Due to limited sample size per individual mouse, blood samples within each group were pooled thus resulting in analysis of one sample per group (wild type non-tumor bearing (**wt-NT**), wild type (**wt**), IFNγR-/- (**gR-/-**), and IFNα/βR-/- (**abR-/-**) per time point. Prior to inoculation, baseline values of CEPs were slightly increased in IFNR-/- mice as compared to wt mice while over the course of tumor progression, CEPs decreased in IFNR-/- to levels lower than that in wt mice. This experiment was performed successfully and to completion one time.

Circulating VEGF

No significant differences in circulating plasma VEGF levels at time of sacrifice were identified between groups.

DISCUSSION

Antitumor and antiangiogenic effects of type I and II interferons (IFNα/β and IFNγ respectively) have been reported previously and more specifically, mechanisms by which these cytokines elicit their effects have also been demonstrated.[17,28-31] Observation of these findings however have typically been either *in vitro* or by induction of supraphysiological interferon levels in vivo either indirectly with interferon inducers or directly by administration of exogenous interferons. The studies presented here sought to and, by enhanced tumor development observed in IFNR-/- mice, have demonstrated a role for endogenous interferons (interferons at normal physiological, unmanipulated levels) in natural tumor surveillance.. Utilization of genetically modified IFNα/βR-/- or IFNγR-/- mice abrogates normal interferon ligand activity thus resulting in effects which would occur in the absence of type I or II interferons respectively. Thus, enhanced tumor growth in the IFNR-/- mice suggests the presence of interferons would prevent such activity, as seen in wt mice, and supports the role of interferons in the defense against tumor development.

To further explore and demonstrate possible mechanisms by which endogenous interferons elicit this antitumor activity, and as they are known inhibitors of angiogenesis, tumor microvessel density (MVD) of all tumors was evaluated. The identification of an increased tumor MVD in IFNR-/- mice, and to a greater extent in the IFNα/βR-/- mice, as compared to tumors of wt mice supports a natural role of these interferons in preventing tumor-associated vascular production. The

dependency of tumor growth on a vascular supply is well accepted thus, it is possible that the increased MVD identified in the IFNR-/- mice correlates with the observed increase in tumor growth rate. It is noted however that there is disparity between the tumor growth rates in IFNα/βR-/- and IFNγR-/- mice when compared to the correlative difference in MVD. More specifically, the tumor growth rate in IFNα/βR-/- mice is remarkably greater than that in IFNγR-/- mice when compared to the difference in MVD between the two knock out strains. This discrepancy suggests that inhibition of tumor growth by enodogenous interferons is likely due, only in part, to their antiangiogenic / antivascular effects. Interferons were first identified for their role in "interfering" with viral replication and were determined to elicit potent antiproliferative activity. Thus, endogenous interferons may additionally inhibit tumor growth by directly inhibiting proliferative activity of the neoplastic cells themselves. While host cells of R-/- mice lack functional interferon receptors, the tumor cells, which were derived in wt mice, can express these receptors and thus can respond to or be affected by ligand activity.

Additional experiments assessed the presence of tumor infiltrating leukocytes (TIL) between groups of mice. The increased presence of dendritic cells identified in tumors of the IFNR-/- mice suggests a role of endogenous interferons in either 1) inhibiting DC recruitment to the tumor site and / or 2) inhibiting DC maturation. What, however, is the potential relevance of tumor associated DCs and how would this interferon-associated DC regulation play a role in tumor immunity? Recent literature has suggested that rather than functioning in a host-favored immune

response, DCs in the tumor microenvironment may actually be redirected / reprogrammed to take on an endothelial cell phenotype and be incorporated into the tumor vasculature.[39] This theory would lend additional support to the increased MVD seen in the IFNR-/- mice which also demonstrated an increased number of tumor associated DCs. Additionally, it has been suggested that the redirection/ reprogramming of DCs is regulated in part by VEGF.[40-42] VEGF is secreted by both tumor cells and cells of the tumor microenvironment and to a large part by tumor infiltrative macrophages (macs).[40,43,44] Infiltrative macrophages represented the highest percentage leukocyte in all tumors. IFNα inhibits VEGF expression, however, as macs of IFNα/βR-/- mice can not respond to IFNα, secretion of VEGF would be uncontrolled and possibly constitutive. This constant VEGF secretion may further potentiate the redirecting of DCs to an endothelial cell phenotype and may provide an additional rationale for the increased tumor MVD in IFNα/βR-/- mice as compared to IFNγR-/- mice (which maintain ability to respond to the IFNa ligand). To lend additional support to this theory, intratumoral levels of VEGF could be analyzed where increased VEGF would be anticipated in tumors of IFNα/βR-/- mice as compared to tumors from IFNγR-/- mice.

Lastly, circulating endothelial progenitor cells which are mobilized from the bone marrow, recruited to the tumor microenvironment, and incorporated in the tumor microvasculature in a process known as vasculogenesis were also analyzed over the course of tumor development.[5,6,8,38] While tumor growth rate and MVD of INFR-/- mice were increased, the numbers of CEPs over the course of tumor development

decreased. This indirect association between MVD and CEPs however may suggest that CEPs were recruited to the tumor microenvironment contributing to an increased vascular network and simultaneously resulting in a decreased number of endothelial progenitor cells in circulation. This finding thus suggests that endogenous interferons may also demonstrate anti-vasculogenic effects by inhibiting recruitment of CEPs to the tumor microenvironment, an observation which, to the author's knowledge, has not yet been reported or suggested in the literature. Factors including stromal derived growth factor – 1 (SDF-1), angiopoietin-1 (Ang-1), GM-CSF, and VEGF have been describe in involvement of bone-marrow derived endothelial progenitor cell mobilization and recruitment however interferons have not been implicated.[45-48] As previously describe, IFNα may play a role indirectly by inhibiting expression of VEGF thus decreasing EPC mobilization and recruitment however, if this was the mechanism, one would expect to see 1) and increased circulating level of VEGF in IFNαβR-/- mice and would expect a lower number of CEPs in IFNα/βR-/- mice as compared to IFNγR-/- mice neither of which were observed. Thus, this suggests that interferons inhibit recruitment of CEPs to the tumor microenvironment by mechanisms not yet determined.

Circulating VEGF levels at time of sacrifice were analyzed yet no difference between mice was identified. It was anticipated that IFNα/βR-/- mice would demonstrate higher levels of circulating VEGF as IFNα inhibits VEGF expression as previously discussed but this was not noted. While circulating VEGF levels were analyzed, intratumoral VEGF levels, which may have varied between groups, were not. It

should be recognized again, however, that tumors in all mice were wt tumors expressing IFN receptors and able to respond to host interferons. Thus, if the primary source of VEGF production was by the neoplastic cells themselves rather than host derived cells, the production level relative to effects of endogenous interferons would be equivalent.

In summary, these studies support our hypothesis that endogenous type I and II interferons play a role in host tumor surveillance. This hypothesis is most strongly supported by the observed tumor growth rate where tumors in IFNα/βR-/- mice grew at a statistically significant increased rate when compared to that of wt mice. The tumor growth rate of IFNγR-/- mice was also increased however was not statistically significant suggesting type I interferons (IFNα/β) have a more significant role in host tumor surveillance than that of IFNγ. These studies also suggest that these interferons elicit their anti-tumor effects, at least in part, by inhibiting the production of the tumor- associated vascular supply. Calculated, yet not statistically significant, differences in MVD support this theory however additional experiments with larger numbers per group should be pursued for further support. Lastly, these studies also suggest that mechanisms by which these interferons regulate the tumor blood supply include the inhibition of DC recruitment and / or differentiation as well as the inhibition of CEP recruitment to the tumor microenvironment, consistent with an anti-vasculogenic role of type I and II interferons. Continued investigation with repeated experiments and increased numbers per group would assist in further substantiating these findings.

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REFERENCES

1. Folkman, J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* **285**, 1182-6 (1971).
2. Folkman, J. What is the evidence that tumors are angiogenesis dependent? *J Natl Cancer Inst* **82**, 4-6 (1990).
3. Folkman, J., Merler, E., Abernathy, C. & Williams, G. Isolation of a tumor factor responsible for angiogenesis. *J Exp Med* **133**, 275-88 (1971).
4. Risau, W. Mechanisms of angiogenesis. *Nature* **386**, 671-4 (1997).
5. Asahara, T. et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* **275**, 964-7 (1997).
6. Asahara, T. et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* **85**, 221-8 (1999).
7. Carmeliet, P. Mechanisms of angiogenesis and arteriogenesis. *Nat Med* **6**, 389-95 (2000).
8. Lyden, D. et al. Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med* **7**, 1194-201 (2001).
9. Hanahan, D. & Weinberg, R.A. The hallmarks of cancer. *Cell* **100**, 57-70 (2000).
10. Carmeliet, P. & Jain, R.K. Angiogenesis in cancer and other diseases. *Nature* **407**, 249-57 (2000).
11. Folkman, J. Incipient angiogenesis. *J Natl Cancer Inst* **92**, 94-5 (2000).

12. Bergers, G. & Benjamin, L.E. Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* **3**, 401-10 (2003).
13. Black, W.C. & Welch, H.G. Advances in diagnostic imaging and overestimations of disease prevalence and the benefits of therapy. *N Engl J Med* **328**, 1237-43 (1993).
14. Hanahan, D. & Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86**, 353-64 (1996).
15. Yancopoulos, G.D. et al. Vascular-specific growth factors and blood vessel formation. *Nature* **407**, 242-8 (2000).
16. Dow, S.W. et al. Lipid-DNA complexes induce potent activation of innate immune responses and antitumor activity when administered intravenously. *J Immunol* **163**, 1552-61 (1999).
17. Albini, A. et al. Inhibition of angiogenesis and vascular tumor growth by interferon-producing cells: A gene therapy approach. *Am J Pathol* **156**, 1381-93 (2000).
18. Whitmore, M.M. et al. Synergistic activation of innate immunity by double-stranded RNA and CpG DNA promotes enhanced antitumor activity. *Cancer Res* **64**, 5850-60 (2004).
19. Blezinger, P. et al. Intravenous delivery of an endostatin gene complexed in cationic lipid inhibits systemic angiogenesis and tumor growth in murine models. *Angiogenesis* **3**, 205-10 (1999).
20. Whitmore, M., Li, S. & Huang, L. LPD lipopolyplex initiates a potent cytokine response and inhibits tumor growth. *Gene Ther* **6**, 1867-75 (1999).
21. Chen, Q.R., Kumar, D., Stass, S.A. & Mixson, A.J. Liposomes complexed to plasmids encoding angiostatin and endostatin inhibit breast cancer in nude mice. *Cancer Res* **59**, 3308-12 (1999).
22. Whitmore, M.M., Li, S., Falo, L., Jr. & Huang, L. Systemic administration of LPD prepared with CpG oligonucleotides inhibits the growth of established pulmonary metastases by stimulating innate and acquired antitumor immune responses. *Cancer Immunol Immunother* **50**, 503-14 (2001).
23. Kamstock, D. et al. Liposome-DNA complexes infused intravenously inhibit tumor angiogenesis and elicit antitumor activity in dogs with soft tissue sarcoma. *Cancer Gene Ther* **13**, 306-17 (2006).
24. Gresser, I., Belardelli, F., Maury, C., Maunoury, M.T. & Tovey, M.G. Injection of mice with antibody to interferon enhances the growth of transplantable murine tumors. *J Exp Med* **158**, 2095-107 (1983).
25. Gresser, I. How does interferon inhibit tumor growth? *Interferon* **6**, 93-126 (1985).
26. Coughlin, C.M. et al. Tumor cell responses to IFNgamma affect tumorigenicity and response to IL-12 therapy and antiangiogenesis. *Immunity* **9**, 25-34 (1998).
27. Kakuta, S., Tagawa, Y., Shibata, S., Nanno, M. & Iwakura, Y. Inhibition of B16 melanoma experimental metastasis by interferon-gamma through direct inhibition of cell proliferation and activation of antitumour host mechanisms. *Immunology* **105**, 92-100 (2002).

28. Oliveira, I.C., Sciavolino, P.J., Lee, T.H. & Vilcek, J. Downregulation of interleukin 8 gene expression in human fibroblasts: unique mechanism of transcriptional inhibition by interferon. *Proc Natl Acad Sci U S A* **89**, 9049-53 (1992).
29. Kato, N. et al. Suppression of gelatinase production with decreased invasiveness of choriocarcinoma cells by human recombinant interferon beta. *Am J Obstet Gynecol* **172**, 601-6 (1995).
30. Singh, R.K. et al. Interferons alpha and beta down-regulate the expression of basic fibroblast growth factor in human carcinomas. *Proc Natl Acad Sci U S A* **92**, 4562-6 (1995).
31. Singh, R.K., Gutman, M., Llansa, N. & Fidler, I.J. Interferon-beta prevents the upregulation of interleukin-8 expression in human melanoma cells. *J Interferon Cytokine Res* **16**, 577-84 (1996).
32. Muller, U. et al. Functional role of type I and type II interferons in antiviral defense. *Science* **264**, 1918-21 (1994).
33. Yin, A.H. et al. AC133, a novel marker for human hematopoietic stem and progenitor cells. *Blood* **90**, 5002-12 (1997).
34. Miraglia, S. et al. A novel five-transmembrane hematopoietic stem cell antigen: isolation, characterization, and molecular cloning. *Blood* **90**, 5013-21 (1997).
35. Gill, M. et al. Vascular trauma induces rapid but transient mobilization of VEGFR2(+)AC133(+) endothelial precursor cells. *Circ Res* **88**, 167-74 (2001).
36. Vasa, M. et al. Increase in circulating endothelial progenitor cells by statin therapy in patients with stable coronary artery disease. *Circulation* **103**, 2885-90 (2001).
37. Shintani, S. et al. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation* **103**, 2776-9 (2001).
38. Rafii, S., Heissig, B. & Hattori, K. Efficient mobilization and recruitment of marrow-derived endothelial and hematopoietic stem cells by adenoviral vectors expressing angiogenic factors. *Gene Ther* **9**, 631-41 (2002).
39. Conejo-Garcia, J.R. et al. Tumor-infiltrating dendritic cell precursors recruited by a beta-defensin contribute to vasculogenesis under the influence of Vegf-A. *Nat Med* **10**, 950-8 (2004).
40. Gabrilovich, D. et al. Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo. *Blood* **92**, 4150-66 (1998).
41. Ohm, J.E. & Carbone, D.P. VEGF as a mediator of tumor-associated immunodeficiency. *Immunol Res* **23**, 263-72 (2001).
42. Laxmanan, S. et al. Vascular endothelial growth factor impairs the functional ability of dendritic cells through Id pathways. *Biochem Biophys Res Commun* **334**, 193-8 (2005).
43. Oyama, T. et al. Vascular endothelial growth factor affects dendritic cell maturation through the inhibition of nuclear factor-kappa B activation in hemopoietic progenitor cells. *J Immunol* **160**, 1224-32 (1998).

44. Mantovani, A., Schioppa, T., Porta, C., Allavena, P. & Sica, A. Role of tumor-associated macrophages in tumor progression and invasion. *Cancer Metastasis Rev* (2006).
45. Young, M.R., Kolesiak, K., Wright, M.A. & Gabrilovich, D.I. Chemoattraction of femoral CD34+ progenitor cells by tumor-derived vascular endothelial cell growth factor. *Clin Exp Metastasis* **17**, 881-8 (1999).
46. Asahara, T. et al. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *Embo J* **18**, 3964-72 (1999).
47. Takahashi, T. et al. Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med* **5**, 434-8 (1999).
48. Moore, M.A. et al. Mobilization of endothelial and hematopoietic stem and progenitor cells by adenovector-mediated elevation of serum levels of SDF-1, VEGF, and angiopoietin-1. *Ann N Y Acad Sci* **938**, 36-45; discussion 45-7 (2001).

CHAPTER 3

ANTITUMOR AND ANTIANGIOGENIC EFFECTS OF IMMUNIZATION WITH XENOGENIC VASCULAR ENDOTHELIAL GROWTH FACTOR IN DOGS WITH SOFT TISSUE SARCOMA

ABSTRACT

This study was designed to investigate the antitumor and antiangiogenic effects of immunization with recombinant human vascular endothelial growth factor (rhVEGF) in a large animal spontaneous tumor model. Nine dogs with naturally occurring soft tissue sarcoma were vaccinated intradermally with xenogeneic rhVEGF for a course of six vaccinations over a sixteen week period. The effects of vaccination on induction of antibodies to human and canine VEGF, circulating VEGF concentrations, tumor microvessel density (MVD), and tumor growth were assessed. Three out of 4 dogs that received 5 or more vaccinations had a significant increase in anti-human and anti-canine VEGF antibodies, while significant decreases in circulating plasma VEGF concentrations were also noted. Tumor MVD was significantly decreased in two dogs at one or more time points during treatment. Three of the 9 enrolled dogs (33%) had partial tumor regression at completion of the study, while the remaining 6 dogs demonstrated progressive tumor growth.

Immunization with xenogeneic rhVEGF was well-tolerated by all dogs and resulted in induction of humoral responses against both human and canine VEGF in animals receiving multiple immunizations. Vaccination was also associated with reduction in circulating VEGF concentrations and inhibition of tumor angiogenesis and tumor growth in some animals. Based on these results obtained in a large animal, spontaneous tumor model, it appears that xenogeneic vaccination against VEGF may be a useful and safe therapy for inhibition of tumor growth and angiogenesis.

INTRODUCTION

Tumor-associated neovascularization is critical for progressive tumor growth as well as metastasis. One of the key regulatory factors involved in this process is vascular endothlelial growth factor (VEGF) as demonstrated in numerous studies.[1-3] For this reason, strategies to abrogate the biologic effects of VEGF have become a primary focus in attempts to inhibit tumor angiogenesis.[4-9] Various approaches have been developed or are currently undergoing further investigation including the use of mAbs to neutralize VEGF or block its receptors, the use of soluble VEGF receptors, and the use of small molecule inhibitors of VEGF receptor kinase activity.[7-11] Notably, inhibition of tumor angiogenesis with a synthetic VEGF mAb has been shown to elicit a significant increase in survival in humans with colon carcinoma treated concurrently with chemotherapy.[12,13]

Active vaccination against pro-angiogenic factors or their receptors is an alternative approach to therapeutic inhibition of angiogenesis.[14-21] For example, it was found that immunization against xenogeneic VEGF via plasmid-DNA could elicit cross-reactive antibodies which neutralized endogenous VEGF resulting in inhibition of both tumor growth and angiogenesis in a mouse model.[22] Similarly, in several different studies, immunization against the VEGF receptor was also found to inhibit tumor angiogenesis in rodent models.[15,23-26] Vaccination against other pro-angiogenic growth factors, such as basic fibroblast growth factor (bFGF), resulted in inhibition of tumor angiogenesis as well.[27] In yet a third approach, immunization with xenogeneic endothelial cells, or more recently with autologous endothelial cells, again demonstrated therapeutic inhibition of angiogenesis.[28-30] The appeal of such approaches is the ability to achieve sustained suppression of VEGF or VEGF receptor signaling without the necessity for long-term drug administration.[17] However, this approach also holds some risk, inasmuch as sustained neutralization of endogenous VEGF may also elicit deleterious side-effects, such as coagulopathy or interference with wound healing.[9]

Therefore, to more fully assess the potential effectiveness and safety of VEGF vaccination as a viable option for treatment in humans, we conducted a pilot study of xenogeneic VEGF vaccination for inhibition of angiogenesis in dogs with cancer. Dogs spontaneously develop a number of cancers that resemble their counterparts in humans.[31,32] Additionally, as dogs are a large and highly outbred species, immunological studies in dogs are likely to reflect more accurately responses in

humans. For this study, we chose dogs with cutaneous soft tissue sarcoma (STS), which is a relatively slow growing and locally invasive tumor which is accessible to repeated biopsy.[31]

The study was designed to determine whether immunization against a xenogeneic form of VEGF could elicit therapeutic anti-VEGF responses. To accomplish this, dogs were immunized against recombinant human $VEGF_{165}$ (rhVEGF) which differs from canine VEGF (caVEGF) at seven amino acids yielding 95.2% homology.[33] While the use of xenogeneic immunotherapy to target angiogenic regulators has been explored, most studies have relied on DNA vaccine approaches.[22,23,30] Immunization with xenogeneic VEGF protein, as demonstrated here, has not been reported. Additionally, this study employs the use of a novel liposome-DNA adjuvant which has previously been shown to elicit effective immunization against protein antigens in mice. [34,35]

In this study of 9 dogs with soft tissue sarcoma, we found that immunization with rhVEGF was able to elicit significant anti-human VEGF (anti-huVEGF) antibody responses in dogs with STS. Moreover, dogs with high anti-huVEGF antibody levels experienced significant declines in their circulating plasma VEGF concentrations which, in some cases, were associated with reduction in tumor size and tumor microvessel density (MVD). High levels of anti-huVEGF antibodies were also associated with modest but detectable anti-caVEGF antibody responses in some dogs. None of the dogs in this study experienced adverse effects such as delayed wound

healing which might be associated with sustained decreases in endogenous VEGF concentrations. We conclude from these results that immunization against xenogeneic VEGF may be a safe and effective treatment option for the induction of antitumor and antiangiogenic activity in some patients with cancer.

MATERIALS AND METHODS

Study design

Client-owned dogs of any age, breed, or gender with spontaneous, initial or recurring, histologically-confirmed, cutaneous soft tissue sarcomas measuring < $125cm^3$ at the time of diagnosis were eligible for entry into the study. Soft tissue sarcomas of any subtype and of any grade were considered eligible for the study. Tumor volume was determined as the product of 3 tumor measurements (length, width, height). Any dog previously treated with radiation therapy or angiogenesis inhibitors and any dog currently receiving corticosteroids or chemotherapy was excluded from entry into the study. Dogs with concurrent disease or evidence of metastatic disease were also excluded from the study. These studies were approved by the Institutional Animal Care and Use Committee at Colorado State University. The study was designed to include a series of 6 immunizations administered over a course of 16 weeks. Animals were enrolled and treated at the Colorado State University Veterinary Teaching Hospital (Ft. Collins, CO) or at the Veterinary Referral Center of Colorado (Englewood, CO).

Preparation of VEGF vaccine

The vaccine was prepared using $rhVEGF_{165}$, which was manufactured by R&D Systems and provided by the NCI Reagent Repository. The liposome-DNA complexes used as the vaccine adjuvant were prepared as described previously.[34] Briefly, liposomes were prepared by dissolving the cationic lipid DOTIM (octadecenolyoxy{ethyl-2-heptadecenyl-3 hydroxyethyl} imidazolinium chloride; Sigma-Aldrich Chemical Co., St. Louis, MO) and cholesterol (Avanti Polar Lipids, Alabaster, AL) in chloroform and adding equimolar concentrations to round bottom 15 ml glass tubes. The solution was then dried overnight in a vacuum dessicator to a thin film and the lipids were rehydrated in 5% dextrose in water at 50°C for 50 minutes, followed by extrusion through a series of 1μm, 0.45μm, and 0.20μm filters to form the final liposomes. To form the liposome-DNA complexes, non-coding plasmid DNA (prepared by Valentis Corp, Burlingame, CA) was added to liposomes in solution (100μl liposomes per ml D5W) with gentle pipetting at a final concentration of 100μg DNA per ml of liposome solution. The vaccine was then prepared by adding rhVEGF directly to the liposome-DNA solution at room temperature. Dogs were immunized within 15 minutes of preparing the VEGF vaccine.

Treatment and monitoring schedule

A series of six immunizations with rhVEGF$_{165}$ and liposome-DNA complex adjuvant were administered intradermally to dogs once every other week for 3 immunizations and then once every 4 weeks for 3 additional immunizations. For the initial immunizations, dogs received 50μg rhVEGF in 600μl liposome-DNA adjuvant (administered in two different sites over the lateral thorax). Subsequent immunizations consisted of 25μg rhVEGF in 300μl liposome-DNA adjuvant administered in one site over the lateral thorax. Dogs enrolled in the study were monitored for adverse effects by physical examination at the time of each immunization. Complete blood counts and serum biochemical profiles were evaluated prior to starting the study and again at week 6 and week 16. The vaccine site was evaluated at each repeat immunization to assess injection site responses. In addition, dogs were carefully evaluated clinically for evidence of coagulopathy (hemorrhage, petechiation) and delayed wound healing at tumor biopsy sites (see below).

Tumor volume was determined using calipers to measure tumor dimensions prior to treatment and at each vaccination. Tumor responses were classified as partial response if tumor volume decreased by > 50% from pretreatment measurements and complete response if the tumor was no longer detectable. Stable disease was defined as tumor volume that did not increase or decrease by > 50% from starting values, whereas progressive disease was defined as tumor volume that increased > 50% from

starting values. For any dog that demonstrated progressive tumor growth, the trial was terminated, the patient was removed from the study, and alternative therapy was implemented.

Assessment of anti-VEGF antibody responses

Plasma and serum samples were obtained from each patient prior to treatment and immediately prior to vaccination at each of the 6 treatments and stored at -80°C. Plasma concentrations of anti-huVEGF antibodies were determined by enzyme linked immunosorbent assay (ELISA). The anti-huVEGF antibody ELISA was prepared by coating Immunolon-3 flat bottom plates (Dynatech Laboratories, Chantilly, VA) with 50ul of 5ug/ml rhVEGF (R&D Systems, Minneapolis, MN) in 50mM carbonate/bicarbonate buffer at a pH of 9.6 incubated overnight at 4°C. Wells were blocked with 200ul of a 5% solution of nonfat dried milk in phosphate buffered saline (PBS) and incubated at 37°C for 60 minutes. Plasma samples were pre-diluted 1:1,000 in buffer (PBS with 1% BSA), added at 100ul per well, and incubated at room temperature for 90 minutes. After washing, plates were incubated with biotinylated-SP-conjugated rabbit anti-dog IgG (Jackson ImmunoResearch, West Grove, PA) at 1:40,000 in buffer (100ul/well) and incubated for 60 minutes at room temperature. After washing, streptavidin-horseradish peroxidase conjugate (Jackson ImmunoResearch) at 1:2000 diluted in PBS was added (100ul/well) for 30 minutes at room temperature. Following a final wash, wells were developed with 3,3',5,5'-tetramethylbenzadine (TMB) liquid substrate system (Sigma, St. Louis, MO), the

reaction stopped with 1M hydrochloric acid, and optical density determined colorometrically at a 405nm wavelength by ELISA plate reader (Thermo LabSystems, Franklin, MA).

Plasma concentrations of anti-caVEGF antibodies were determined following the same protocol as described above however with the substitution of 50ul of 5ug/ml of recombinant canine VEGF (rcaVEGF) (R&D Systems) for rhVEGF as the coating (detection) reagent. Additionally, mouse anti-canine VEGF antibody (R&D Systems) at .5ug/ml incubated with biotinylated-donkey anti-mouse IgG (Jackson ImmunoResearch) at 1:20,000 as secondary antibody was utilized for assay validation.

Measurement of plasma VEGF concentrations

Plasma VEGF concentrations in dogs were determined using a human VEFG ELISA kit (R&D Systems) in accordance with the manufacturer's directions. Validation of this kit for the detection of canine VEGF has been previously documented. [36-38]

Tumor biopsy and assessment of tumor microvessel density, VEGF expression, and IgG deposition.

Tumor biopsies were obtained under local anesthesia by wedge biopsy immediately prior to the first treatment, at week 6, and at week 16. Biopsy sites were sutured and biopsy specimens were embedded in Tissue-Tek OCT compound (TedPella Inc.,

Redding, CA), snap frozen in isopentane and dry ice, and stored at -80°C. Tissues were cryosectioned to a thickness of 4μm and adhered to positively charged glass slides. For identification of tumor microvessels, a mAb specific for human CD146 (clone P1H12, Chemicon, Temecula, CA) which cross reacts with canine endothelial cells was utilized as described previously.[36,39] Tissues were fixed in acetone, blocked for endogenous peroxidase activity with 3% hydrogen peroxide (H_2O_2) for 10 minutes at room temperature and blocked for non-specific binding with 5% normal donkey serum. Slides were then incubated with the primary antibody (mouse anti-human P1H12) for 30 minutes at room temperature and washed in 1x PBS for 10 minutes prior to a 25 minute incubation with the secondary donkey anti-mouse IgG antiserum antibody (Jackson ImmunoResearch). Slides were then treated with streptavidin–HRP followed by 3-amino-9-ethylcarbazole (AEC) substrate (Vector Laboratories, Burlingame, CA), counterstained with hemtoxylin, air dried, and coverslipped with aqueous based crystal mount. Negative controls included incubation with irrelevant mAb and omission of the primary antibodies. Normal canine spleen was utilized as positive control tissue.

To quantitate tumor MVD, four digital photomicrographs at 10X magnification were obtained (Leica Microscope equipped with digital camera in conjunction with SPOT Advanced Imaging software), as described previously.[36] Adobe Photoshop and Reindeer Graphics Quantitative Analysis Plug-ins (Asheville, NC) were utilized to convert the photomicrographs to binary images and the number of microvessels per section, more specifically analyzed as number of pixles, were determined. The final

MVD value for each tumor section was calculated as the mean (± SEM) number of vessels for the four 10X fields.

To assess tumor associated VEGF expression, cryosectioned slides were first fixed in 10% formalin for 5 minutes rather than fixation in acetone. Prior to staining, slides were treated in target retrieval solution (Dako, Carpinteria, CA) pH 6.0 at 125°C for one minute. Staining was then performed utilizing a Dako autostainer in conjunction with Dako reagents (LSAB+ Link System HRP, Dako K0679) according to manufacturer's directions. Goat anti-canine VEGF antibody (R&D Systems) at 1:25 was utilized as the primary antibody and diaminobenzidine (DAB) was utilized as substrate. Following staining, slides were then dehydrated through graded alcohol followed by xylene and coverslipped with permanent based mount. All sections were subjectively evaluated microscopically to assess the percentage of VEGF-positive cells and relative intensity of staining. Sections were assigned a score of 0 to 3 where 0 represented no detectable immunoreactivity and 3 represented intense and generalized imunoreactivity.

For assessment of IgG deposition in tumor tissues, cryosectioned biopsy specimens were prepared as described above however with acetone rather than formalin fixation. Slides were incubated with peroxidase-conjugated rabbit anti-dog IgG (Jackson ImmunoResearch) for 30 minutes at room temperature, followed by incubation with AEC substrate for 15 minutes at room temperature. Incubation with irrelevant primary antibody (rabbit anti-mouse IgG) and omission of primary antibody were

used as negative controls while normal canine splenic tissue was utilized as a positive control. All sections were subjectively evaluated microscopically and assigned a score of 0 to 3 based on overall IgG immunoreactivity and intensity of staining similar to that as described above for VEGF immunoreactivity.

Statistical analyses

Paired samples were compared statistically using the Student's t-test. Comparisons between more than two treatment groups or more than two time points were done using one way analysis of variance (ANOVA), followed by Tukey's multiple means comparisons test. Analysis was done using GraphPad Prism statistical software (San Diego, CA). For all analyses, p values <0.05 were considered statistically significant.

RESULTS

Effects of vaccination on anti-VEGF antibody induction

A total of 9 dogs with soft tissue sarcoma were enrolled in the study (Table 1). Of these 9 dogs, 4 dogs remained in the study long enough to receive 5 or more immunizations, while 5 dogs received 3 or fewer immunizations. Treatment was discontinued at various time points in 5 dogs once it had been determined that the

tumor was growing progressively. For the 4 dogs that received 5 or 6 immunizations, antibody concentrations generally increased over time, with 3 of the 4 dogs mounting a significant increase in anti-VEGF antibody responses (Figure 1). None of the 5 dogs receiving 3 or fewer vaccinations mounted a significant anti-VEGF antibody response (Table 1). Thus, it appeared that immunization with rhVEGF was capable of eliciting anti-VEGF antibody responses, but that multiple immunizations (at least 5 or more) were required to elicit significant increases. Significant antibody responses ranged from four-fold to ten-fold increases in anti-huVEGF antibody when compared to pre-treatment values.

Dog	Dx/Grade	Clinical Response	α-huVEGF Ab	Plasma VEGF	MVD	# of Vacc.
1	PNST / I	PR	+	Decreased	NC	6
2	PNST / I	PR	+	Decreased	Decreased	6
3	PNST / II	PR	-	Decreased	NC	6
4	PNST / I	PD	+	NC	Decr/Incr	5
5	PNST / I	PD	-	Decreased	NA	3
6	PNST/ R-II	PD	-	NC	NA	3
7	PNST / II	PD	-	NC	NA	2
8	Undiff Src / III	PD	-	NC	NA	2
9	PNST / R-I	PD	-	NC	NA	1

Table 1. Clinical responses in 9 dogs immunized with rhVEGF. Nine dogs with soft tissue sarcoma were treated with repeated rhVEGF vaccination. Clinical responses were classified as partial response (**PR**; greater than 50% decrease in tumor volume) or progressive disease (**PD**; greater than 50% increase in tumor volume). **NC** = no change; **NA** = not available for evaluation. **α-huVEGF Ab: (+)** = a 3-fold increase or greater when comparing antibody levels at the latest time point to pre-vaccination antibody levels. **Plasma VEGF: Decrease** = a statistically significant decrease in circulating [...] when comparing levels at the latest time point to pre-vaccination levels. **MVD: Decreased** or **Increased** if $p < .05$ at one or more time points during treatment when compared to pretreatment MVD. **# of Vacc.** = the # of vaccinations each dog received. **Dx/Grade:** Tumor type (diagnosis) and grade where **PNST** = peripheral nerve sheath tumor, **Undiff Src.** = undifferentiated sarcoma, **I,II, III =** grade, and **R =** recurrence.

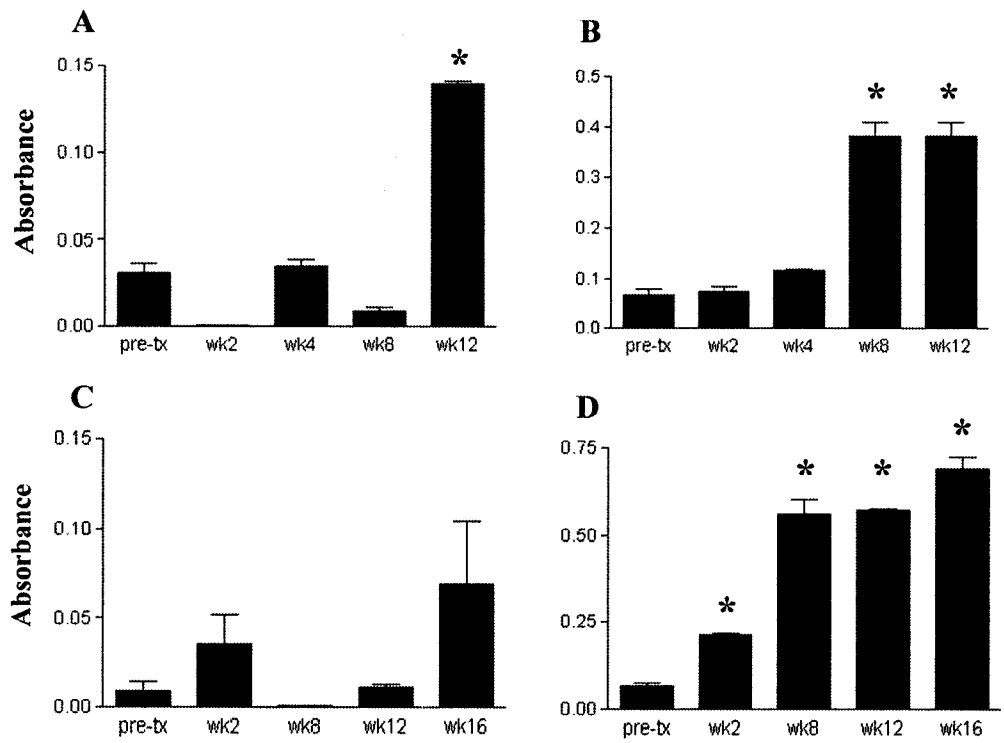

Figure 1. Anti-huVEGF antibody responses following vaccination against recombinant human VEGF (rhVEGF) in dogs. Nine dogs with STS were enrolled in a study to evaluate the effects of vaccination against xenogeneic (human) VEGF. Plasma samples were collected prior to immunization (pre-tx) and at several different time points after initial immunization with rhVEGF. Plasma samples were assayed via ELISA for detection of anti-huVEGF antibodies as described in materials and methods. Anti-huVEGF antibody responses over time were plotted for the 4 dogs that received 5 or more immunizations (A-D, which correlates with dogs #1-4 in Table 1 respectively). A significant increase in anti-huVEGF antibody levels following immunization with rhVEGF was noted in 3 of the 4 dogs (panels A, B, & D).In all three dogs, elevated levels were detected following the 4^{th} or 5^{th} vaccine, however, dog 4 (panel D) additionally had significantly elevated levels after the first immunization. * denotes a significant ($p < 0.05$) increase in anti-huVEGF antibody response compared to pre-treatment values, as analyzed by ANOVA. The y-axis is provided as absorbance (OD) as the assay was not performed with a commercialized kit (see materials and methods) and there was no available antibody on which to perform serial dilutions from a known concentration thus precluding generation of a standard curve from which to calculate concentrations.

Effects of vaccination on circulating plasma VEGF concentrations

We next assessed the effects of VEGF immunization on circulating VEGF concentrations utilizing a human VEGF ELISA kit as described in materials and methods. A significant decrease in plasma VEGF concentrations was observed at two or more time points in 3 of the 4 dogs that received 5 or more VEGF immunizations (Figure 2). In the fourth dog, no significant changes in circulating VEGF concentrations were noted despite a significant increase in anti-huVEGF antibodies (see dog 4 Table 1, Figure 1& 2). In contrast, VEGF concentrations did not decrease in 4 of the 5 dogs that received 3 or fewer VEGF immunizations (Table 1). The one exception was the dog (dog #5) that was immunized 3 times, where a significant decrease in VEGF concentrations was noted (data not shown). Thus, in some dogs, repeated immunization with rhVEGF induced a significant anti-huVEGF antibody response and was associated with a significant decline in VEGF concentrations (dogs 1 and 2, Table 1). In two other dogs (dogs 3 and 5, Table 1) there was a significant decrease in circulating VEGF despite the lack of a significant anti-huVEGF antibody response. These results suggest that in some dogs, VEGF vaccination may have induced a decrease in circulating VEGF concentrations by immunological mechanisms other than only induction of anti-VEGF antibody responses.

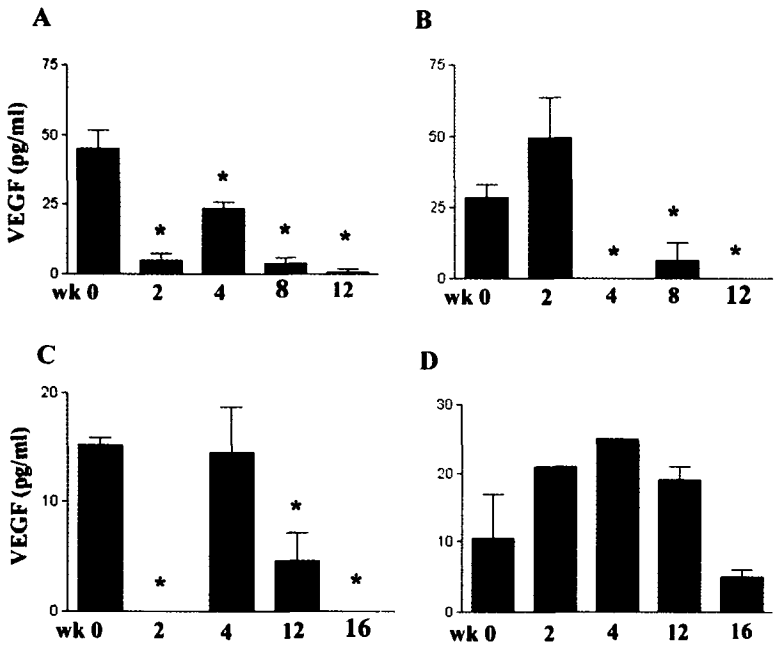

Figure 2. Effects of vaccination on plasma VEGF concentrations in dogs. Circulating VEGF concentrations were measured in dogs prior to vaccination (week 0) and at twice weekly intervals following vaccination with rhVEGF. VEGF concentrations were determined by VEGF ELISA, utilizing a human VEGF ELISA kit as described in materials and methods. In four dogs (A-D) that received more than 5 immunizations, VEGF concentrations were plotted over time. Significant decreases in plasma VEGF levels are noted in panels A, B, & C (dogs 1, 2, & 3) which correlate with significant increases in anti-huVEGF Abs at the completion of immunization in dogs 1 & 2 (Figure 1). There is a significant derease in plasma VEGF levels at week 16 when compared to week 4 in dog 4 (panel D) however this is not a significant decrease when compared to pre-tx levels at week 0. Changes in VEGF levels do not correlate with changes in anti-huVEGF Ab levels in dogs 3 & 4 (panels C & D). Rationale for these results is addressed in the discussion. * denotes a significant ($p < 0.05$) decrease in VEGF concentration, compared to pre-treatment (wk 0) concentrations, as determined by ANOVA.

Effects of vaccination on induction of an anti-canine VEGF antibody response

Next, studies were conducted to determine whether vaccination with rhVEGF was capable of eliciting cross-reactive antibodies against endogenous canine VEGF (caVEGF). Such a response would be expected if immunization against xenogeneic VEGF was capable of breaking tolerance. Plasma samples from each of the four dogs

that received five or more rhVEGF immunizations were assessed by ELISA for the presence of antibodies recognizing caVEGF. We found that anti-caVEGF antibody levels demonstrated a statistically significantly increase in 3 of the 4 multiply vaccinated dogs that had also mounted strong anti-huVEGF responses (Figures 3 & 1). However, the antibody responses that were detected against caVEGF were weak compared to the responses detected against huVEGF.

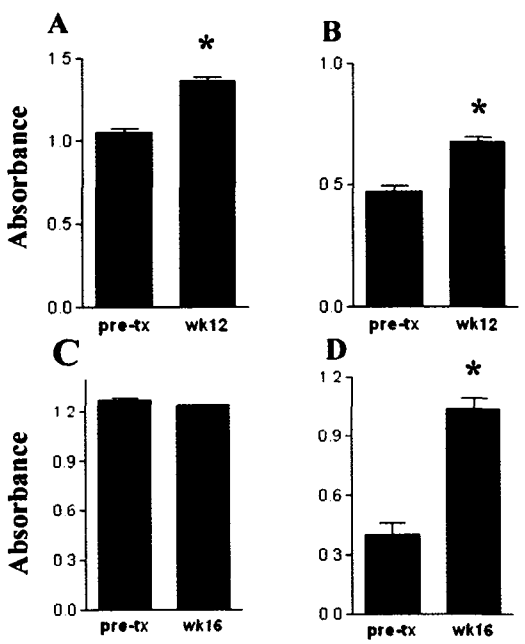

Figure 3. Detection of anti-caVEFG antibodies in dogs immunized with rhVEGF. Dogs were immunized against rhVEGF as described in Figure 1 and plasma was collected for analysis of anti-caVEGF antibody responses by ELISA, as described in materials and methods. For the 4 dogs (A-D) that received 5 or more VEGF vaccines, anti-caVEGF antibody responses were measured prior to treatment and again at the completion of the immunization period (week 12 or week 16). Pre-treatment and post-treatment antibody responses were compared by t-test and significant differences ($p < 0.05$) were denoted by *.

Effects of vaccination on tumor MVD

Tumor microvessel density (MVD) was evaluated in biopsy specimens obtained pre-treatment, on week 6, and on week 16 of the study from the 4 dogs that received multiple VEGF immunizations. Due to the design of the study, tumor biopsies could 0not be obtained from the 5 dogs that received 3 or fewer immunizations since they did not remain in the study long enough to reach the first biopsy time point. Two of the 4 multiply vaccinated dogs demonstrated a significant decrease in tumor MVD at one or more time points when compared to pre-treatment MVD (Figures 4 and 5). It should be noted however that in one of these dogs, tumor MVD actually increased at a later time point, coincident with progressive growth of the tumor (see dog 4 in Table 1). In the other 2 dogs, tumor MVD remained relatively constant after VEGF immunization was initiated. Thus, it appeared that repeated VEGF immunization was capable of inhibiting tumor angiogenesis in some of the multiply vaccinated dogs. Due to inadequate tissue sample, MVD analysis at week 6 for dog #1 was not attainable.

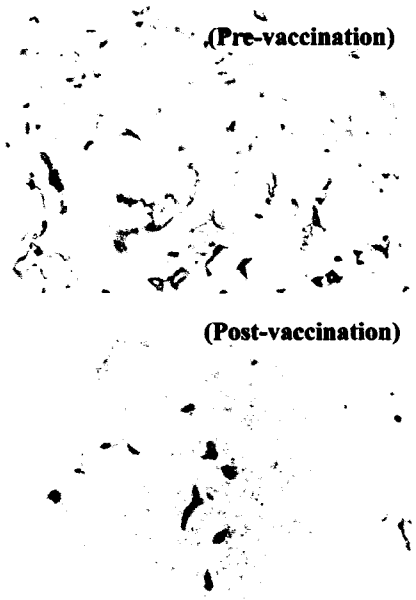

Figure 4. Determination of tumor microvessel density by immunohistochemistry. Two 20x photomicrographs of immunohistochemicaly stained sections from tumor biopsy samples obtained at week 0 (pre-vaccination) and week 16 (post-vaccination) from dog #2 (see Table 1; B in Figures 1-3). Sections were immunostained with a murine mAb specific for human CD146 (Clone P1H12, Chemicon) as described in Methods. Tumor MVD was markedly decreased at week 16 (post-vaccination), compared to MVD at week 0 (pre-vaccination).

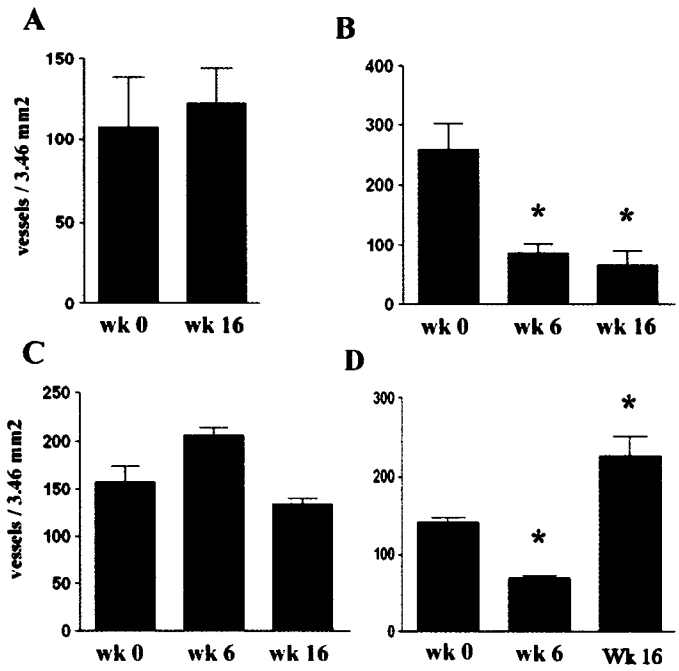

Figure 5. Effects of vaccination on tumor microvessel density in dogs with soft tissue sarcomas. Tumor biopsies were obtained from 4 dogs (A-D) before treatment, at 6 weeks, and again at 16 weeks after initiation of the VEGF vaccine trial. Tumor microvessel density (MVD) was quantitated using CD146 immunohistochemistry, as described in materials and methods. Pre- and post-treatment tumor MVD was compared by ANOVA. Significant differences ($p < 0.05$) are denoted by *. A significant decrease in MVD was noted in dogs 2 & 4 (B & D respectively) following initiation of rhVEGF vaccination however, in dog 4 (D) a significantly increased MVD, coincident with tumor progression, was noted thereafter.

Effects of vaccination on intratumoral VEGF expression and IgG deposition

We also investigated the possible effects of repeated VEGF vaccination on intra-tumoral VEGF concentrations. Immunohistochemistry was used to evaluate intracellular expression of VEGF by tumor cells and other cells within the tumor from pre- and post-vaccination tumor biopsies from 4 dogs that received more than 5 immunizations. In all 4 cases, we did not note a significant difference in the extent or intensity of tumor VEGF staining when pre- and post-vaccination biopsies were

compared (data not shown). Of note, however, is that immunohistochemical expression of VEGF was performed on cryosections as described in materials and methods which, to the author's knowledge, has not been previously described in the literature.

Immunohistochemical assessment of IgG complex deposition within the tumor or tumor vasculature was also evaluated as deposition has been noted in tumors of mice immunized with xenogeneic VEGF previously.[23] We did not find any evidence of a vaccine effect when pre- and post-vaccination tumor biopsies were evaluated (data not shown). These results suggest that the major effect of immunization on canine VEGF probably occurs systemically, rather than within the tumor itself.

Clinical responses in vaccinated dogs

Tumor responses were assessed in all dogs enrolled in the study by repeated measurements of tumor volume (Figure 6). A partial tumor response (> 50% decrease in tumor volume) was observed in 3 of the 9 enrolled dogs. In the other 6 dogs, progressive tumor growth was observed and all were disenrolled from the study before completing their full series of rhVEGF immunizations. The 3 dogs in which tumor regression was noted were also those that received the full course of 6 immunizations (Table 1). One explanation for these results is that receiving the full course of VEGF immunizations was more likely to induce tumor regression.

However, we cannot exclude the possibility that dogs with inherently less aggressive tumors were those that were able to complete the vaccination regime.

Finally, all treated dogs were carefully evaluated for adverse effects associated with repeated VEGF immunization. Vaccine site reactions were not observed in any of the dogs. Additionally, no clinical evidence of coagulopathies (eg, excessive bleeding from venipuncture or biopsy sites) or changes in complete blood counts or serum biochemistries were observed (data not shown). We also did not observe any interference with wound healing at the tumor biopsy sites in the 4 dogs that had repeated tumor biopsies performed. Thus, it appeared, based on this small series of dogs, that repeated immunization against xenogeneic VEGF did not elicit serious or unanticipated side-effects.

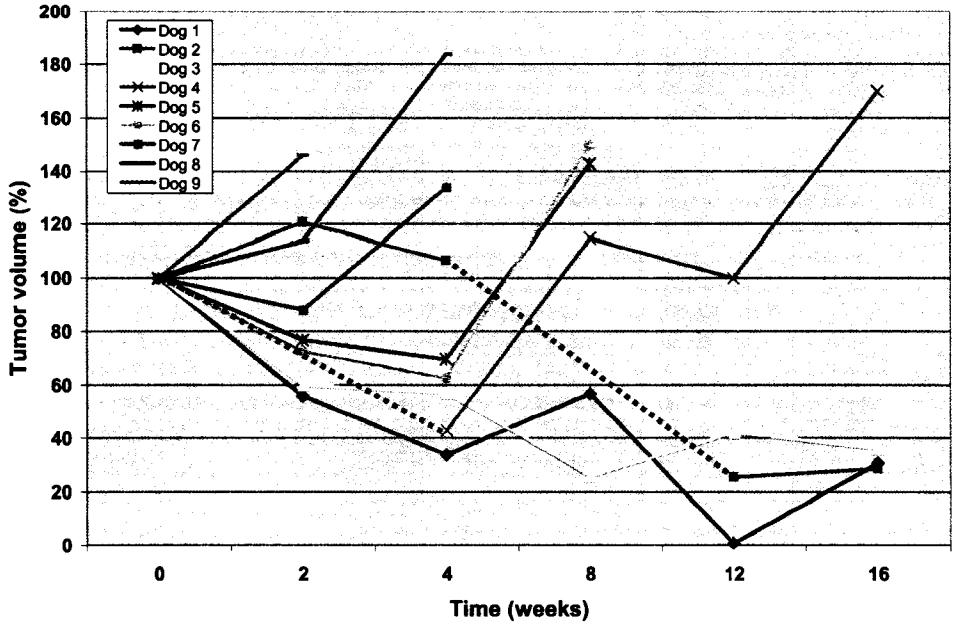

Figure 6. Tumor response in all dogs immunized with rhVEGF. Clinical tumor response was determined by measurements of tumor volume at the time of entry into the study (week 0) and over the course of vaccination (weeks 2, 4, 8, 12, & 16). Tumor volume at time of entry (wk 0) was considered as 100% and changes in tumor volume over time were calculated (and plotted) as respective percent increases or decreases. Dogs 1 through 3 demonstrated a partial tumor response (>50% decrease in tumor volume) at completion of the study (week 16) while dog 1 additionally had close to a complete response (no detectable tumor) with a tumor volume of .0125cm^3 at week 12. Dog 4 had an initial partial response (wk 4) which was followed by progressive disease (>50% increase in tumor volume). Dogs 5 through 9 had progressive disease and were subsequently disenrolled. Dog 7 was disenrolled at week 8 due to progressive disease however tumor volume was not recorded and thus this time point is not represented on the graph above. Dotted lines (dogs 2 & 4) connect time points for which the tumor volume at the interposed time point (wk 8 & wk 2 respectively) was not available.

DISCUSSION

This study successfully demonstrates that repeated immunization of dogs with recombinant human VEGF, which is highly homologous to canine VEGF, is able to induce anti-huVEGF humoral responses. This generation of antibody responses

against human VEGF was additionally associated with significant reductions in circulating VEGF concentrations, induction of antitumor activity, and reduction of tumor microvessel density in two of the four dogs where post-immunization biopsies were available for analysis. We also observed weak recognition of caVEGF by plasma from dogs with significant anti-huVEGF antibody responses. These studies, which are the first involving xenogeneic immunization with VEGF protein in a large animal spontaneous tumor model, indicate that this approach is technically feasible, may inhibit angiogenesis, can stimulate anti-tumor activity, and appears to be a safe therapeutic strategy in animals with cancer.

From the study, it was apparent that the generation of anti-VEGF humoral responses was critically dependent on the number of immunizations an animal received. Dogs that received fewer than 5 immunizations generally failed to produce significant anti-huVEGF antibody responses whereas significant antibody responses were observed in dogs that received 5 or 6 immunizations (see Figure 1). In 2 of 3 dogs with significant anti-huVEGF antibody responses, significant decreases in circulating plasma VEGF concentrations were also noted (see Table 1). In contrast, circulating VEGF concentrations were significantly reduced in two dogs that did not mount statistically significant anti-huVEGF antibody responses. We believe, though it cannot be proven from this study alone, that vaccination against VEGF is likely to be the primary reason for the decrease in VEGF concentration. We base this belief in part on the fact that in a previous study in 13 dogs with the same tumor type undergoing nonspecific immunotherapy, we did not observe any significant change in

circulating VEGF concentration on repeated analyses over a period of 12 weeks despite observing antitumor activity.[36]

Examination of tumor MVD in the 4 dogs that received 5 or more vaccinations, revealed a significant decrease in one dog (dog 2, Table 1) during and at the end of treatment, no change in MVD in 2 dogs, and a decrease in tumor MVD in one dog followed by an increase (dog 4, Fig. 4). This late increase in MVD following the initial decrease in MVD could possibly be due to tumor-associated production and secretion of alternative pro-angiogenic factors, such as bFGF or interleukin-8, subsequent to the selective pressure of anti-VEGF antibodies. [27]

We also evaluated tumor responses clinically by serially measuring tumor volume. From analysis of this data (Table 1 & Figure 6), it was apparent that number of vaccinations correlated closely with clinical tumor responses. For example, 3 of 4 dogs that received 5 or more immunizations had objective clinical tumor responses. In contrast, all 5 dogs that had 3 or fewer vaccinations experienced progressive tumor growth. Tumor responses and number of vaccinations however may have also been influenced by tumor grade. While entry into this study required a diagnosis of soft tissue sarcoma (STS), there was no restriction with regard to tumor grade or recurrence. Three of the 4 dogs that received 5 or more immunizations were diagnosed with grade I tumors while the fourth dog was diagnosed with a grade II STS (Table 1). In contrast, in the 5 dogs that received fewer than 4 vaccinations, due to progressive disease, diagnoses included 2 dogs with grade I tumors (one of which

was recurrent), 2 dogs with grade II tumors (also one of which was recurrent), and one dog with a grade III STS (Table 1).

It is also important to note that circulating anti-VEGF antibodies were evaluated in plasma rather than in serum. Acquisition of serum requires in vitro coagulation however, when blood samples clot, platelets can release substantial amounts of VEGF.[40-42] This aberrant release of VEGF could potentially react with any serum associated anti-VEGF antibodies thereby falsely lowering the anti-VEGF antibody concentrations that were detected. This, in fact was found when we initially compared VEGF antibody results from paired serum and plasma samples from some dogs in the study (data not shown). Therefore, for the studies shown here, plasma was used for both antibody and circulating VEGF concentration studies.

One of the endpoints of this study was to assess the ability of the xenogeneic vaccine to overcome self-tolerance against endogenous VEGF. Though the vaccine utilized a potent vaccine adjuvant and did elicit substantial antibody responses against the xenoantigen (huVEGF) in some dogs, there was no direct data from the study that strong antibody responses were generated against caVEGF (see Figures 1 and 3). This is reflected in the results obtained from direct ELISA where the recognition of caVEGF by plasma from multiply-immunized dogs, although statistically significant, was very weak when compared to anti-huVEGF antibody responses. However, it is possible that anti-canine VEGF antibodies were elicited but that they were no longer present in plasma for detection due to complexing with VEGF. For example, in 3 of

the 4 multiply vaccinated dogs there was a significant reduction in circulating VEGF concentrations (Figure 1 and 2) despite lack of marked anti-caVEGF antibodies (Figure 3). These results suggest the possibility that cross-reactive antibodies against caVEGF were generated, but that they were rapidly eliminated from circulation following binding to circulating caVEGF. Differences in antibody affinity or specificity for huVEGF versus caVEGF may also help explain the difference in levels of anti-huVEGF and anti-caVEGF antibodies detected. For example, antibodies elicited against unique determinants on huVEGF not expressed on caVEGF would still be detected using the huVEGF ELISA, but would not be detected using the caVEGF ELISA. Also, if higher affinity antibodies were elicited against huVEGF than against caVEGF after vaccinating dogs with rhVEGF, this would also be reflected in the higher antibody concentrations detected using the huVEGF ELISA. In support of the idea that the reduction of circulating VEGF had a functional effect, we noted that 3 of the 4 dogs that had a reduction in circulating plasma VEGF concentrations also had objective tumor responses (Table 1). The fourth animal with reduced circulating VEGF levels however, which did not have a tumor response, did not have detectable anti-VEGF antibodies (Table 1).

Another major endpoint of the study was to determine whether neutralization of VEGF by vaccination was a safe approach clinically. By evaluating this approach in a spontaneous tumor model in dogs, which are very similar physiologically and immunologically to humans, we hoped to provide a realistic assessment of potential safety in humans. We did not observe obvious adverse effects in this study. In

particular, we did not observe interference with normal angiogenic processes, such as wound healing. More specifically, in dogs that underwent multiple tumor biopsies over the course of the study, we did not observe any problems with healing of the tumor biopsy sites. In addition, we also did not note obvious clotting disturbances on physical examination or following blood collection by venipuncture. Thus, by standard clinical criteria we did not observe that vaccination against xenogeneic VEGF inhibited processes of normal angiogenesis or clot formation, though these were not directly evaluated by laboratory testing. Also, we cannot comment on the possible effects of VEGF vaccination on fertility, as all of the female dogs in this study were neutered.

One of the major determinants as to whether or not a treatment effect was observed appeared to be the number of vaccines that could be administered before progressive tumor growth developed. This might reflect primarily the inherent growth rate and/or aggressiveness of the tumor, or might reflect how quickly antibody responses could be elicited by the vaccine. Thus, anything that could increase the efficiency of immunization might improve treatment outcomes. For example, immunization with an increased dose of VEGF antigen, vaccination by a different route, or use of an alternative adjuvant may elicit greater or more rapid antibody responses. Of note, prior to this study, a smaller pilot study of xenogeneic VEGF vaccination in 4 dogs immunized with rhVEGF$_{165}$ plasmid DNA, delivered by the intramuscular route was completed in our lab. Only very weak anti-huVEGF antibody responses and no objective tumor responses were observed (Dow, SW; unpublished data). Therefore,

we predict that the use of conventional plasmid DNA immunization in dogs is unlikely to offer any significant improvement over the current immunization protocol. Additionally, cellular immune responses to VEGF were not evaluated in this pilot study and therefore the role cellular immunity might have played in some of the observed responses is unknown. We did however note that there were no obvious increases in mononuclear cell infiltration (CD4 and CD8 T-cells) via immunohistochemistry in pre- and post-vaccination tumor biopsy specimens (data not shown).

Of note, it is recognized, similar to that in chapter 1, that this study does not include a placebo-treated group. It is also recognized that inclusion of such a group could strengthen and further validate the abovementioned conclusions. Inclusion of such a group however would require the absence of treatment in the face of disease thus presenting potential ethical concerns. This component thus becomes an inherent problem or issue in lieu of clinical trials as demonstrated both here and in the clinical trial presented in chapter 1. An additional group which could arguably be implemented into this study to strengthen results could be an adjuvant-only treated group such that these patients would receive the liposome-DNA adjuvant devoid of the rhVEGF protein. This would help to further define what, if any, responses were elicited by the adjuvant alone. A third group receiving the rhVEGF protein in an inert adjuvant would further round out the study allowing for evaluation of the effects elicited by each vaccine component individually as well as in combination.

In summary, this pilot study demonstrates that repeated vaccination with xenogeneic VEGF protein in a liposome-DNA adjuvant is well-tolerated clinically in 9 dogs with cutaneous soft tissue sarcoma. Moreover, in animals that received 5 or more immunizations, there was induction of strong anti-huVEGF antibody responses. Induction of this humoral response was also associated with a significant decrease in circulating VEGF concentrations, a reduction in tumor volume, and, in some patients, a reduction in tumor MVD. These data, generated in an outbred species, suggest that xenogeneic vaccination against pro-angiogenic growth factors has great potential as an alternative or adjunctive therapy to controlling tumor growth and angiogenesis.

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REFERENCES

1. Carmeliet, P. VEGF as a key mediator of angiogenesis in cancer. *Oncology* **69 Suppl 3**, 4-10 (2005).
2. Millanta, F. et al. The role of vascular endothelial growth factor and its receptor Flk-1/KDR in promoting tumour angiogenesis in feline and canine mammary carcinomas: A preliminary study of autocrine and paracrine loops. *Res Vet Sci* (2006).
3. Parikh, A.A. & Ellis, L.M. The vascular endothelial growth factor family and its receptors. *Hematol Oncol Clin North Am* **18**, 951-71, vii (2004).
4. Benouchan, M. & Colombo, B.M. Anti-angiogenic strategies for cancer therapy (Review). *Int J Oncol* **27**, 563-71 (2005).
5. Cao, Y. Antiangiogenic cancer therapy. *Semin Cancer Biol* **14**, 139-45 (2004).
6. Cardones, A.R. & Banez, L.L. VEGF inhibitors in cancer therapy. *Curr Pharm Des* **12**, 387-94 (2006).
7. Ferrara, N. VEGF as a therapeutic target in cancer. *Oncology* **69 Suppl 3**, 11-6 (2005).
8. Glade-Bender, J., Kandel, J.J. & Yamashiro, D.J. VEGF blocking therapy in the treatment of cancer. *Expert Opin Biol Ther* **3**, 263-76 (2003).
9. Verheul, H.M. & Pinedo, H.M. Inhibition of angiogenesis in cancer patients. *Expert Opin Emerg Drugs* **10**, 403-12 (2005).
10. Sandler, A.B., Johnson, D.H. & Herbst, R.S. Anti-vascular endothelial growth factor monoclonals in non-small cell lung cancer. *Clin Cancer Res* **10**, 4258s-4262s (2004).
11. Zhang, H.T. & Bicknell, R. Therapeutic inhibition of angiogenesis. *Mol Biotechnol* **25**, 185-200 (2003).
12. Salesi, N. et al. Clinical experience with bevacizumab in colorectal cancer. *Anticancer Res* **25**, 3619-23 (2005).
13. Sanborn, R.E. & Sandler, A.B. The safety of bevacizumab. *Expert Opin Drug Saf* **5**, 289-301 (2006).
14. Okaji, Y. et al. Vaccines targeting tumour angiogenesis--a novel strategy for cancer immunotherapy. *Eur J Surg Oncol* **32**, 363-70 (2006).
15. Niethammer, A.G. et al. A DNA vaccine against VEGF receptor 2 prevents effective angiogenesis and inhibits tumor growth. *Nat Med* **8**, 1369-75 (2002).
16. Li, Y. et al. Active immunization against the vascular endothelial growth factor receptor flk1 inhibits tumor angiogenesis and metastasis. *J Exp Med* **195**, 1575-84 (2002).
17. Li, Y., Bohlen, P. & Hicklin, D.J. Vaccination against angiogenesis-associated antigens: a novel cancer immunotherapy strategy. *Curr Mol Med* **3**, 773-9 (2003).
18. Moniz, M., Yeatermeyer, J. & Wu, T.C. Control of cancers by combining antiangiogenesis and cancer immunotherapy. *Drugs Today (Barc)* **41**, 471-94 (2005).

19. Rafii, S. Vaccination against tumor neovascularization: Promise and reality. *Cancer Cell* **2**, 429-31 (2002).
20. Reisfeld, R.A., Niethammer, A.G., Luo, Y. & Xiang, R. DNA vaccines suppress tumor growth and metastases by the induction of anti-angiogenesis. *Immunol Rev* **199**, 181-90 (2004).
21. Reisfeld, R.A., Niethammer, A.G., Luo, Y. & Xiang, R. DNA vaccines designed to inhibit tumor growth by suppression of angiogenesis. *Int Arch Allergy Immunol* **133**, 295-304 (2004).
22. Wei, Y.Q. et al. Immunogene therapy of tumors with vaccine based on Xenopus homologous vascular endothelial growth factor as a model antigen. *Proc Natl Acad Sci U S A* **98**, 11545-50 (2001).
23. Liu, J.Y. et al. Immunotherapy of tumors with vaccine based on quail homologous vascular endothelial growth factor receptor-2. *Blood* **102**, 1815-23 (2003).
24. Lu, F., Qin, Z.Y., Yang, W.B., Qi, Y.X. & Li, Y.M. A DNA vaccine against extracellular domains 1-3 of flk-1 and its immune preventive and therapeutic effects against H22 tumor cell in vivo. *World J Gastroenterol* **10**, 2039-44 (2004).
25. Keke, F., Hongyang, Z., Hui, Q., Jixiao, L. & Jian, C. A combination of flk1-based DNA vaccine and an immunomodulatory gene (IL-12) in the treatment of murine cancer. *Cancer Biother Radiopharm* **19**, 649-57 (2004).
26. Luo, Y. et al. Immunotherapy of tumors with protein vaccine based on chicken homologous Tie-2. *Clin Cancer Res* **12**, 1813-9 (2006).
27. Plum, S.M. et al. Administration of a liposomal FGF-2 peptide vaccine leads to abrogation of FGF-2-mediated angiogenesis and tumor development. *Vaccine* **19**, 1294-303 (2000).
28. Scappaticci, F.A. & Nolan, G.P. Induction of anti-tumor immunity in mice using a syngeneic endothelial cell vaccine. *Anticancer Res* **23**, 1165-72 (2003).
29. Okaji, Y. et al. Vaccination with autologous endothelium inhibits angiogenesis and metastasis of colon cancer through autoimmunity. *Cancer Sci* **95**, 85-90 (2004).
30. Wei, Y.Q. et al. Immunotherapy of tumors with xenogeneic endothelial cells as a vaccine. *Nat Med* **6**, 1160-6 (2000).
31. Vail, D.M. & MacEwen, E.G. Spontaneously occurring tumors of companion animals as models for human cancer. *Cancer Invest* **18**, 781-92 (2000).
32. Porrello, A., Cardelli, P. & Spugnini, E.P. Oncology of companion animals as a model for humans. an overview of tumor histotypes. *J Exp Clin Cancer Res* **25**, 97-105 (2006).
33. Scheidegger, P. et al. Vascular endothelial growth factor (VEGF) and its receptors in tumor-bearing dogs. *Biol Chem* **380**, 1449-54 (1999).
34. Zaks, K. et al. Efficient immunization and cross-priming by vaccine adjuvants containing TLR3 or TLR9 agonists complexed to cationic liposomes. *J Immunol* **176**, 7335-45 (2006).
35. Mueller, R.S., Veir, J., Fieseler, K.V. & Dow, S.W. Use of immunostimulatory liposome-nucleic acid complexes in allergen-specific

immunotherapy of dogs with refractory atopic dermatitis - a pilot study. *Vet Dermatol* **16**, 61-8 (2005).
36. Kamstock, D. et al. Liposome-DNA complexes infused intravenously inhibit tumor angiogenesis and elicit antitumor activity in dogs with soft tissue sarcoma. *Cancer Gene Ther* **13**, 306-17 (2006).
37. Clifford, C.A. et al. Plasma vascular endothelial growth factor concentrations in healthy dogs and dogs with hemangiosarcoma. *J Vet Intern Med* **15**, 131-5 (2001).
38. Wergin, M.C. & Kaser-Hotz, B. Plasma vascular endothelial growth factor (VEGF) measured in seventy dogs with spontaneously occurring tumours. *In Vivo* **18**, 15-9 (2004).
39. St Croix, B. et al. Genes expressed in human tumor endothelium. *Science* **289**, 1197-202 (2000).
40. Verheul, H.M. et al. Platelet: transporter of vascular endothelial growth factor. *Clin Cancer Res* **3**, 2187-90 (1997).
41. Banks, R.E. et al. Release of the angiogenic cytokine vascular endothelial growth factor (VEGF) from platelets: significance for VEGF measurements and cancer biology. *Br J Cancer* **77**, 956-64 (1998).
42. Webb, N.J., Bottomley, M.J., Watson, C.J. & Brenchley, P.E. Vascular endothelial growth factor (VEGF) is released from platelets during blood clotting: implications for measurement of circulating VEGF levels in clinical disease. *Clin Sci (Lond)* **94**, 395-404 (1998).

CHAPTER 4

DISCCUSION, CONCLUSIONS, FUTURE DIRECTIONS

General Discussion

Most commonly, the literature refers to the development of a tumor-associated blood supply as tumor angiogenesis. Angiogenesis, however, is just one means by which this blood supply develops and, as previously discussed, refers specifically to the sprouting of new capillaries from a pre-existing vascular network. Vasculogenesis, is an additional mechanism by which the tumor associated blood supply develops and involves the recruitment of bone-marrow derived endothelial precursor cells. More recently, the concept of vascular mimicry, which reflects the actual involvement of the neoplastic cells themselves as part of the tumor vasculature, has been introduced and the incorporation of leukocytes into the tumor vasculature has now been recognized as well. While research in the field of "tumor angiogenesis" has catapulted, it appears the appropriate terminology to reflect these respective discoveries and mechanisms of tumor-associated blood vessel production has somewhat fallen behind.

It is for these reasons I have entitled this dissertation and, where appropriate, utilized "tumor-associated neovascularization" as this terminology reflects the development of a new tumor-associated blood supply however is not specific for the exact method by which it is occurring. When a specific mechanism is identified or focused upon within a specific project, it then seems appropriate to utilize the respective terminology as has been applied within the contents of this dissertation.

As indicated in the introduction, it should be reiterated that "vasculature" is not only limited to the blood supply but also encompasses lymphatic vessels. Thus, perhaps over time when investigations into the area of tumor-associated lymphatics captures the interest and passion of the scientific community to the degree in which tumor-associated blood vessels have, even more appropriate terminology may evolve to include tumor-associated hematologic or lymphatic neovascularization. While none of the work within has addressed the lymphatic vasculature, it is recognized that lymphatic vessels are lined by endothelial cells which are also regulated by VEGF. More specifically, the lymphatic endothelial cells express VEGFR-3 which is specific for VEGF C and D (discussed in the introduction). Similar to the blood-associated vasculature, the lymphatic vasculature can and does provide a route for tumor metastasis. Currently, it seems this is an under-explored area in cancer research however this is likely to change in the coming years and decades.

While the work within focuses on neovascularization as it relates to tumor progression, the benefits of understanding neovascularization are not just limited to

cancer. Multiple diseases exist, progress, or are exacerbated by an increased vasculature (i.e. cancer, rheumatoid arthritis, vascular retinopathies inducing blindness, psoriasis, etc.) however, conversely, multiple diseases exist, progress, or are exacerbated by a decreased vasculature (i.e. heart disease, stroke, ulcers, infertility, etc.). Thus, uncovering, discovering, and elucidating the mechanisms which regulate blood vessel formation in general provides a foundation, not only for the treatment and prevention of cancer, but for potential therapeutic and preventative applications for a number of diseases which could carry tremendous impact.

Conclusions and Future Directions

As has been stated multiple times throughout the presented work, tumor growth and metastasis are dependent on the development of a tumor-associated blood supply. The identification of this critical vascular component and its paramount role for the existence of cancer (clinical disease) created a new perspective in the method by which cancer therapy could be approached. In contrast to conventional / traditional cancer therapy which has predominantly focused on direct attack of the tumor cells themselves, research has significantly shifted to exploit the tumor-associated blood supply in attempts to indirectly affect the neoplastic cells by interrupting, inhibiting, or abolishing this vascular network. In order to devise therapies to intervene with and prevent the process of tumor-associated neovascularization, the pathogeneses of such processes must first be identified. Over the last decade, investigations into both these areas have occurred simultaneously with remarkable progress and discovery. In

2004, the first anti-angiogenic drug bevacizumab (Avastin) a synthetic anti-VEGF monoclonal antibody was approved by the FDA for use in patients with colon cancer. The development of this drug was dependent on the recognition and discovery of VEGF's role in tumor associated neovascularization and subsequently in investigating the effects of VEGF inhibition.

Studies presented here focused on both therapeutic strategies and further elucidation of mechanisms involved in tumor-associated vessel development in the normal physiological tumor-bearing state. The primary aim presented in chapter one was to assess the antitumor and antiangiogenic effects of intravenous gene therapy in dogs with spontaneous soft tissue sarcomas utilizing plasmid DNA encoded for the antiangiogenic protein endostatin. Antitumor and antiagniogenic effects were observed but, ultimately, it was not in response to the endostatin but rather to the vehicle by which the plasmid DNA was administered. Results from these studies demonstrated that CLDCs (cationic liposome DNA complexes) themselves, result in antitumor and antiangiogenic activity regardless of what the associated DNA is encoded for. The subsequent question which then developed was by what mechanism? As CLDCs induce a potent innate immune response and as cytokines associated with innate immunity (i.e. type I and II interferons) have recognized anti-angiogenic and anti-proliferative properties it was suggested that CLDCs induce these antitumor and antiangiogenic effects, in part, through the induction of innate immunity. Controlled studies which could be pursued to further address or support this theory could evaluate tumor associated CLDC effects in the face of a suppressed

innate immune response (i.e. depletion of key leukocytes which produce the cytokine of interest, treatment with antibodies against the cytokine of interest, or utilization of knock out tumors which lack functional receptors to the cytokines of interest). The project within did demonstrate direct endothelial cell cytotoxicity by CLDCs in vitro thus this is also suggested to be a mechanism by which antitumor and antiangiogenic effects were observed in vivo.

As has been indicated in the above study and as has been reported in the literature, activation of an innate immune response can result in antitumor activity. It is also known that in the normal host, numerous physiological mechanisms for tumor surveillance, thus preventing tumor development, exist. These may include regulation by p53, a well recognized tumor suppressor gene, which can induce cell cycle arrest and allow for gene repair, or if unrepairable can induce apoptosis. Other mechanisms involve host immunity where the expression of tumor antigens, if recognized as foreign, may induce a host immune response or if tumor cells lack MHCI expression they may be susceptible to attack by NK cells. Many natural mechanisms against tumor development exist but, for purposes presented here, we were interested in exploring natural mechanism associated with tumor neovascularization and innate immunity. Controlled studies carried out in IFNR-/- mice allowed us to evaluate the effects of endogenous type I and II interferons under physiological tumor-bearing conditions. Results from these studies supported a role for endogenous interferons in natural tumor surveillance. More specifically, the antitumor effects appeared to be, at least in part, due to interferon-associated

inhibition of the tumor vasculature and additionally, results suggested the method by which this occurs may be due in part to inhibition of DC and CEP recruitment to the tumor microenvironment.

To validate these findings additional studies are necessary which, at minimum, should employ a greater number of mice per treatment group to strengthen results identified in future *in vivo* studies. In the current study, IFNR-/- mice demonstrated decreasing numbers of CEPs over the course of tumor development in the face of an increased MVD. Thus it is presumed that CEPs decreased due to recruitment from circulation to the tumor microenvironment and incorporation into the vasculature (vasculogenesis). While this study demonstrated the former (identified CEPs via immunostaining and FACS analysis), the latter (demonstration of these cells in the tumor microenvironment) is presumed and was not confirmed. FACS analysis of tumor digest for endothelial cells via immunostaining with various EC markers was carried out however, upon recruitment and incorporation of endothelial progenitor cells (EPCs) phenotype changes rapidly. Thus, a more appropriate means by which to demonstrate recruitment and incorporation of CEPs to the tumor vasculature may be to employ methods by which cells can be fluorescently labeled in the bone marrow to allow for tracking to the final destination. These labels would need to target cell surface markers constitutively expressed during both immature and mature stages (i.e. CD34 and or CD144) or be independent of surface markers targeting another cellular component which would be maintained throughout recruitment and maturation (i.e. genetically tagged cells with GFP). Similar techniques could also be employed to

further evaluate the involvement of leukocytes similar to experiments performed by Coukos et al. at the Abramson Family Cancer Research Institute at the University of Pennsylvania (*The role of dendritic cell precursors in tumor vasculogenesis*. Brit J. of Cancer 2005, 92:1182-87). While many studies exploring the recruitment of progenitor cells to the tumor vasculature exist in the literature, the potential role(s) endogenous interferons may play in this arena is not reported.

The final study (chapter 3) constituting this body of work, explored the antiangiogenic and antitumor effects of xenogeneic vaccination with recombinant human VEGF (rhVEGF) in dogs with spontaneously occurring soft tissue sarcoma. While immunotherapy targeting either VEGF or its respective receptor VEGFR-2 has been explored, this study was novel for two reasons. One, this study was performed in a large animal spontaneous tumor model and two, this study employed the use of xenogeneic VEGF protein for vaccination. One other known immunotherapy study targeting VEGF utilized plasmid DNA encoding xenogeneic VEGF but there are no known reports exploring immunotherapy with xenogeneic VEGF protein (Wei, YQ., Zhang, W., et al., *Immunogene therapy of tumors with vaccine based on Xenopus homologous vascular endothelial growth factor as a model antigen.* Proc.Natl.Acad.Sci. USA 98:11545-550). The goal of immunotherapy is to induce an immune response against the target of interest and in this case, and many cases, requires breaking or overcoming immune tolerance.

Results from this study showed that vaccination with rhVEGF in dogs can induce a humoral response and the antibodies produced are specific for and react with both human and canine VEGF. Identification of antibody production however is not sufficient to determine effective immunotherapy. Effective immunotherapy demonstrates the presence of neutralizing antibodies and an associated desired clinical effect. Antitumor and antiangiogenic effects were identified in some patients in this study and additionally, decreasing levels of circulating VEGF were identified in the face of increasing anti-VEGF antibody levels. These findings are supportive for and suggestive of neutralizing antibody effects however we were unable to demonstrate antibody neutralization in vitro. Endothelial cell proliferation assays utilizing plasma from treated dogs were pursued however plasma contains numerous growth factors in addition to VEGF. Thus, the absence of growth inhibition does not indicate lack of neutralization as these finding may be due to the presence of other mitogenic factors. An alternative and perhaps more reliable method to demonstrate antibody neutralization in this study or in future studies would be to perform cell proliferation assays following IgG purification from serum. These samples could then be applied to the proliferation assays of cultured canine endothelial cells in the face of varying concentrations of recombinant canine VEGF (rcVEGF).

While the primary focus of this study was to determine if vaccination in dogs with xenogeneic VEGF could elicit a humoral response ultimately resulting in antitumor and/or antiangiogenic effects, further evaluation to determine the induction of a potential cellular response could also be considered. If present, these findings would

strengthen the argument for implementing such therapies in dogs and would suggest similar responses with xenogeneic protein vaccination are possible. Evaluation of a cellular response could be pursued by employing lymphoproliferative assays utilizing patient PBMCs and rcVEGF. Lastly, in a bigger picture, this study could basically be transferred to mice in its entirety utilizing wild type mice with syngeneic wild type tumor cells for tumor induction. Vaccination with rhVEGF could be maintained as there is marked VEGF homology across all species. Transferring this experiment to mice would allow for much more controlled parameters and, in addition to IgG purification studies for in vitro neutralization, adoptive transfer studies could also be performed. Upon completion of that work, further studies to address different question could utilize various knock out stains of mice or various types of tumors. The possibilities are endless.

Ultimately, continued discovery in the field of tumor associated neovascularization holds great promise in the future of diagnosing, treating, and potentially preventing cancer in both human and veterinary patients. It should be recognized however that due to the complexities of the pathogenesis of neovascularization and the normal physiological compensatory processes (i.e. suppression of one factor may result in compensatory promotion / increased production of another factor) anti-angiogenic / anti-vasculogenic therapy as a single modality is likely unreasonable. Instead, optimal therapies, while including consideration of the patient (age, gender, reproductive status, concurrent disease) and tumor type, will likely involve multiple therapeutic modalities including those of conventional based cancer therapy in

conjunction with one or more anti-vascular therapy. Additionally, as has been discussed in the introduction but should not go without mention in these final comments, is the awareness and concern of potential deleterious or adverse effects in the face of angiogenic therapies predominantly as they relate to undesirable effects on the normal vasculature. Greatest concerns exist with regard to wound healing and the reproductive system where active, new vessel formation is necessary. Otherwise, in general, the vasculature is present in a quiescent state and less susceptible to the effects of such therapies. Again, consideration of the patient, concurrent diseases and tumor type prior to initiating therapy will assist in addressing these concerns.

With the continued quest for research in this field, the concepts of 1) cancer without disease and 2) cancer as a chronic, manageable disease are more and more likely to become reality.