DISSERTATION

THE ROLE OF INNATE IMMUNITY AND ANGIOGENESIS IN OSTEOSARCOMA GROWTH AND METASTASIS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY JOSEPH L. SOTTNIK ENTITLED THE ROLE OF INNATE IMMUNITY AND ANGIOGENESIS IN OSTEOSARCOMA GROWTH AND METASTASIS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

THE ROLE OF INNATE IMMUNITY AND ANGIOGENESIS IN OSTEOSARCOMA GROWTH AND METASTASIS

Osteosarcoma is the most common primary bone tumor in dogs and humans. Novel therapeutics are required since a number of patients will succumb to metastatic disease. Currently, it is known that a neoplastic mass contains more than transformed cells, but requires the presence of immune cells to create a supportive environment, and endothelial cells to form blood vessels to deliver oxygen and nutrients. Not only do these cells play a role in primary tumor growth, but they create an atmosphere conducive to invasion and metastasis, the primary cause of death for osteosarcoma patients. Therefore, better understanding of how immune and endothelial cells support metastatic growth is crucial for better understanding osteosarcoama.

Utilizing murine tumor models, we have been able to explore the observation that patients with post-surgical infections have increased time to metastasis and increased survival. We have determined that this effect is mediated by NK cells and monocytes, which inhibit tumor growth through restriction of angiogenesis. We have also investigated the role of an anti-inflammatory therapeutic, tepoxalin, which leads to tumor growth inhibition. These observations explain the paradox of inflammation, where the type and timing of inflammation may inhibit or promote an anti-tumor effect.

Due to the importance of angiogenesis and metastasis in osteosarcoma, we sought to develop new models and techniques to assess these processes. Fine needle aspiration coupled with flow cytometry accurately measures angiogenesis in cutaneous tumors, thereby allowing for repeated assessment of angiogenesis in a minimally invasive manner. We have also developed a novel post-surgical model of luciferase transfected murine osteosarcoma that grows orthotopically and spontaneously metastasizes, allowing us to non-invasively investigate the development of metastases. These tools will allow for investigation into novel anti-angiogenic and anti-metastatic compounds.

Novel prognostic markers are required to better determine the outcome of cancer patients. We have determined that monocyte and lymphocyte counts from a pre-treatment complete blood count are prognostic for disease free interval in dogs with osteosarcoma. These data describe the interactions between immune infiltrate and metastasis. The combination of the studies presented herein provides evidence for the interactions between the immune system and angiogenesis in the process of metastasis.

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Dedication

This dissertation is dedicated to the campers and counselors of Sky High Hope Camp, for they foster the inspiration and determination to find a cure for cancer.

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Literature Review and Project Rationale

Osteosarcoma

Overview

Osteosarcoma (OSA) is the most common primary bone tumor in humans and dogs [1, 2]. OSA affects approximately 1,000 human patients and approximately 8,000 canine patients every year [2-4]. Tumor metastasis is the most common cause of death from OSA [5-8]. OSA typically metastasizes to the lung, brain, and other bone sites [2, 5-8]. There is no specific etiology of disease, although theories range from ionizing radiation to mutations in the p53 and Rb genes [9-12]. There are numerous subtypes of OSA, such as osteoblastic, chondroblastic, and fibroblastic, which characterize the predominant matrix and cells present within the tumor [13]. The 5-year survival in

humans is approximately 60% with surgery and adjuvant chemotherapy [14]. However, in the dog, long term survival is only 20% with comparable treatment [5].

The primary modalities of treatment for OSA are surgery and chemotherapy. Radiation therapy, by external beam radiation, is sometimes used for the treatment of OSA in conjunction with surgery; however, the efficacy of this combination is still controversial across species [15-19]. Surgery is required to remove the primary tumor and to attempt to inhibit dissemination of disease and decrease pain.

The first method developed for treatment of appendicular disease was amputation. It was later described that a limb-sparing surgery, which involves removal of the tumor-bearing bone and replacement using a cortical allograft, could be used to successfully remove the primary tumor and allow the patient to retain function of the limb [20]. Techniques such as rotationplasty (eg. Van Nes Rotation) have also been developed to allow patients to retain limb function and remove the primary tumor [21]. However, 10-20% of patients presenting with OSA have clinical metastases at diagnosis, with a much greater number of animals having occult micro-metastatic disease [7, 8]. Therefore, systemic chemotherapy is required to treat this disease. The standard of care for OSA is currently amputation of the tumor bearing limb and adjuvant chemotherapy, typically consisting of doxorubicin and carboplatin in dogs. In humans, the standard of care is typically removal of the primary tumor and chemotherapy with cisplatin, methotrexate, and doxorubicin [22]. The introduction of liposomal muramyl tripeptide phosphatidyl ethanolamine (L-MTP-PE; discussed below) as an immunotherapeutic has been a promising new approach in human and canine OSA [2, 8, 22-30].

Canine OSA as a Translational Model of Human OSA

The dog is a superlative model for human OSA due to numerous similarities in the diseases [2]. The recent elucidation of the canine genome and the subsequent realization of the greater degree of homology to that of the human genome than that of the mouse has also fostered interest in the use of the canine model [2, 31-33]. Indeed, dogs share a common environment with humans and have a relatively long period of disease progression when compared to rodent models [2, 13]. Furthermore, the biology of canine OSA is similar to that of humans in that it metastasizes to similar organs, spontaneously occurs, and has similar pathology to the human condition [2, 4, 5, 34]. The large number of canine patients that develop spontaneous disease is also beneficial because it allows for large numbers of animals to be studied in a relatively short period of time. Due to these factors, a number of individuals and consortia, specifically the Comparative Oncology Trials Consortium (COTC) have pooled their resources to determine methods to better integrate canine cancer patients into human clinical trials and drug development [2].

It has been suggested that canine cancer patients can be used to help translate pre-clinical research. Normal beagle dog studies are already a staple of the drug development process, but further integration of pet dogs with spontaneous disease into the drug development pipeline could help bolster these studies [2]. This work has been exemplified in the process of moving L-MTP-PE from dogs into humans, and shows the interplay of how the dog may be integrated into human clinical trials [23-30, 35]. Therefore, it is plausible that dogs with spontaneous disease may be integrated to better define pharmacokinetic and pharmacodynamic endpoints before movement of novel

therapeutics into the human setting. It is also believed that modulation of shared cancer biomarkers between species may be used to better understand the mechanisms of disease and efficacy of these novel compounds [2, 5]. Lastly, the ability to study the efficacy of novel compounds in a condensed time frame allows for decreased screening time, and thus less time to approve compounds for human use as long as due diligence is performed in human testing. Therefore, integration of the dog model is a welcome and efficacious step juxtaposing rodent/laboratory pre-clinical models and human clinical trials.

Orthotopic Cancer Models in Mice

There have been numerous murine tumor models developed for the study of OSA [36-47]. The use of syngeneic and immunocompetent mouse tumor models bridges numerous mouse strains, thereby yielding flexibility to investigators interested in testing hypotheses involving genetically modified mice. For example, the DLM8 (C3H) and K7M2 (BALB/c) models are the most widely used [43, 48, 49]. Interestingly, there does not appear to be a model of murine OSA that grows in C57BL/6 mice, although descriptions of spontaneous tumors in this strain exist [50, 51]. Furthermore, there have been a number of human and canine xenograft models that have been created to allow for the study of OSA as it pertains directly to these species, allowing for the investigation of novel therapeutics when the endpoints are specific to the xenogeneic tumor cells [36, 38, 40, 41, 45, 47]. Human examples include the SAOS-2 and KRIB cell lines, whereas Abrams is a canine OSA cell line often used.

Murine OSA models can further be described by those that are implanted orthotopically, in the bone, the tissue of origin [36, 38, 40, 42, 43, 45-47]. Orthotopic

tumors are superior to tumor models grown in subcutaneous tissues because they preserve the natural environment in which tumors arise, and better recapitulate the progression of disease [52-54]. For example, it has previously been described that tumors implanted orthotopically and heterotopically grow and metastasize differently, and it is plausible that changes in the tissue micro-environment account for this difference [42, 44, 55-57]. Furthermore, syngeneic tumor models are superior to models utilizing nude mice in that they retain a more natural interaction between the tumor cells and host [49, 58]. Recently, a transgenic model of spontaneous murine OSA has been created through engineered mutations of the p53 and Rb genes in a conditional knock-out [59, 60]. Syngeneic tumor models allow for natural interactions in cytokines released from the tumor and host animal to interact. However, the interaction of human tumor cells and murine stromal cells not interact properly unless they are highly conserved, thereby altering tumor growth. Furthermore, these data would suggest that spontaneous tumors developing in dogs, and syngenic tumor models in mice, are the most valuable models [1, 49, 56, 58]. Therefore, novel mouse models that are being defined, which better mimic the clinical subtext of cancer patients, are important in helping to understand the basis of metastasis biology and testing of novel therapeutics.

Metastasis

Overview

The term metastasis is used to describe a process as much as it describes the presence of disseminated disease. Delay or prevention of metastatic disease is used as a measure of therapeutic success, and metastasis is typically the reason patients succumb to cancer as a disease. The need to better understand the metastatic cascade and the inhibition of this process is thought to be one of the requirements in cancer therapy. This is of considerable importance in OSA because it is believed that while only 10-20% of patients have clinically detectable metastatic disease at the time of diagnosis, the number of patients with occult micrometastatic disease that is not evident at the time of diagnosis is considerably higher [7, 8]. Strikingly, 30-40% of human patients with OSA will die from these metastases despite best available care [6-8]. Therefore, it is of great importance to understand the process of metastasis in order to more effectively develop interventions to inhibit metastatic disease and increase survival for patients with OSA and other types of cancer.

Metastatic Initiation

Tumor cells metastasize as they grow and invade, and acquire mechanisms by which to invade surrounding tissues. This process is of such importance that it is considered a 'hallmark of cancer' [61]. Tumor cells and their supportive stromal components release a number of matrix metalloproteinases (MMP) in order to break down the extracellular matrix and allow tumors to invade into this environment [62].

This process, coupled with the increased motility of the malignant cells, provides one of the first steps in the metastatic cascade allowing tumor cells to begin to metastasize. Considerable research has been performed to help describe those tumors that are likely to metastasize, as well as the factors responsible for this phenotype. Ezrin, a cytoskeletal linker protein, is one of the primary players in OSA metastasis [42, 63, 64]. Ezrin is involved in the reorganization of the cytoskeleton and cell adhesion molecules allowing cells to become more invasive and potentiating metastasis [63, 65]. Exacerbated tumor growth and metastasis require angiogenesis, blood vessel growth. The presence of blood vessels and lymphatics in a tumor provides a mechanism to deliver oxygen and nutrients and transport waste away from a tumor, but they also allow for tumor cells to move into these vessels and disseminate systemically [57, 66]. Tumor invasion and migration into vasculature are required for the dissemination of malignant tumors, and are perceived as the early steps leading to systemic metastases.

Routes of Metastasis

The two primary routes of metastasis are lymphatic and hematogenous. Lymphatic metastasis occurs when tumor cells extravasate from the tumor environment, enter lymphatic vessels, and migrate to a draining lymph node [67, 68]. Lymphatic vessels are an important part of tumor growth as they are a constituent of normal tissues, thereby allowing some tumors to colonize draining lymph nodes during the metastatic process. A primary example of this process is breast cancer, in which a finding of tumor cells in draining lymph nodes is a known negative prognostic factor [68-70]. The metastasis to lymph nodes is of such importance in breast cancer that a number of these

nodes are removed as a precautionary measure when a mastectomy is performed to remove possible micrometastatic disease which may be present in these tissues. Lymph node metastasis also opens the possibility of systemic dissemination of disease due to drainage of lymphatics into venous circulation allowing for systemic circulation of tumor cells in the blood. However, the route of metastasis from a given tumor is described by the method the tumors cells exit from the primary tumor, and not the route in which they enter distant tissues [71].

The other primary route of metastasis is hematogenous metastasis since tumor cells are able to directly enter the systemic circulation of blood vessels. One of the primary examples of hematogenous metastasis has been well described by *in vivo* mouse models of pancreatic cancer. Dr. Isiah Fidler has described this process through the implantation of orthotopic models in which tumor cells are injected into the pancreas or colon and subsequently, and spontaneously, metastasize to the liver [72-75]. The tumor cells are established as a primary tumor in the pancreas before undergoing the metastatic process and reaching the liver, where they form new metastatic nodules.

The hematogenous route of metastasis can also be studied by challenging mice with tumor cells by intravenous injection, leading to seeding of cells in the lung and disease formation [76]. However, these models do not replicate the entire metastatic process as previously described, for they do not account for intravasation to the vasculature and escape from the primary tumor environment. These models typically use a large number of tumor cells, and do not form metastases by extravasation from the vasculature, but may create focal disease by embolizing in the vessel, and growing,

before invading into the lung parenchyma. However, these models are useful in that they allow for the study of tumor formation in a site of typical of metastasis, such as the lung.

Metastatic Clonality

There have been a number of questions concerning the clonality of individual metastases and their relationship to the primary tumor. Since changes in the tumor microenvironment can greatly alter the expression of genes within tumor and stromal cells, these questions have increased in complexity [42, 52-54]. It was previously believed that a single cell could circulate systemically before coming to rest in a distant tissue, and then create metastatic foci [77]. However, the primary tumor itself is composed of a mosaic of tumor cells, with each of these subpopulations having a varied gene expression all its own [78]. This may explain in part some of the variability in the genetic makeup of metastases [78-80]. Furthermore, this process is likely beneficial to the tumor, in that it provides numerous mechanisms by which to disseminate and sustain growth of positively selected cells [78, 80]. This evolutionary selection process for clones which are able to intravasate into the circulation, extravasate, and survive in a distant tissue, may be one of the reasons metastases are more aggressive than their primary tumor counterparts. Indeed, it is likely that selection of those cells that can survive and grow in hostile foreign environments propagates the aggressiveness of tumors. Metastatic cells have already been selected for to evade immune recognition and organize a variety of cellular players to promote their own survival [78, 81]. However, it has been argued that metastatic clones are able to adapt to their environment [80]. Thus, it is thought that a clonal population of cells is released from the primary tumor, and

those that can adapt to their new environments develop into metastases while those that cannot are selected against. The underlying argument is that the cells best able to adapt to their environment may have been selected for by the process of metastasis [82]. Therefore, it is a difficult question to discern the establishment and mechanisms associated with the clonality of metastasis, even though it is an integral question to metastasis biology.

Pre-Metastatic Niche and Metastatic Development

The pre-metastatic niche is a term developed to describe a location in a distant tissue that is susceptible to metastatic development, and may be required to precede the seeding of tumor cells in this environment. The seed-and-soil hypothesis was first postulated by Paget in 1889 to describe the site (the soil) for proper growth of the tumor cells (the seed) [83]. There are numerous explanations and arguments for and against this hypothesis, and this subject is still extensively argued [57, 78, 84-86]. However, the cells that make-up this pre-metastatic niche are thought to derive from the bone marrow, and act in locations of previous damage that tumor cells may exploit in order to grow in distant tissues [57, 84-86]. For example, Harold Dvorak has previously described tumors as “wounds that will not heal” [87]. The location of a microscopic injury that is being repaired by leukocytes may create an immunosuppressed and pro-angiogenic environment which may benefit the implantation and early growth of metastatic clones. The presence of inflammatory cells in the pre-metastatic niche assists with the aggregation of tumor cells into a large and developed primary tumor, thus enabling promotion of tumor seeding and growth [88].

It is believed that a subset of hematopoietic stem cells (HSC) that are bone marrow derived play a role in this process by homing to areas of repair and regeneration to create a supportive environment for tissue repair [84]. Tumor cells exploit these niches, which provide protection from the foreign tissue environment [84]. It will be discussed later how leukocytes play a large part in tumor formation and growth, and it is plausible that HSC play a similar role in preparing an environment for tumor generation in distant tissues. The expression of cytokines such as VEGF, bFGF, and Angiopoietin-1 play a role in aiding tumor growth, and thus likely play a role in enabling metastasis [57]. The cytokines expressed in the pre-metastatic niche may even allow for homing or direction of tumor cells to these sites as cells in these locations express these cytokines to elicit their repair/regenerative functions. Once in these locations, tumor cells are nurtured by these cells, and allowed to grow in a less hostile environment than the rest of the tissue. Since tumor cells may have been selected in the primary tissue, this step is important because it provides a barrier against more hostile surrounding environments, and allows the primordial metastasis to develop, until it is of reasonable size to promote its own growth and conduct changes in the tumor microenvironment [78, 80]. Once these changes occur, a metastasis develops, and this new tumor can plausibly spur its own metastases. Furthermore, the selection and adaptation to a new environment may make for more aggressive progeny since numerous anti-tumor obstacles were overcome in the formation of the original metastases.

Hypoxia

HIF-1α

Hypoxia inducible factor-1α (HIF-1α) is a transcription factor and is one of the primary means by which cells respond to hypoxia, or a decrease in tissue pO_2. HIF-1α is continually expressed and rapidly turned over in cells, making it a prime regulator of rapidly changing oxygen conditions. In the presence of oxygen, HIF-1α is ubiqutinated by the Von-Hippel Lindau (VHL) tumor suppressor protein, which leads to the degradation of HIF-1α and its inability to act as a transcription factor [89]. In the presence of oxygen, VHL is hydroxylated leading to formation of the active binding site, thereby allowing for ubiquitination of HIF-1α [90]. Under hypoxic conditions, this hydroxylation does not occur, and thus the ubiquitin ligase function of VHL is not present [89, 90]. Stabilization of HIF-1α allows it to translocate to the nucleus and bind to hypoxia response elements (HRE) leading to the activation and production of numerous hypoxia-related genes [91-99]. It has been shown that HIF-1α is at least partially responsible for activating genes such as mTOR, VEGF, c-myc, and p53 which lead to a phenotype conducive to living in the hypoxic environment [96-99]. Therefore, HIF-1α is an important mediator in the regulation of cellular metabolism and signaling, and plays a crucial role in how cells interact with their environment.

Hypoxic Adaptation of Tumor Cells

Hypoxia is an important process in tumor growth because it acts to alter the cellular metabolism of tumor and stromal cells [100]. Tumor cells require nutrients and

oxygen to survive like any other cell in the body. In fact, tumors are not able to grow more than a few millimeters in size without a sufficient blood supply [101, 102]. Without the delivery of oxygen to these cells, they institute a number of molecular and cellular changes in order to survive. In the presence of hypoxia, a metabolic shift occurs away from oxygen dependent processes, such as oxidative phosphorylation, and toward processes that do not require free oxygen, such as glycolysis [103]. It is believed that hypoxia plays a role in helping to maintain the Warburg effect, which was first described by Dr. Otto Warburg in 1956 to describe the metabolic changes that tumor cells must undergo to survive in hypoxic and stressful environments [104]. Hypoxia, like metastasis, is believed to be of such importance that it was also denoted as one of the "hallmarks of cancer" [61].

Hypoxia leads to a number of cellular changes that can induce more aggressive changes in tumor cells. For example, it has been shown that hypoxia can not only alter angiogenesis but alter the translation of specific proteins dependent upon the presence of 5' mRNA cap [105]. This observation suggests that a switch occurs in tumor cells promoting a mechanism that allows for the synthesis of a greater number of mRNA's and removes a key regulatory mediator in translation. Another group has recently shown that VHL plays a much larger role than simply regulating HIF-1α concentrations by also modulating microtubule formation [106]. Recently, it has also been shown that hypoxia may be a contributing factor to the cancer stem cell phenotype, a population of cells believed to be involved in chemotherapy resistance and metastasis [107, 108]. Therefore, the effects of hypoxia go much further than simple oxygen deprivation of cells.

Modulation of the Microenvironment

Tumors are composed of a variety of stromal components, including immune cells, endothelial cells, fibroblasts, and tumor cells, all of which may be impacted by the hypoxic environment present in tumors. For example, it has been suggested that an oxygen gradient may be a chemoattractant leading to enhanced monocyte migration [109]. It has been shown that tumor associated macrophages (TAM) preferentially accumulate in hypoxic regions of tumors, likely due to a phenotype which promotes repair of hypoxic wounds [110, 111]. Once in this environment, monocytes/TAM alter their gene expression in response to the hypoxic environment, making them more suited to survive [111, 112]. These changes are partially responsible for the promotion of pro-angiogenic factors released from TAM [113, 114]. In this environment, TAM also release a number of anti-inflammatory molecules, such as TGF-β and IL-10 [111, 115]. However, new work suggests that hypoxia can also lead to increased IFN-γ production, which may promote an anti-tumor phenotype, suggesting that there are a number of factors impacting TAM function in the hypoxic environment [116]. The presence of the hypoxic environment also leads to an upregulation of NF-κB, leading to increased survival of TAM [111, 117].

These changes manifest themselves at the cellular level, as they promote tumor angiogenesis and invasion into surrounding tissues, thereby increasing tumor aggressiveness [118, 119]. It is not only TAM that alter their phenotype in response to hypoxia; these changes also occur in neutrophils [120] and T cells [121, 122]. Thus, it is very apparent that the presence of hypoxia modulates the tumor microenvironment in

many ways, and can significantly alter the way in which cells respond to their surroundings.

Angiogenesis

A Brief History and Overview

Angiogenesis is the process of blood vessel growth, and is of such importance to tumors that it has been described as a "hallmark of cancer" [61]. The process of angiogenesis as it pertains to tumors was first described by Dr. Rudolf Virchow in the late 1800's when he observed that tumors were highly vascularized [123]. Dr. Judah Folkman later elaborated upon these observations to describe the presence of pro-angiogenic factors [101, 102]. Folkman described how angiogenesis was required for tumor growth and that tumors could not grow larger than two millimeters without recruiting blood vessels. Angiogenesis is required in that it allows for the supply of nutrients and oxygen to tumors and removes waste and metabolic by-products from cells. Angiogenesis is a global term that encompasses a number of processes that are involved in blood vessel growth. For example, the term angiogenesis specifically applies to the branching of new blood vessels from a common progenitor which then invade and supply blood to a specific tissue [61]. Conversely, the process of vasculogenesis requires precursors, typically circulating in the blood, to move into tissues and create new vessels [124, 125]. Further divisions of the types of angiogenesis have also been termed to

explain specific phenotype that correspond to blood vessel growth, such as sprouting angiogenesis and intussusceptive angiogenesis [126].

Tumors themselves have also evolved new mechanisms to create blood supplies in order to increase oxygenation of tissues, such as vascular mimicry in which tumor cells create patent openings that lead to established conduits without endothelial cells, allowing blood to pass through as if they were actual vessels [127, 128]. The process of vessel co-option occurs when tumor cells grow along established vessels in an attempt to disseminate [126]. Tumors themselves have developed a number of mechanisms to 'flip the angiogenic switch,' and thus it is easy to discern the importance of angiogenesis in tumor growth [129-133]. Nevertheless, tumor endothelial cells may be more nefarious than simply supplying the tumor with oxygen and nutrients, and may also play a role in immune suppression [134].

Angiogenic Cytokines

One of the primary molecules mediating angiogenesis is vascular endothelial growth factor (VEGF). VEGF was first described in 1983 by Senger and Dvorak who named it vascular permeability factor (VPF) [135]. VPF was implicated in making vasculature leaky to promote increased movement of leukocytes from the blood into peripheral tissues. It was not until later that VPF was also implicated as a growth factor for endothelial cells, and was renamed VEGF once it was determined that the two molecules were the same [136].

VEGF-A was the first isoform of VEGF described, and subsequent isoforms were consecutively lettered to differentiate them (e.g. VEGF-A, VEGF-B, VEGF-C) [137].

VEGF signals through three VEGF receptors (VEGFR1, VEGFR2, and VEGFR3), each of which has a specific tissue distribution and function [137-140]. For example, VEGFR1 is expressed on endothelial cells and monocytes/macrophages and is responsible for cellular motility and extravasation [139], whereas VEGFR2 is expressed on the same cells and induces mitotic changes and vessel permeability [138], while VEGFR3 is primarily involved in lymphoangiogenesis [140]. Lymphoangiogenesis is the process of new lymphatic vessel development from pre-existing lymph vessels in a similar matter to that of blood vessel growth [141]. Each of these receptors is a receptor tyrosine kinase that acts through various pathways to elicit the above noted actions [142]. Activation of VEGFR is important in maintaining the vascular endothelium and promoting vessel invasion. Tumors and stromal cells can secrete high concentrations of VEGF which may act as a chemoattractant for cells expressing VEGFR, and VEGFR2 more specifically. However, the interplay of these receptors is also important, as alternatively spliced forms of VEGFR2 may be stimulatory for vascular growth and inhibitory of lymphatic growth [143].

The signaling and effects of VEGF remain difficult to discern and at times contradictory due to the variety of roles VEGF can play given various environments, isoforms, and receptors activated. For example, VEGF has been shown to be necessary for maintenance of vasculature due to autocrine signaling [144]. However, it has recently been postulated that VEGF may also serve as an antagonist to vascular growth under certain microenvironmental pressures and its absence may actually increase tumorigenesis if removed from myeloid cells [145, 146]. Therefore, the role that VEGF plays in maintaining and initiating angiogenesis has yet to be fully defined, although its

importance in angiogenesis cannot be dismissed. Furthermore, increased circulating levels of VEGF have been found to be a negative prognostic factor in human and canine OSA [147-150].

It has also been shown that interleukin-8 (IL-8) is a powerful initiator of angiogenesis [151-153]. IL-8 signals through CXCR1/2, which also responds to various other CXC-type chemokines, and feeds into to common pathways involved with VEGFR signaling [154-156]. Furthermore, activation of these pathways, primarily PI3-K and AKT, lead to signals that also promote cell survival, proliferation, and invasion [153]. It is also believed that the induction of neutrophil and monocyte/macrophage chemotaxis leads to the promotion of a pro-angiogenic phenotype in these target cells by degrading the extracellular matrix and stimulating blood vessel invasion [153, 157]. The ability of IL-8 to promote angiogenesis, chemotherapy resistance, and tumor cell survival make it a potent modulator of tumor development and dissemination.

Circulating Endothelial Cells

Circulating endothelial cells (CEC) are a generic term used to describe any cell in circulation that may give rise to new vasculature. These cells were first described in 1997 by Asahara et al. who showed that CEC could be isolated from the blood and induced to form vascular endothelium when provided with the proper growth factors [124, 158]. Since this time, much work has been done to better characterize these cells, their function, and role in vascular growth in a number of diseases [159-167] including cancer [168-174]. CEC are currently defined as cells that shed from mature vasculature in the body and circulate before initiating branching and new vessel formation elsewhere

in the body. It is further believed that tumors themselves can induce shedding of CEC to promote vascularization in other parts of the tumor. However, it is also thought that there may be a common bone marrow progenitor cell (e.g. a circulating endothelial progenitor [CEP]), which is released from the bone marrow to circulate and initiate angiogenesis at distant sites. It is believed that CEP play a greater role in development, but it has also been suggested that tumors may elicit responses that lead to increased CEP release from the bone marrow to stimulate angiogenesis. CEP can also be elicited by vascular disrupting agents used in cancer therapy, possibly acting as a mechanism to initiate angiogenesis after ablation with these therapies [175].

Due to the division of CEC and CEP in circulation, much work has been afforded to characterizing these cells for their potential to act as a biomarker for diseases such as cancer. It has only recently been suggested that a standardized method of characterizing CEC for analysis in humans is required [176]. Mancuso et al. have suggested that cells that are $DNA^{+}CD45^{-}CD31^{+}CD146^{+}$ may be identified as CEC in humans. It is imperative that $CD45^{-}$ cells are selected since those cells that are $CD45^{+}$ may also express these markers, such as monocytes and neutrophils. Since some of these markers (e.g. CD146) are also present on platelets, a stain for DNA is required to identify CEC. Previously, a number of different markers, including CD133, CD34, CD117, and VEGFR2/Flk-1 were suggested to define CEC in humans and mice [170, 177, 178]. However, a number of these markers are general hematopoietic stem cell (HSC) markers, and may thus not truly be labeling CEC unless used in coordination endothelium specific markers such as CD31 [170]. It has been further suggested that CEC and CEP can be distinguished by their differential expression of these markers, such as the presence of

CD133 (human) or CD117 (mice) on CEP and lack of these markers on CEC [179, 180]. However, others have shown that the presence of these markers may define a subset of CEC rather than truly identifying CEP [170].

It has previously been suggested that CEC may act as a pharmacodynamic surrogate suggestive of changes in systemic angiogenesis. Indeed, it has been shown that cancer patients have increased CEC [172]. For this reason, many have been interested in tracking CEC over time to determine whether changes in CEC can be used as a marker for changes in tumor angiogenesis and a measure of therapeutic efficacy. The use of CEC as a biomarker has been best described by specific anti-angiogenic compounds such as VEGFR inhibitors and "metronomic" chemotherapy, the practice of giving small doses of cytotoxic drugs more frequently to decrease toxicity while maintaining a positive therapeutic index [173, 181-184]. The ability to monitor angiogenesis using a blood test would be valuable in the clinic and drug development sectors as it could provide insight into changes in angiogenesis and possibly tumor inhibition or progression.

Measuring Angiogenesis

There are numerous methods used to evaluate changes in angiogenesis using *in vitro* and *in vivo* based assays [185]. *In vitro* assays are typically based on using primary or immortalized endothelial cell (EC) lines that are conducive to cell culture principles and thus may not be representative of EC *in vivo*. However, HUVEC (human umbilical vein endothelial cells) are non-immortalized cells that can be used in these types of experiments and were first isolated in 1973 [186]. Assays concerning the relative growth and migratory capacity of EC lines have been in the forefront of study [185, 187].

Further studies have also been performed to recapitulate the *in vivo* scenario by allowing EC to differentiate and form tubes to better understand the mechanisms of vessel formation in an isolated system [185, 188-190]. Even though these systems are beneficial to understanding the properties of EC in defined systems, they lack the complexity of the microenvironment and growth factors which are present in animal models.

The ability to use *in vivo* models to study angiogenesis has allowed for a more relevant context into the study of angiogenesis as a biologic process. One of the simplest models is the chick chorioallantoic membrane (CAM) assay. Since the embryo is immunotolerant, tumor cells can be introduced into the system to study the invasion of EC into the tumor in a simple model system [191]. However, this is system only allows for short term analysis of angiogenesis, and thus may be more beneficial for screening anti-angiogenic compounds rather than understanding changes occurring over time [185]. Another model system involves the use of the cornea as a measurement for changes in vessel growth [192, 193]. The cornea can be damaged or tumor tissue/cells implanted into the tissue and observation of blood vessels into the area assessed [194-196]. This technique has been used to describe changes that occur in the presence of tumor cells and various cytokines and growth factors. The chamber assay is another assay in which angiogenesis can be studied by placing a skin fold from a mouse in a frame which can be illuminated and vessels observed [197]. The advantage of this model is that it can be used for longer periods of time than the methods previously described, and can be used to describe vessel formation into a region that can be easily monitored [185]. This model has the advantage that it does not initiate angiogenesis on its own, and thus changes in

angiogenesis are due to the tumor cells implanted or cytokines/growth factors placed into the frame [198]. The other advantage is that it allows for repeated analysis of a single animal over time [198]. As good as these models are they do not take into account the variable microenvironment present in tumors, and the changes that may be associated with orthotopic tumor sites.

The description of vasculature in the tumor microenvironment is an important aspect for understanding the role of angiogenesis in tumor biology. For many years, subcutaneous tumor models have been the preferred method of studying tumors in mice due to their easily accessible location; however, non-invasive techniques to study angiogenesis are typically time and cost prohibitive [199-201]. The primary problem with this system is that it does not allow for repeated measurements of angiogenesis within the tumor unless expensive methodologies such as ultrasound, CT, or angiography are utilized [185]. Furthermore, characterization of the amount of angiogenesis requires sacrifice of the animal and subsequent immunohistochemical analysis by immunostaining with CD31 and manual determination of micro-vessel density (MVD) [202, 203]. Large biopsies are required in dogs and humans with subsequent staining using CD31 or CD146, respectively [185, 204]. However, it is likely that vessels are not evenly distributed throughout a tumor, and thus a method of "hot-spot" analysis may be performed in which fields with only dense vasculature are counted in order to select for the most angiogenic regions of a tumor [202]. This method may not be representative of the overall tumor burden, and is therefore subject to bias. Lastly, the interaction between human/canine tumor xengorafts and the stromal components of the host nude mouse may alter vascular modeling and maintenance [49, 52-54, 58]. It is plausible that the

cytokines/growth factors between the species may not interact properly with their xenograft counterpart, which may lead to artifacts not present in syngeneic studies. The ability to properly characterize angiogenesis in mouse models is critical, as novel therapeutics seek to target angiogenesis as a means to inhibit tumor growth.

Anti-Angiogenic Therapeutics

The development of compounds that are approved as anti-angiogenic compounds for cancer has only occurred recently with the acceptance of Avastin® (bevacizumab). Avastin is a humanized monoclonal antibody targeted against VEGF-A. Avastin was first approved for metastatic colon and non-small cell lung cancer along with standard of care therapy in each of these diseases [205-207]. However, Avastin was recently (2008) approved for use against metastatic breast cancer even though it failed to significantly prolong survival and only moderately decreased the rate of tumor growth [208]. Additional studies are investigating the efficacy of Avastin in glioblastoma, renal cell carcinoma, and other tumors [209-213]. The ability to inhibit angiogenesis while concurrently inhibiting tumor growth with more conventional cytotoxic compounds suggests that inhibition of tumor growth by a variety of mechanisms may increase survival in patients with various tumor types.

There have been numerous ideas on the best way to inhibit angiogenesis. There has been interest in directly inhibiting the receptor for VEGF as an anti-angiogenic strategy. One of these compounds is ZD6474, which acts as a VEGFR2 and EGFR inhibitor [214-216]. It is thought that the inhibition of multiple pathways involved in angiogenesis and tumor progression may be a better approach to anti-tumor therapy than

selective inhibition of a single pathway. However, a recent opposing view is that this pan-receptor form of inhibition may lead to myelosuppression due to the indirect inhibition of development pathways, particularly c-kit and Flt-3 [217]. It has also been suggested that metronomic chemotherapy, the practice of giving small doses of cytotoxic therapy continuously, may inhibit angiogenesis by the inhibition of CEC [170, 173, 218]. The primary compound investigated in this paradigm has been cyclophosphamide, with an inhibition of CEC and decreased tumor vasculature reported [173, 219]. The promise of novel anti-angiogenic compounds suggests that angiogenesis may be a potential mechanism to inhibit tumor growth.

The first anti-angiogenic compound approved for therapy in dogs was Palladia® (toceranib). Palladia is a tyrosine kinase inhibitor (TKI) that specifically targets c-kit, but has also been found to inhibit PDGFR and VEGFR [220-223]. Palladia is currently approved as a therapeutic for mast cell tumors in dogs and is under evaluation for efficacy against other tumor types in combination with standard cytotoxic chemotherapy. A number of other TKIs have been used in canine cancer treatment, although exploitation of these compounds as a possible therapeutic has only recently begun to be explored [224]. Thus, it is easy to see how anti-angiogenic therapies may play a role in helping to control tumor growth and synergize with other therapeutics to increase efficacy and survival in cancer therapy.

Inflammation and Cancer

A Brief History and Overview

The observation that tumors contained large leukocytic infiltrates was first made by Dr. Rudolf Virchow in 1863 [225, 226]. Since then, the importance of inflammation in tumors has waxed and waned through scientific literature for much of the past century. It has now become accepted that inflammation plays an important role in cancer initiation and progression [227]. Dvorak stated that "tumors are wounds that do not heal," thus leading to a chronic inflammatory response in the presence of a tumor [87]. The presence of infectious agents, both bacterial and viral, has been implicated in creating and sustaining chronic low-level inflammation that perpetuates oncologic changes in cells; these studies will be discussed later in this chapter. Inflammation is a double-edged sword in biology because it allows the immune system to coordinate an attack against foreign pathogens, yet if not kept in check, the process of inflammation itself can cause damage to cells that cannot be repaired, thus leading to other problems. Chronic inflammatory diseases are of great concern to human health, and thus may be viewed in a much wider context than that of cancer.

Inflammatory Mediators

The mediators of inflammation are not only immune cells, but the inflammatory factors produced by normal cells in response to damage and/or foreign pathogens. Inflammatory cytokines, such as the interferons, interleukins 1, 2, 8, 12, 18, and tumor necrosis factor-α (TNF-α) may be released by resident cells in response to stresses [228-

234]. The release of these inflammatory mediators trigger a cascade of other cytokines and processes that promote the invasion of immune cells into target areas [235]. For example, IL-8 and CCL2 promote the infiltration of neutrophils and monocytes, respectively [153, 236-238]. Soluble immune mediators act as signals between and among cells to coordinate activities that remove pathogens and repair damage caused by various types of injury. The production of prostaglandins and leukotrienes coordinate the inflammatory environment, and recruit cells that remove foreign material, clean up the debris, and repair the site of damage [239]. If these systems are not inter-related and balanced, auto-immunity may develop as a consequence of the inflammatory cascade. Therefore, the importance of inflammation extends beyond that of the cancer phenotype, and persists as a foundation of human health when properly controlled and implemented.

Cycloxygenase

The cyclooxygenase (COX) family is a group of enzymes primarily responsible for the formation of a number of inflammatory mediators, primarily prostaglandins and their derivatives. The COX pathways convert arachadonic acid to prostaglandins [240]. The primary COX enzymes involved are COX-1 and COX-2 [241]. The distribution of these enzymes varies across tissues, but primarily resides in blood vessels, stomach, and kidneys. COX-1 is considered to be constitutively active in most normal tissues; whereas, COX-2 is a more inducible form of the enzyme that is expressed in areas of high inflammation by mediators such as macrophages [242]. Increased prostaglandin concentrations do more than regulate inflammation, they also lead to changes in vascular smooth muscle tension, platelet aggregation, hormone control, control cell growth, and

regulate calcium movement (an important signaling factor) [243-248]. It has also been suggested that COX-2 expression may be prognostic in dogs with OSA, and inhibition of COX-2 may lead to decreased pain in these patients [249, 250]. Indeed, it is easy to see the importance of the COX enzymes and their contribution to inflammation and its regulation.

Lipoxygenase

Other important mediators of inflammatory signals are the lipoxygenase (LOX) enzymes. These are a family of iron containing enzymes that mediate the formation of leukotrienes from arachadonic acid [239]. Leukotrienes derived their names from the large expression within from leukocytes [251]. The primary mediator of leukotriene synthesis is 5-lipoxygenase (5-LOX) [239]. Leukotrienes act as important inflammatory mediators, and have been best studied in the field of asthma [252, 253]. Leukotrienes are also important chemoattractants for neutrophils [254]. Leukotrienes have been implicated in increasing vascular permeability, and thus may play a role in increased leukocyte migration into areas of inflammation.

NSAID Inhibitors of Inflammation

Numerous compounds have been developed for the treatment and inhibition of inflammation. One of the most popular classes of these compounds is the non-steroidal anti-inflammatory drugs (NSAIDs). These drugs act primarily by inhibiting the function of the COX/LOX enzymes. Inhibition of the production of anti-inflammatory mediators has been used for the treatment of both acute and chronic inflammation. However,

NSAIDs constitute a broad class of drugs that differentially inhibits COX-1 and COX-2 [255, 256]. For example, aspirin is a salicylate that irreversibly binds to COX-1 and modulates the activity of COX-2 [257]. However, newer compounds such as rofecoxib were created to specifically inhibit COX-2, although these drugs have met with some unanticipated side-effects requiring their withdrawal from the commercial market [258]. Compounds such as indomethacin, nordihydroguaiaretic acid (NDGA), and piroxicam differentially inhibit COX-1 and COX-2, leading to a variety of effects and uses based on these differences [255, 256, 259-261].

Inhibition of LOX pathways is slightly different since leukotrienes retain their fatty-acid characteristics, and thus inhibition of leukotrienes are typically independent of COX inhibition. However, the compound tepoxalin, a dual COX-1/COX-2 inhibitor that also inhibits 5-LOX, inhibits the production of both prostaglandins and leukotrienes, providing a broader inhibition of inflammation [262]. Targeting of multiple pathways may be a better strategy in cancer therapy because it decreases the possibility that tumor cells will evade the effects of the compound. The primary toxicity associated with NSAID therapy is gastrointestinal in nature, and decreasing this toxicity is important if NSAIDs will be used for long periods of time, as is necessary for them to be most effective [263-267].

Importance of COX/LOX in Cancer

Increased expression of COX-2 has been studied in a variety of tumors, and has been found to have prognostic value in various tumor types including OSA [249, 268-271]. Therefore, a number of investigations have been performed to better classify the

expression and possible inhibition of COX-2 in cancer and its related processes. Logically, tissues normally expressing COX-2 are preferential targets for this type of treatment since tumors arising from these tissues typically over express these enzymes. It has also been shown that long-term treatment with an NSAID may decrease the risk of specific tumor types due to the anti-inflammatory properties, such as colon cancer [272]. It has recently been shown that inhibition of COX-2 may lead to inhibition of breast cancer formation in a murine model [273]. Inhibition of COX/LOX can influence various processes such as the inhibition of metastasis, reduction in bone pain and tumor growth, and inhibition of tumor formation and promotion in a colon tumor model [250, 274-276]. In veterinary medicine, piroxicam has been studied as a therapeutic for bladder cancer in dogs [277, 278]. It is easy to see the importance that inflammation plays in tumor growth and promotion, and the possibilities that are present through the modulation and of inflammatory mediators.

Infection and Cancer

A Brief History and Overview

It has long been established that inflammation is a plausible cause of cancer progression. One of the most well documented examples is that of *Helicobacter pylori*, a bacterial pathogen present in the stomach [279-282]. Chronic infection with this pathogen leads to low levels of inflammation in the stomach wall, and may subsequently give rise to gastric carcinoma and mucosa-associated lymphoid tissue lymphoma [279-

285]. It has also been shown that removal of the inflammatory stimuli can lead to tumor regression. Another example is osteomyelitis, or infection of the bone [13, 286]. Studies speculate that the chronic low level infection associated with osteomyelitis may lead to carcinogenic changes possibly leading to tumor formation. Recently, prostate cancer was implicated as having a possible infectious etiology. However, it is not just bacterial pathogens that can lead to tumor formation, for there is a wide body of literature that has shown that a number of viruses can incite pro-oncologic changes in target cells [287-290]. In this case, virally infected cells are re-programmed by the virus to survive in the presence of an immune assault, and continue to replicate. These factors lend themselves to a pro-tumor environment, in which few other changes are necessary for the development of a tumor.

The antithesis of the above information was proposed in the 1893 when Dr. William Coley observed that a head-and-neck cancer patient developed an infection that subsequently lead to tumor regression [291]. This observation, and anecdotal evidence collected over the next decade (including a passage by Paget suggesting infection may play a role in tumor regression), suggested that infection could lead to tumor growth inhibition [292, 293]. Coley went as far as to prepare a mixture of live bacteria which he injected into the tumors of patients with inoperable disease, with mixed success [294, 295]. A number of Coley's patients succumbed to the effects of acute infection; however, there are also reports of miraculous cures [292]. Interestingly, Coley's inspiration may have been derived from the death of patients with metastatic bone tumors (including OSA), and he believed that his unique form of immunotherapy may be especially potent against this type of tumor [295]. Nevertheless, Coley never published

the recipe to his so called “Coley’s toxin,” although it is believed he never stopped altering the recipe. Due to these observations and experiments, Coley is thought of as the father of immunotherapy in a time when germ theory was being postulated [296].

Modern Mechanisms of Coley’s Toxin

Exploration into the mechanisms behind Coley’s toxin rapidly fell out of favor due to the mixed results of his experiments and contradictions in experimental setup [295]. It was not until the 1990’s and discovery of toll-like receptors (TLRs) did the scientific community began to approach Coley’s work again. It was thought that targeting of TLRs could activate the immune system in a similar manner to that of the infectious agents used by Coley, but in a safer manner. Numerous individuals and companies formulated mixtures and compounds to simulate the response of Coley’s infectious agents [294]. Soluble agents of infection have also been studied, and treatment of bladder cancer with BCG has become an effective therapeutic for some patients [297-299]. Murine models investigating a number of infectious agents as anti-tumor therapeutics have also been created, although many of these are more focused on the mechanisms of growth inhibition than the use of pathogens as clinical agents [227, 300-303].

One of the greatest advancements in recapitulating Coley’s toxin while decreasing the toxicity of the compound is liposomal muramyl tripeptide (L-MTP-PE). This compound increases monocyte accumulation in the tumor as one of the mechanisms of tumor and metastasis inhibition [304]. It is thought that activation of monocytes homing to the tumor and tumor associated macrophages become activated in the presence of L-

MTP-PE thus leading to tumoricidal properties and an increased inflammatory response against the tumor [305-307]. It has also been shown that combination of L-MTP-PE with conventional chemotherapy may be an efficacious therapy for OSA [308]. Furthermore, this therapy has found a home in treating OSA, in humans and dogs [23-30, 35]. Another mechanism of exploiting the effects of Coley's toxin without treating patients with infectious pathogens utilizes CpG pathogens. CpG islands are unique to bacterial DNA, and administration of these molecules has been shown to activate the immune system non-specifically and may act as a potent adjuvant in cancer vaccines [309-311]. Clinical trials have also been performed with these molecules to some success [312-314]. Therefore, it is plausible that the type and timing of inflammation can direct tumor growth promotion or inhibition. It is interesting in that Coley first explored the use of immunotherapy in bone tumors, and this is one of the most successful venues for this type of therapy.

Pathogens as Delivery Systems in Cancer

Due to the unique ability of specific bacteria, such as *Salmonella* species, to migrate towards areas of hypoxia, it has been suggested that these pathogens act as a vehicle for anti-tumor compounds [315, 316]. Thus, individuals have engineered a variety of therapeutics into these pathogens to exploit these effects, such as IL-2, CCL21, Fas Ligand (FasL), IL-18, TRAIL, and endostatin [315-326]. However, it is likely, that initiating an immune response to bacteria present in a tumor will lead to indirect killing of tumor cells and thus decreased tumor growth. For example, *Salmonella* migration to a tumor incites an immune response against the bacteria. Subsequent infiltration of

neutrophils and monocytes into the tumor microenvironment leads to lysis of the bacteria and indirect killing of the tumor cells due to the release of cytotoxic compounds from both of these cell types. However, this role has also been attributed to some viruses, and may be a mechanism of delivering normal genes to tumor cells to correct tumor promoting mutations [327, 328]. There has been interest in mechanisms to exploit the observations originally made by Coley, and make these therapeutics more efficacious with fewer toxicities.

Innate Immunity/Myeloid Cells

Monocytes

Monocytes, or mononuclear cells, are bone marrow derived leukocytes that play a role in guarding against foreign pathogens and initiating repair mechanisms for damaged tissues. This wide variety of roles has made monocytes of great interest to tumor biology, since they are the pre-cursor cell to both macrophages and dendritic cells (DC). Monocytes are harbored in bone marrow and are released due to systemic changes in the concentration of MCP-1 (monocyte chemoattractant protein-1; CCL2) and attracted into tissues by MCP-1, CSF (colony stimulating factor), IL-17, Galectin-1, RANTES/CCL5, and other danger signals such as the chemokines CX(3)CL1 and CXCL16 [329-337]. However, there are believed to be multiple types of monocytes in circulation, which can be recruited to tissues dependent on signals sent from these tissues to perform a number of jobs [338-340]. Once monocytes migrate out of the blood and into peripheral tissues,

they rapidly undergo phenotypic changes to become macrophages or DC. Therefore, the interesting aspects of these cells involve the small differences which persist while they are in circulation, and what these changes mean for these cells once they extravasate from the blood into peripheral tissues.

The introduction of flow cytometry and identification of specific markers tied to their cellular phenotype has greatly enhanced the characterization of circulating leukocytes. Typical surface markers present on murine monocytes are $Ly6G^{-}$ $CD11b^{+}CD115^{+}Ly6C^{+/-}$ [340-342]. Ly6C differentiates monocytes from neutrophils despite the expression of very similar markers (eg. Ly6G) on these two cell types [342]. The differential size of these two cells type also enhances their identification by flow cytometry. Interestingly, the ability to use Ly6C (monocyte/macrophage differentiation antigen) in the analysis allows for characterization of two distinct subsets of monocytes that cannot be differentiated by cellular morphology, but do have phenotypic differences [339, 340]. However, it is not well understood how expression of Ly6C is regulated, and how cells coming out of the bone marrow are led to express this marker or not [340, 343]. However, it is well established that the lack of Ly6C (steady state monocyte) or presence of Ly6C (inflammatory monocyte) helps distinguish the phenotypic function of these cells [344].

Inflammatory Monocytes

Inflammatory monocytes are characterized as $Ly6C^{+}$, which helps differentiate them as having a pro-inflammatory bias in response to inflammation. It has been shown that $Ly6C^{+}$ monocytes are more activated and differentiate into activated macrophages

once undergoing extravasation at target tissues [340]. Furthermore, it has been shown that $Ly6C^+$ cells preferentially differentiate into DC once they extravasate. DC are professional phagocytic cells which phagocytize debris and transport it to a lymph node to help prime an adaptive immune response. DC induction is critical in the clearance of pathogens because they help coordinate innate and adaptive immune responses and provide an inflammatory environment [345].

It is believed that the circulation of high numbers of inflammatory monocytes allows for rapid migration of a large number of cells into damaged tissues in response to inflammatory signals. In order to contain, damage, and sequester possible pathogens, large numbers of activated cells are required. However, $Ly6C^+$ monocytes also have a shorter half-life, and are thus present as a rapidly deployable defense in response to inflammatory stimuli [339, 340].

Tumors employ mechanisms to dampen the inflammatory properties of $Ly6C^+$ monocytes since inflammatory monocytes are potentially harmful to tumor development. It has been shown that the bone marrow can down-regulate $Ly6C^+$ expression on monocytes due to a variety of stimuli, and it is plausible that a similar mechanism may be present in tumors that nullify the inflammatory properties of these cells and turn them into more tumor supportive monocytes [346].

Steady State Monocytes

Steady state monocytes are characterized as being $Ly6G^-CD11b^+CD115^+Ly6C^-$ cells when characterized by flow cytometry [340]. These cells have also been referred to as resident monocytes, since they may be found attached to the luminal side of blood

vessels and remain in a partially inactivated state until stimulated by inflammatory mediators [339, 347]. Not as much is known about the role of $Ly6C^-$ monocytes, but in a mouse model of cardiac damage, it was shown that after the inflammatory period and migration of $Ly6C^+$ monocytes into the area of damage, there was an influx of $Ly6C^-$ monocytes [348]. Other models have shown that $Ly6C^-$ monocytes may be sequestered in the bone marrow, and upon receiving inflammatory stimuli, quickly become activated and potentially increase $Ly6C^+$ expression [343, 346]. Once present, these steady state monocytes took over the role of repairing the damage done by the inflammatory process. These changes were characterized by the release of anti-inflammatory compounds and increased expression of VEGF, a potent stimulator of angiogenesis [348]. Therefore, it stands to reason that a similar process may be occurring in tumor tissues, in which inflammatory mediators are released that recruit these less inflammatory cells to quell the process and begin to repair the affected tissues and support tumor growth.

Macrophages

Macrophages are derived from monocytes which have recently extravasated from the circulation upon movement into the extracellular environment. The context of this environment is thought to impact how these cells change phenotypically, and the response and interactions which they have with their local environment. Macrophages have classically been subdivided into two groups: the classically activated macrophage (M1) that is inflammatory and acts against foreign pathogens, and the reparative macrophage involved in repairing damaged tissues (M2) [235]. These two types of macrophage have been defined in numerous models, including cancer, but are best

described in a variety of infectious disease models where their phenotypes are more sharply contrasting and straightforward [349]. In tumors, the phenotypic designation of these cells is not as straightforward, and there exists a continuum upon which different "flavors" of these cells reside [349]. It is no longer thought that there are simply two types of macrophages, or that there are inflammatory and anti-inflammatory cells. Current studies show that a mixture of factors in the tumor micro-environment can create a variety of phenotypes for these cells that cannot be easily defined. However, for simplicity, many people still refer to macrophages as M1 or M2 to describe their overall functions, sometimes further characterizing them into more precise groups by defining M2a, M2b, and M2c phenotypes to describe large differences in the non-inflammatory population of cells [350].

Inflammatory macrophages (M1)

Inflammatory macrophages are thought to be derived from inflammatory monocytes and have an anti-tumor role [340]. M1 macrophages are also called classical macrophages in that they were first reported as scavengers of bacterial pathogens, and play a role in controlling infections [340]. The cytotoxic role of these cells is not reserved only for pathogens, but it is thought that macrophages could also inhibit tumor cell growth [351, 352]. It is now believed that macrophages produce an environment inhibitory to tumor growth by promoting inflammatory responses that place various stresses on tumor cells [350]. Furthermore, the presence of an inflammatory process is stimulatory to other immune cells and effectors, and thus leads to an anti-tumor phenotype. Although, this process may be beneficial in inhibiting growth of established

tumors, it may also lead to tumor promotion. It has been well studied that macrophages may promote certain types of cancer if this inflammatory response is prolonged, leading to the accumulation of mutations in cells and resulting in their transformation [353-356]. It is thought that production of ROS and RNS may lead to mutation accumulation in cells leading to transformation and increased invasion [354, 355, 357]. Thus, the duality of this process is likely tumor type and environment specific. M1 macrophages are typically inflammatory and promote this phenotype through the production of IL-1β, IFN-γ and TNF-α [358-360]. The production of these cytokines also promotes a TH1 type response, thereby helping to activate T cells against the tumor [349].

By altering the tumor microenvironment, it is possible to change the polarization of macrophages a pro-tumor (M2) to an anti-tumor (M1) phenotype [361, 362]. This is one mechanism by which M1 macrophages may inhibit tumor growth. Since tumors become reliant on the pro-tumor phenotype of macrophages, it is plausible that peripheral activation of monocytes and their subsequent macrophages may inhibit tumor growth [363, 364]. It has been previously shown that increased numbers of TAM is prognostic in certain cancers; however, the results are conflicting and tumor type dependent [365-367]. Therefore, further classification of macrophages and their responses are required to differentiate these effects.

Alternatively Activated Macrophages (M2)

There have been many classifications concerning the phenotype of the alternatively activated (M2) macrophage; however, many of differences focus on the anti-inflammatory and repair roles of these cells. Therefore, for the purpose of this review,

attention will be focused on the general classification of these cells as M2 macrophages, and not into individual subgroups. However, it is important to remember that the differences between M1 and M2 macrophages are a continuum rather than a black-and-white comparison [349].

M2 macrophages are characterized by their propensity to promote tumor growth, and are involved in wound healing and tissue repair in other disorders [349]. These cells are thought to be derived from steady state monocytes, but the tumor microenvironment is the primary mechanism determining the phenotype of these cells [349]. M2 macrophages create an environment of immune inhibition by promoting regulatory T cells and are themselves stimulated in the presence of T_H2 responses [349, 368-372]. This inhibition of immune responses helps protect the tumor from adaptive immune responses that may be activated against the tumor, and provide a basis for immune evasion. Anti-inflammatory cytokines such as IL-4 and IL-10 help promote M2 formation and activities [373-375]. Interleukin-10 also inhibits macrophage differentiation into DC, inhibiting future immune responses [372]. Furthermore, production of TGF-β promotes macrophage repair activities, and is also a potent inhibitor of immune responses [376, 377]. Up-regulation of the mannose receptor in these cells is thought to promote debris clearance and initiate repair of their environment [375].

In order to help repair damaged areas, as suggested by Dvorak, macrophages break down the extracellular matrix; however, this leads to increased tumor invasion, and is supportive of tumor growth [378]. Hypoxia is also thought to induce an M2 phenotype in macrophages since this leads to the production of VEGF and other pro-angiogenic factors [111]. Macrophages produce an environment conducive to tumor growth by

limiting the intrusion of immune effectors, and promoting tumor growth by breaking down the extracellular matrix and providing oxygen and nutrients to tumor cells by stimulating angiogenesis and metastasis [379, 380]. Indeed, it is easy to see how some tumors can accumulate a large number of these cells, and how macrophage density can lead to a poor prognosis when macrophages are activated in this manner. It has therefore been suggested that depletion of macrophages may be a viable method for the inhibition of tumor growth [303, 381].

Neutrophils

Neutrophils are one of the first-responders to damage within the body. This damage leads to a release of inflammatory mediators which act as a chemoattractant for neutrophils. One of the primary roles of neutrophils is to remove infectious organisms from a specific area [382, 383]. They are able to perform this role by entering tissues using IL-8 as a gradient and releasing a number of inflammatory compounds, which leads to a hostile environment in which invading organisms cannot survive [382-385]. However, in this process, neutrophils typically die in their release of these compounds, requiring that more neutrophils invade the area. The short lived but highly cytotoxic role neutrophils have makes them proficient at clearing infectious agents, but also leads to a large degree of collateral damage, although in a tumor these effects may be beneficial to control of the disease.

Neutrophils may have a multiple phenotypic characterizations much like macrophages [386-392]. Furthermore, it has been suggested that neutrophils may undergo a type of differentiation akin to macrophages that may lead to a type of duality in

their final function [393]. Indeed, there has been contrasting information in the literature suggesting antagonistic roles for neutrophils and their role in cancer disease progression [390, 394-396]. It has been suggested that one role for this change is the persistent hypoxic environment in the tumor that may lead to decreased apoptosis of neutrophils, in turn leading to pro-tumor phenotypic changes [120]. Therefore, our understanding of the role of neutrophils in cancer biology is still relatively unclear at this, time, but due to the large component that these cells compose of peripheral blood and indirect connections to disease outcome, it is plausible that neutrophils play a role in maintaining a pro-tumor environment in many patients and possibly mediate monocyte/macrophage function [397-399]. It has recently been suggested that an immature subset of neutrophils, the myeloid derived suppressor cell, may be the effector portion of the neutrophil population [400].

Myeloid Derived Suppressor Cells

Myeloid derived suppressor cells (MDSC) are a recently discovered type of immune cell. MDSC are thought to be similar to immature monocytes and neutrophils, and may be an immature circulating precursor of these cells [400-403]. They have risen to greater importance in cancer due to their increased numbers in human patients and murine models of cancer [400-402, 404-406]. It is believed that these cells accumulate in the spleen of tumor bearing human patients and mice leading to immunosuppression via T-cell inhibition by releasing arginase and nitric oxide which may lead to the formation of regulatory T-cells; an inhibitory type of T cell prior implicated in being pro-tumorigenic [407-409]. Phenotypic differentiation of these cells is difficult and requires complex multi-color flow cytometry. However, numerous studies describe these cells as

being $CD11b^{+}Gr\text{-}1^{+}$ cells in mice [400, 410] and $CD11b^{+}CD14^{-}CD33^{+}$ and possibly $CD15^{+}$ in humans [400, 411]. The concern is that both CD11b and Gr-1 are expressed ubiquitously on mature circulating leukocytes, specifically monocytes and neutrophils, and that more work is necessary to differentiate this specific cohort of cells from other cells in circulation. Further characterization of these cells will allow for increased research into targeting these cells as a possible therapeutics and determining the role that the play in cancer biology.

Natural Killer Cells

Natural killer (NK) cells are thought to be a mediator between innate and adaptive immunity. The term NK cell was coined in 1975 when it was determined that these cells have the ability to kill tumor cells innately, and without pre-conditioning [412-414]. However, the ability for NK cells to have memory, such as that of T or B cells, was only recently described, and only expressed in a very limited capacity [414-416]. The primary difference is that NK cells encode a number of receptors that do not undergo specific rearrangement like T and B cells [417]. However, the presence of cells that express markers of both NK and T cells have previously been described, and are thought to act as a mediator between the innate and adaptive branches of immunology; however, these cells are not classified as conventional NK cells, but as NKT cells [418].

NK cells mediate their effects primarily through the release of large amounts of inflammatory cytokines, which help to elicit an immune response and attract other leukocytes to the area of inflammation. For example, NK cells can express large amounts of IFN-γ, a potent inflammatory cytokine [419]. When NK cells are challenged with

foreign bodies, they release large amounts of perforin and granzyme that create pores in target cells membranes and induce apoptosis [420, 421]. It has been suggested that subsets of NK cells can undergo a more vigorous response to re-challenge with specific pathogens, akin to T and B cells, and become tolerant to specific stimuli [414]. These properties make NK cells important mediators in the priming of an immune response to a potential pathogen, as well as coordinating future responses to the possible recurrence of a given pathogen.

The role that NK cells play in cancer is also quite varied. NK cells have been implicated in causing the direct lysis of tumor cells and activation of T cells, thus creating a bridge between the innate and adaptive immune systems [422]. The NK cell receptor NKG2D is believed to be expressed on almost all activated NK cells [417]. Cells expressing NKG2D are implicated in immune surveillance and are thought to be one of the defenses against cancer [423, 424]. It has even been shown that NK cells are able to respond to the presence of DNA damage and lyse these damaged cells [425]. Due to the inherent genetic instability present in many tumor cells and their metastatic variants, surveillance by NK cells is an important phase in detecting neoplastic cells by the immune system. Finally, the stress response present in tumor cells leads to the up-regulation of heat shock proteins (HSP). NK cells are able to survey for these changes and lyse these stressed cells, therefore suggesting an additional method for NK cell control of cancer [417]. Indeed, it is easy to see the many facets involved in NK cell biology and their interactions with tumor cells and inhibition of them.

Cancer Biomarkers

A biomarker is a relevant biological marker that can be used as an indicator of a given biological state or process. Biomarkers are of significant importance in cancer because they allow scientists and clinicians to help characterize a specific type of tumor, understand the processes occurring due to specific treatments, and even prognosticate the possible outcome of patients based on previously described data. For example, CEC can act as a biomarker because they describe the current state of angiogenesis within a patient [170]. Since increased CEC are present in patients that have a poor prognosis, CEC may be associated with disease outcome [426, 427]. Furthermore, since CEC are an indicator of angiogenesis, they can be measured over time, and thus changes in CEC due to anti-angiogneic therapies are used to understand the status of a patient's disease. Other groups have shown that circulating tumor cells may be prognostic in that they may be a surrogate for distant metastases and metastatic potential of tumors [428, 429]. Therefore, biomarkers are of absolute importance to understanding the underlying biology present in cancer patients and acting as a window into the disease process.

The possibilities associated with a field as wide as biomarkers have led to a number of approaches for the identification and characterization of biomarkers and their relative utility. Both genomic and proteomic approaches have been used to identify individual genes and proteins which may be involved in specific types of cancer [430-436]. One of the best understood biomarkers is HER2/neu on breast cancer cells. Expression of HER2 on breast cancer cells has been correlated with increased aggressiveness and consequentially decreased survival [437]. However, it is believed

that the combination of many of these markers may be cancer specific, and may be compounded so that a greater prognostic index can be made by combining a number of risk factors. For example, it has been recently postulated that a unique 70-gene signature may be prognostic in breast cancer [438]. However, the problem with some of these approaches is that they are time consuming, require specialized resources and skill sets, and are not always economically feasible.

Diagnostic tests that are relatively inexpensive and straightforward are necessary to allow wide acceptance of specific biomarkers. For example, circulating levels of serum alkaline phosphatase have been associated with decreased survival in humans and dogs with OSA [439-441]. This is a relatively simple test that can be taken from peripheral blood and analyzed in a cost and time efficient manner. However, it should not be believed that simple tests are automatically superior to those that are more consuming. Comparative analyses must be performed in large cohorts of patients to better understand the relative effectiveness of biomarkers. These studies are typical in human patients, in which a number of factors can be analyzed to determine which provide significant prognostic information, and if a combination of these factors lends itself to a greater understanding of the disease state. In the future, studies will have to be done to not only validate novel biomarkers discovered by a variety of approaches, but to determine the validity of biomarkers in a greater context. These markers should not only be implemented to characterize disease, but would optimally be able to be used as a marker of treatment efficacy.

Project Rationale

Tumors are composed of a complex and dynamic mix of many components. Interactions between tumor cells and these other components, such as macrophages and endothelial cells, provide the foundation for numerous interactions that propagate tumor growth, invasion, and metastasis. Therefore, there is intense interest in how these mechanisms interact, and how they can be disrupted in order to exploit the weaknesses of tumors, and possibly eradicate the disease. The interaction of immunosuppressive cells and tumor cells provides a supportive environment for tumor growth by inhibiting immune responses and enhancing invasion and migration. Endothelial cells form vessels deliver oxygen and nutrients to tissues while removing waste products. Tumors exploit this process, not only for the reasons explained above, but to metastasize to distant organs. The overall goal of this dissertation is to explore some of these mechanisms and better understand the clinical observations. The other goal is to develop new models and techniques to better explore these observations and interactions, and further the development of novel methods to understand tumor biology and metastasis.

The first project presented in this dissertation was based upon a clinical observation made in dogs with OSA and later confirmed in human patients [442, 443]. That observation was that dogs with OSA that were treated by a limb-spare and subsequently developed an infection at the surgical site lived significantly longer than patients not developing infections, owing to a significant delay in metastasis development. Local recurrence of the disease was not impacted by the infection. Therefore, we hypothesized that we would be able to recapitulate these observations in a

mouse model of osteomyelitis where mice were challenged with OSA **(Chapter 2: Chronic Bacterial Osteomyelitis Suppression of Tumor Growth Requires Innate Immune Responses)**. We further hypothesized that the effects would be mediated by inhibition of angiogenesis as was suggested by contemporary literature in related models [227, 300]. However, there are a number of mechanisms that could induce the anti-tumor response, and thus we planned to also study the role that IFN-γ and immune cells play in this system.

Chapter 2 explains the development of a model of murine osteomyelitis based on a similar model previously developed. Once a model of infection was developed, and was found to be chronic and localized, work was performed to determine if the infection could inhibit growth of a primary tumor located at a distant location. With a model that recapitulated the clinical observations, we sought to determine the mechanism of action behind the observed tumor growth inhibition. Furthermore, to determine if the effect is tumor type specific, we investigated three different tumor types in three different mouse strains, and two different bacterial pathogens. We first investigated the role of angiogenesis in the presence of infection, and the possible role that circulating endothelial cells play in angiogenic development. Due to previous literature concerning the role of IFN-γ, and the ability of NK cells to produce large amounts of this cytokine, we depleted these cells to determine if they were required for tumor growth inhibition. We then sought to characterize changes in circulating monocyte populations and subsequent changes on tumor associated macrophages. Thus, our goal was to better describe the mechanism of action associated with the inhibition of systemic tumor due to distant and localized infections.

The process of infection leading to tumor growth inhibition is an inflammatory process, and thus we sought to study the counter-role that anti-inflammatory compounds play in tumor growth. There have been numerous examples of how anti-inflammatory compounds (NSAIDs) can inhibit tumor growth [250, 274-278]. Even though many of these observations were made in colon cancer, it was hypothesized that other high COX-2 expressing tumor cells could be similarly vulnerable to these compounds [249]. The ability to modulate inflammation reduces the supportive environment for the tumor thus leading to growth inhibition. Therefore, we hypothesized that a dual COX-1/COX-2 and 5-LOX inhibitor would inhibit tumor growth **(Chapter 3: Induction of VEGF by Tepoxalin Does Not Lead to Increased Tumor Growth in a Canine Osteosarcoma Xenograft)**.

We first performed *in vitro* assays to determine if tepoxalin inhibited cellular viability on a number of different canine tumor cell lines. Further investigation into what appeared as a slight stimulation of growth lead us to investigate possible growth factors, such as VEGF. We then observed that tepoxalin stimulated VEGF production from these cells. We then hypothesized that this increase in VEGF is due to the unique structure of tepoxalin acting as an iron chelator, and investigated the stabilization of HIF-1α in response to tepoxalin treatment by western blot analysis. These observations led us to hypothesize that tepoxalin would lead to increased tumor growth in a tumor xenograft model. In order to test this hypothesis, we investigated the role that tepoxalin has in a canine xenograft model using pharmacologically achievable concentrations that are comparable to those found in dog, the target species of the drug. Pharmacokinetic analysis was performed to determine an appropriate dose for these studies. *In vivo*

experiments were then performed to investigate the effects of tepoxalin on tumor growth, and explore changes in systemic VEGF in response to tepoxalin. Finally, we sought to determine the effects of short term tepoxalin administration on changes in systemic VEGF concentrations in normal dogs.

The first two studies performed thus far have investigated the role of inflammation on changes in tumor angiogenesis. However, analysis of angiogenesis could only be performed by euthanizing mice, removing the tumor, cryosectioning, and immunostaining for the endothelial cell marker CD31. This process is time consuming and requires the use of large numbers of mice. Furthermore, there is no technique that could be used to investigate changes in angiogenesis in a single tumor over time [185]. These experiments are typically performed by selecting an arbitrary time point at which mice are sacrificed and analyzed, or groups of mice are serially sacrificed to reveal trends associated with changes in angiogenesis. However, both of these techniques are time consuming and require large numbers of mice. Therefore, we sought to develop a novel technique to characterize angiogenesis in tumors over time that was minimally invasive. Clinical use of fine needle aspiration (FNA) allows for small samples of tumor to be obtained, and analyzed by microscopy. In this process, there is typically a large amount of immune infiltrate and other tumor components present. Therefore, we hypothesized that FNA could be used to obtain small samples of EC by a minimally invasive method. Characterization of these EC could then be performed by flow cytometry **(Chapter 4: Minimally Invasive Assessment of Tumor Angiogenesis by Fine Needle Aspiration and Flow Cytometry)**.

In order to determine if FNA was a feasible method to identify EC, we first used a variety of techniques to determine the needle type that would provide a sufficient number of cells for analysis while not causing irreparable harm to a tumor bearing animal. Once this was performed, we sought to identify EC in tumors and compare the results of an FNA to digest of the whole to tumor to determine if the FNA was representative of the overall tumor burden due to variations in angiogenesis. In order to validate the technique, we compared the FNA technique to the current gold standard of IHC to determine if the two approaches gave similar results. In order for this technique to be feasible we insured that repeated FNA did not induce an artifact and alter angiogenesis. This then allowed us to investigate if we could detect changes in angiogenesis as a function of time, and as a consequence of anti-angiogenic therapy. These experiments provided evidence that FNA coupled with flow cytometry is a reliable technique to serially measure angiogenesis in tumor growth. The creation of a technique to better monitor angiogenesis is beneficial because it allows for removing experimental bias through repeated measures, and helps decrease the animal burden required to investigate changes over time using *in vivo* tumor models.

The development of a novel model of angiogenesis is important to investigating changes in tumor angiogenesis; however, there remains a need for more realistic tumor models that more closely approximate the clinical scenario. The use of primary tumor models in heterotopic sites is common practice for *in vivo* tumor biology even though these conditions do not mimic the relationships of the tumor to its natural environment [42, 44, 55-57]. It has been previously shown that an orthotopic location can greatly alter tumor growth, metastasis, and response to therapy [52-54]. Therefore, we sought to

create a novel model of osteosarcoma that could be implanted orthotopically. We also sought to luciferase transfect the tumor cells so the progression of systemic metastases could be non-invasively measured. Furthermore, treatment in clinical patients typically includes surgical removal of the tumor prior to the initiation of systemic chemotherapy, and thus we created a model in which the primary tumor implanted orthotopically could spontaneously metastasize before amputation of the tumor bearing leg **(Chapter 5: An Orthotopic, Postsurgical Model of Luciferase Transfected Murine Osteosarcoma with Spontaneous Metastasis)**. We thus hypothesized that a novel murine model of OSA would allow us to investigate the role of novel therapeutics for OSA in a micrometastatic setting independent of the pressures of the primary tumor.

We first utilized a plasmid system that would allow us to transfect the luciferase gene into the DLM8 murine osteosarcoma cell line and would be constitutively active. We then selected for luciferase positive cells that metastasized to the lung from an orthotopic site. Characterization of the orthotopic tumor and its metastases was performed using micro computed tomography, luciferase imaging, and immunohistochemistry. There are a number of orthotopic tumor models, but this is the first using this cell line and C3H mice. Therefore, we sought to more closely resemble the clinical treatment paradigm by amputating the tumor bearing leg. A surgical adjuvant model was created, and experiments were performed to determine when 100% of the mice developed micrometastatic disease. Using this model, we were then able to investigate the role of conventional and novel chemotherapeutics for OSA. The development of an orthotopic OSA tumor model in immunocompetent mice that spontaneously metastasizes in a postsurgical setting will allow for the screening of novel

compounds in a model system similar to that which human and canine cancer patients endure.

After defining a more relevant model system for OSA, we sought to explore the role of circulating leukocytes as a prognostic in clinical disease. Observations from the osteomyelitis study suggested that changes in circulating leukocytes may be mediators of tumor growth in OSA. We also investigated changes in response to the anti-inflammatory compound tepoxalin. However, it would be beneficial to determine if circulating leukocytes could be prognostic in OSA. The need exists to define simple tests that can cost-effectively prognosticate outcomes in OSA. Therefore, we investigated the role that a pre-treatment complete blood count may play in determining OSA prognosis **(Chapter 6: Increased Circulating Monocytes and Lymphocytes are Associated with Decreased Disease Free-Interval in Dogs with Osteosarcoma)**.

In chapter 6 we determined that changes in circulating leukocytes were prognostic in OSA. Changes in leukocytes may suggest more about the biology of the tumor and disease progression. We hypothesized that differences in monocytes would be prognostic in OSA due to the observed role that these cells play in mediating tumor biology. To answer this question, we screened 313 clinical OSA cases presenting to the Colorado State University Veterinary Teaching Hospital between 2003 and 2006. Patients were selected based on surgery type received, lack of detectable metastases at presentation, and treatment with conventional chemotherapy. We then performed statistical analyses on these dogs to determine if circulating leukocytes or other variables assessed were prognostic in OSA. Conclusions from the first population of dogs were then tested and reproduced in a second independent population of dogs also treated at CSU. Therefore,

we concluded that increased numbers of circulating monocytes and lymphocytes at the time of presentation were a prognostic factor for disease-free interval (time to clinical metastatic development).

The goal of the work presented in this dissertation is to better describe the processes of angiogenesis and innate immunity in OSA. This exploration begins with the observation that localized osteomyelitis can increase survival in patient dogs. Thus we sought to develop a model of this process in mice to better define the mechanism by which this effect may occur. We then explored the antithesis of this in determining what the role of an anti-inflammatory compound may have on OSA biology. Interestingly, both of these techniques led to decreases in tumor growth, and thus the complex duality of inflammation in different forms was shown in this approach. Exploration of methods to better characterize changes in angiogenesis, and development of models for which to investigate OSA biology and novel therapeutics were also undertaken to improve the understanding of OSA. Lastly, we went back to the clinic to explore the role of circulating leukocytes in naïve OSA patients. This dissertation concerns the interactions of the many factors implicated in tumor biology, and seeks to explain a few of these factors and add to the expansive field of tumor biology as a whole, and more specifically the field of OSA.

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Chapter Two

Chronic Bacterial Osteomyelitis Suppression of Tumor Growth Requires Innate Immune Responses

Abstract

Clinical studies over the past several years have reported that metastasis free survival times in humans and dogs with osteosarcoma are significantly increased in patients that develop chronic bacterial osteomyelitis at their surgical site. However, the immunological mechanism by which osteomyelitis may suppress tumor growth has not been investigated. Therefore, we used a mouse model of osteomyelitis to assess the effects of bone infection on innate immunity and tumor growth. A chronic Staphylococcal osteomyelitis model was established in C3H-HeN mice and the effects of infection on tumor growth of syngeneic DLM8 osteosarcoma were assessed. The effects of infection on tumor angiogenesis and innate immunity, including NK cell and monocyte responses, were assessed. We found that osteomyelitis significantly inhibited the growth of tumors in mice, and that the effect was independent of the infecting

bacterial type, tumor type, or mouse strain. Depletion of NK cells or monocytes reversed the antitumor activity elicited by infection. Moreover, infected mice had a significant increase in circulating monocytes and numbers of tumor associated macrophages. Infection suppressed tumor angiogenesis but did not affect the numbers of circulating endothelial cells. Therefore, we concluded that chronic localized bacterial infection can elicit significant systemic antitumor activity mediated primarily by NK cells and monocytes/macrophages.

Introduction

The ability of bacterial infections to inhibit tumor growth was first described over a century ago by Dr. William Coley [1]. Coley suggested that the fever accompanying the infection led to a warming of the tumor which triggered tumor regression [2]. More recent studies have shown that systemic infection with non-bacterial pathogens is capable of inhibiting tumor growth. For example, infection with *Toxoplasma gondii* has been shown to suppress tumor growth via induction of IFN-γ release, suppression of tumor angiogenesis, and leukocyte recruitment [3, 4].

Models of tumor localized infection, where bacteria home directly to the tumor, have been used as one approach for tumor immunotherapy [5-8]. The homing of the pathogen to the tumor triggers leukocyte recruitment to tumor tissues, which is believed to stimulate non-specific antitumor immunity. Genetically altered bacteria and viruses have also been used to deliver immunostimulatory cytokines and other anti-tumor molecules to the tumor microenvironment [6, 7, 9-13]. Studies in various tumor models have shown that these approaches elicit leukocyte recruitment to the tumor and promote anti-tumor activity.

Osteosarcoma is the most common primary bone tumor in humans and dogs, and the tumor in dogs is widely viewed as the most relevant animal model for human osteosarcoma [14, 15]. Tumor metastasis is the most common cause of death from osteosarcoma [16]. For this reason, both humans and dogs with osteosarcoma typically receive adjuvant chemotherapy [14, 17]. Despite the use of chemotherapy, the long term survival of dogs with osteosarcoma remains less than 20% [16]. In humans, the overall

5-year survival is 60% with surgery and adjuvant chemotherapy as the standard of care [17].

It was recently reported that dogs with osteosarcoma developing localized osteomyelitis following limb-sparing surgery had significantly increased metastasis-free intervals and survival times compared to dogs that did not develop infections [18]. Survival times were nearly doubled in dogs that developed osteomyelitis. Even when adjusted for confounding variables, bone infection was significantly associated with inhibition of metastasis. Furthermore, the authors noted that disease progression and survival effects were due to the delay in metastasis, rather than to local recurrence of disease [18]. Similar findings were reported in humans with osteosarcoma that developed osteomyelitis at the site of limb sparing surgery [19]; although, it should be noted that a second study in humans with osteosarcoma failed to find an association with bone infection and survival times [20]. These findings suggest that localized osteomyelitis may elicit an immune response that is associated with the inhibition of tumor growth and metastasis.

Therefore, we hypothesized that localized bacterial osteomyelitis was capable of eliciting systemic antitumor immunity. To test this hypothesis, we developed a mouse model of chronic osteomyelitis and subcutaneously implanted syngeneic DLM8 osteosarcoma cells [21]. Using this model, we found that localized bone infection could suppress tumor growth through sustained activation of innate immune responses. These findings are important because they suggest that chronic, sustained, low level inflammation could be used therapeutically to control tumor growth.

Materials and Methods

Cell Lines

The mouse osteosarcoma tumor cell line, DLM8, was generously provided by Dr. Eugenie Kleinerman (MD Anderson Cancer Center) and was maintained in C/10 Dulbecco's modified Eagle media [DMEM (Lonza, Walkersville, MD) supplemented with 1X MEM vitamin solution (Cellgro, Henderson, VA), 2 mM L-glutamine (Cellgro), 1 mM sodium pyruvate (Cellgro), 1X non-essential amino acid solution (Cellgro), 1X antibiotic/antimycotic (Cellgro), and 10% heat inactivated fetal bovine serum (FBS, Atlas, Fort Collins, CO)]. The murine CT26 (colon carcinoma) and B16 (melanoma) cell lines were maintained in C/5/5 MEM media [MEM (Lonza) and supplemented as described above with 5% FBS and 5% heat inactivated newborn calf serum (Hyclone, Logan, UT)].

Bacteria

Staphylococcus aureus stably expressing both the luciferase and luciferin genes (XEN36) was purchased from Xenogen/Caliper Life Sciences (Hopkinton, MA). *Pseudomonas aeruginosa* engineered to stably express luciferase and luciferin was kindly provided by Dr. Herbert Schweizer (Colorado State University). Bacteria were grown to log phase in LB Broth (USB Corporation, Cleveland, OH), then bacterial stocks were tittered and frozen at -80ºC prior to use.

Animals

All animal studies were performed in an AALAC-approved facility, with approval of the Colorado State University Institutional Animal Care and Use Committee. Female mice 8-10 weeks of age were used for all experiments, and were purchased from Harlan Sprague Dawley (Indianapolis, IN). C3H-HeN-Hsd mice were used for all experiments involving the DLM8 tumor cell line, BALB/c-Hsd mice were used for all studies involving the CT26 tumor cell line, and C57BL/6-Hsd mice were used for experiments involving the B16 tumor cell line. Five mice per group were used for all *in vivo* experiments.

Bacterial Osteomyelitis Model

Osteomyelitis was induced in mice, using a modification of a previously reported technique [22]. Briefly, bacteria were cultured in sterile LB broth at 37°C in a shaking incubator for 4-6 hours to log growth. Bacteria were diluted to a concentration of $1x10^6$ CFU per mL in LB broth, and 3 mm segments of 3-0 braided silk suture (Syneture, Norwalk, CT) were incubated in the bacteria for 2 hours at 37°C on a shaking incubator. To induce biofilm formation on the suture material, suture segments were then transferred to sterile LB broth and incubated for an additional 36 hours in a shaking incubator, with the culture medium changed every 12 hours. Control suture segments were prepared similarly, except without bacterial infection.

Mice were anesthetized using isoflurane (Minrad, Bethlehem, PA) for the procedure and the surgical site shaved and cleaned. An incision was made over the proximal tibia, and a 25g needle (Becton Dickinson, Franklin Lakes, NJ) was used to drill

a perpendicular hole in the proximal tibia. The hole was then enlarged through the use of a 23g needle (Becton Dickinson) to facilitate insertion of the suture segment. Suture segments were placed into the medullary cavity of the tibia. Mice receiving infected and sham treated suture material received buprenorphine (0.05 mg/kg) administered subcutaneously every 12 hours for 72 hours post-surgery.

***In Vivo* Imaging of Infected Bone**

The bone site of infection was evaluated 2 to 3 times weekly to assess the intensity of bacterial infection by quantifying luciferase expression intensity. Imaging was performed using an IVIS 100 imaging system and Living Image version 2.50.1 software (Xenogen). Mice were anesthetized with isoflurane and imaged. A one minute exposure time with high sensitivity binning was used to enhance quantification of the infection. The minimum intensity was set at 10% of the maximum, and a contour ROI plot with default parameters (ROI edge value of 5%) chosen to increase objectivity. Total flux of the ROI was recorded as photons/sec for each sample.

Determination of Bacterial Burden in Tissues

To determine whether bacterial infection had disseminated from the site of bone infection to other tissues, mice were sacrificed at the peak of bone infection, 10 days post-infection, and blood, spleen, liver, lung, infected tibia, opposite tibia, and tumor tissue were dissociated for determination of bacterial counts (colony forming units; CFU). Tissues were subjected to collagenase digestion and trituration before serial dilutions of supernatants were plated on LB agar plates (Fisher, Fair Lawn, NJ) and

incubated overnight at 37°C. Bacterial colonies were counted visually and CFU per organ were determined. Plates were imaged using the IVIS system described above to identify luiferase-positive colonies and exclude contaminating organisms.

Tumor Challenge and Growth Model

Three days after establishment of bone infection, mice were injected with $2x10^6$ DLM8 tumor cells subcutaneously (s.c.) on the contralateral flank to the bone infection. In other experiments, B16 and CT26 tumor cells were injected s.c. at a concentration of $5x10^5$ cells per mouse. Mice were then imaged as previously described, and tumors measured two to three times a week using calipers. Mice were tail bled weekly by lateral tail vein incision to assess changes in circulating cell populations by flow cytometry. All mice were euthanized when the tumor of the first mouse in the control group reached a tumor diameter of 10 mm, except in survival experiments where each mouse was sacrificed when the individual tumor reached a size of 10 mm.

NK Cell Depletion

NK depletion was performed by intraperitoneal (i.p.) injection of 50 μL rabbit anti-asialo GM1 antiserum (Wako Pure Chemical Industries, Inc., Osaka, Japan), as described previously [23]. Treatment was initiated three days post tumor challenge and continued weekly. This treatment resulted in depletion of 73% of $NKG2D^+$ cells in the spleen **(Figure 2.7)**.

Macrophage Depletion

Monocytes and macrophages were depleted by intravenous (i.v.) injection of liposomal clodronate, as reported previously [24-27]. Liposomal clodronate was prepared as described previously [28]. Control liposomes were prepared similarly, except that phosphate-buffered saline (PBS) was used instead of clodronate. Treatment with liposomal clodronate was initiated 3 days after tumor challenge and continued weekly. The efficiency of monocyte depletion was assessed by flow cytometry; i.v. injection of liposomal clodronate was found to deplete 71% of circulating monocytes **(Figure 2.11)**. Injection of PBS liposomes did not deplete monocytes **(Figure 2.11)**.

Flow Cytometry

Leukocytes in the blood, tumor, spleen, and lymph node tissues were quantified using flow cytometry. Blood was lysed in ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA) to remove red blood cells from analysis. Cells were then washed in FACS buffer (1x PBS with 2% FBS and 0.1% sodium azide). Tumors were prepared by collagenase digestion and the single cell suspensions were washed once in FACS buffer. Spleen and lymph node samples were prepared by pushing sample through a 10 μm nylon cell strainer. Spleen samples were then lysed with ACK lysis buffer while lymph node samples were not. Spleen and lymph node samples were then washed in FACS buffer.

Single cell suspensions, at a concentration of $5x10^5$ to $1x10^6$ cells per well, were immunostained with the following antibodies: anti-mouse CD3-APC-alexa fluor 750

(clone 17A2), anti-mouse NKG2D-APC (clone CX5), anti-mouse CD4-PB (clone RM4-5), anti-mouse CD8-PE/Cy7 (clone 53-6.7), anti-mouse CD11b-biotin (clone M1/70), anti-mouse CD3-biotin (clone ebio500A2), anti-mouse CD31-FITC (clone 390), anti-mouse CD11b-APC/Cy7 (clone M1/70), anti-mouse CD115-PE (clone AFS98), anti-mouse F4/80-APC (clone BM8), and anti-mouse Gr-1-PE/Cy7 (clone RB6-8C5) were purchased from eBioscience (San Diego, CA); anti-human CD45-Pacific Orange (clone HI30) and nuclear stain (LDS-75) were purchased from Caltag/Invitrogen (Eugene, OR); and anti-mouse Ly6G-FITC (clone 1A8) and anti-mouse Ly6C (clone AL-21) were purchased from BD Pharmingen (San Jose, CA). For biotinylated antibodies, streptavidin conjugates were used to provide flourophores for analysis (eBioscience and Invitrogen).

Neutrophils were defined as being $Ly6G^{+}CD11b^{+}$. Monocytes were defined as being $Ly6G^{-}CD11b^{+}CD115^{+}Ly6C^{+}$ and were further subdivided into steady state ($Ly6C^{lo}$) and inflammatory ($Ly6C^{hi}$) monocytes. Macrophages were defined as $Ly6G^{-}CD11b^{+}F4/80^{+}$. NK cells were defined as $CD3^{-}CD4^{-}CD8^{-}NKG2D^{+}$. Circulating endothelial cells were defined as $CD45^{-}CD11b^{-}CD3^{-}CD31^{+}LDS\text{-}751^{+}$ events. Endothelial cells in tumor tissues were identified as $CD45^{-}CD11b^{-}CD3^{-}CD31^{+}$.

Prior to immunostaining, cells were first blocked for non-specific staining using unlabelled anti-mouse Fcr III antibody (CD16/32; clone 93; eBioscience) diluted in normal mouse serum (Jackson Immunoresearch). Immunostaining was performed at room temperature for 30 minutes, and then the samples were washed. Spleen and lymph node samples were fixed in 1% paraformaldehyde prior to flow cytometric analysis; blood and tumor samples were not fixed but analyzed immediately. Samples were

analyzed using a CyAn ADP flow cytometer (Beckman-Coulter, Fullerton, CA) and analysis performed using Summit software v4.3 (Beckman-Coulter).

Immunohistochemistry

The following antibodies were used for immunohistochemistry: anti-mouse purified CD31 antibody (clone 390; eBioscience), anti-mouse purified CD68 antibody (clone FA-11; AbD Serotec, Raleigh, NC), biotinylated donkey anti-rat (Jackson ImmunoResearch). Tumor tissues were embedded in OCT embedding medium (Sakura, Torrance, CA) and cryosectioned into 4 μm sections. Slides were rehydrated, non-specific binding blocked with appropriate serum, then incubated with appropriately diluted primary antibodies. After washing, sections were incubated with appropriate biotinylated secondary antibody described above. A Vectastain ABC kit (Vector) and subsequent AEC peroxidase substrate kit (Vector) were used according to manufacturer's instructions. Slides were then counterstained with hematoxylin and crystal mount (Biomeda, Foster City, CA) applied.

Immunohistochemical staining was analyzed in a randomized and blinded fashion. Microvessel density (MVD) was analyzed by manually counting the number of microvessels per 20X high power field, with five random fields counted per tumor section. Macrophage density was determined using five random fields and Carl Zeiss AxioVision Software v4.6 (Zeiss, Thornwood, NY). Computerized determination of positively staining cells was performed by blanking against sections stained with an irrelevant isotype control antibody. The average number of vessels or macrophages per high power field was determined.

Assessment of Tumor Associated Macrophage Cytotoxicity

Tumor tissues from infected and control mice were removed and prepared by collagenase digestion. Single cell suspensions were prepared in sort buffer (1x PBS with 2% FBS) and TAM were immunostained using CD11b and F4/80, as described above. Cells were then sorted using a MoFlo Flow Cytometer (Beckman-Coulter). The purity of the recovered cells was determined by flow cytometry to be 85%.

The sorted TAM were admixed with DLM8 tumor cells at macrophage to tumor ratios from 1:2 to 1:8. The cells were incubated for 24 hours and cytotoxicity was assessed using an LDH release assay, according to manufacturer's directions (Promega, Madison, WI). As a positive control for macrophage cytotoxicity, thioglycolate-elicited peritoneal macrophages were used, following overnight activation with 20 ng/mL of recombinant murine IFNγ (Prepotech, Rocky Hill, NJ).

Statistical Analysis

Statistical analysis was performed using Prism 5 (GraphPad Software, La Jolla, CA). Tumor growth experiments with two groups were compared using two-way repeated measures ANOVA, and those with three or more groups were analyzed using repeated measures one-way ANOVA and Bonferroni post-test. For all tumor growth experiments, day zero was not included in the statistical analysis since this was the day of tumor challenge and no mice had measurable disease; this point is present for graphical depiction only. Data from two groups were compared using a two-tailed Student's t-test and data with three or more groups was compared using one-way ANOVA with

Bonferroni post-test. Survival analysis, as described as time to a specific tumor diameter, was performed using Kaplan-Meier log-rank analysis. Comparison of monocytes over time was performed using a two-way ANOVA with Bonferroni post-test. For all analyses, p-values of less than 0.05 were considered statistically significant.

Results

Establishment of Mouse Model of Chronic Osteomyelitis

To study the effects of a localized bone infection on distant tumor growth, we first established a mouse model of chronic bacterial osteomyelitis, using a modification of an earlier protocol [22]. One of the primary modifications in our model was to establish bacterial biofilms on the suture prior to implantation in the marrow cavity of the tibia. This was done by prolonging the length of incubation in bacterial cultures, along with continuous shaking. We found that biofilm sutures led to more reliable infections with greater sustained luciferase intensity over time than did sutures prepared using the original method **(Figure 2.1)**. We also observed a 4-fold increased mean infection intensity at the bone site in mice receiving biofilm infected suture, compared to mice implanted with non-biofilm suture at 49 days after suture implantation **(Figure 2.1)**.

Luciferase expressing *Staphylococcus aureus* biofilms were established on silk suture and placed in the medullary cavity of C3H-HeN mice (n=5 per group). Following placement of the suture, the intensity of luciferase expression was determined in each infected mouse (**Figure 2.2**). With this infection model, we observed that there

was a progressive increase in the intensity of bone infection, with the peak of infection developing 10 days post challenge **(Figure 2.2)**. The bone infection was also sustained, with live bacteria still detectable by luciferase imaging at the bone infection site over 50 days post-challenge**.**

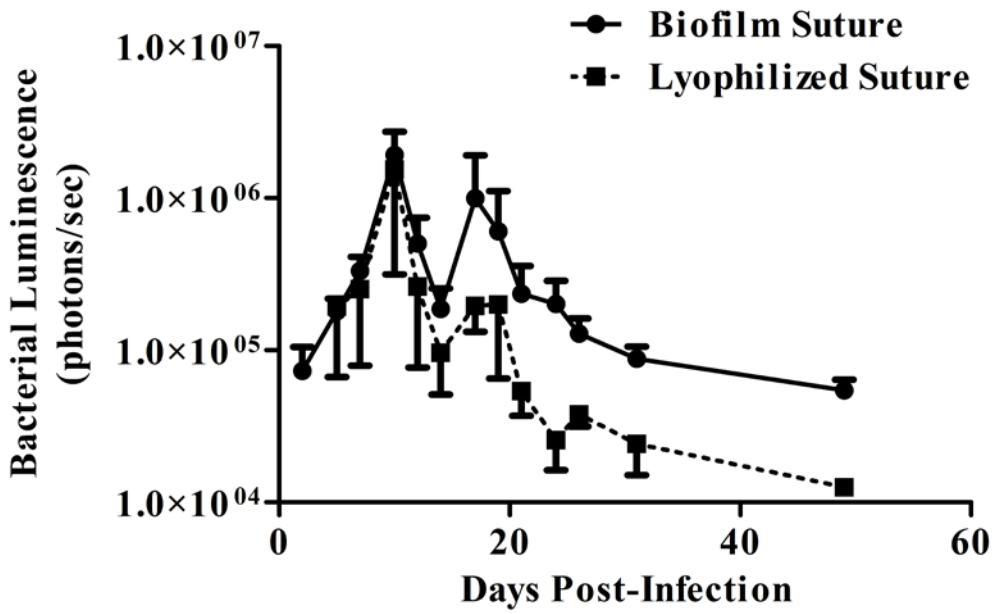

Figure 2.1: *Biofilm suture provides a more robust infection than lyophilized suture.* Biofilm and lyophilized suture segments were established as described in the Methods. Sutures were implanted in C3H mice (n=5 per group) and serially imaged using the IVIS system described in the Methods. Biofilm established osteomyelitis had a significantly ($p<0.01$) greater intensity over time as measured by repeated measures two-way ANOVA. Data are representative of two independent experiments and mean (± SEM) are depicted.

We also determined whether the infection remained localized in the tibia or whether the Staphylococci became disseminated after bone infection; no signs of illness were noted during the infection. By Xenogen imaging we did not detect a luciferase signal at any sites besides the bone inoculation site **(Figure 2.2)**. However, to increase the sensitivity of detection, we also performed quantitative cultures of various organs

from mice with infected limbs. We did not detect bacterial colonization of any tissues other than the inoculated bone at any time during the infection studies. Therefore, we concluded that in this model, the infection remained localized to the site of infected bone.

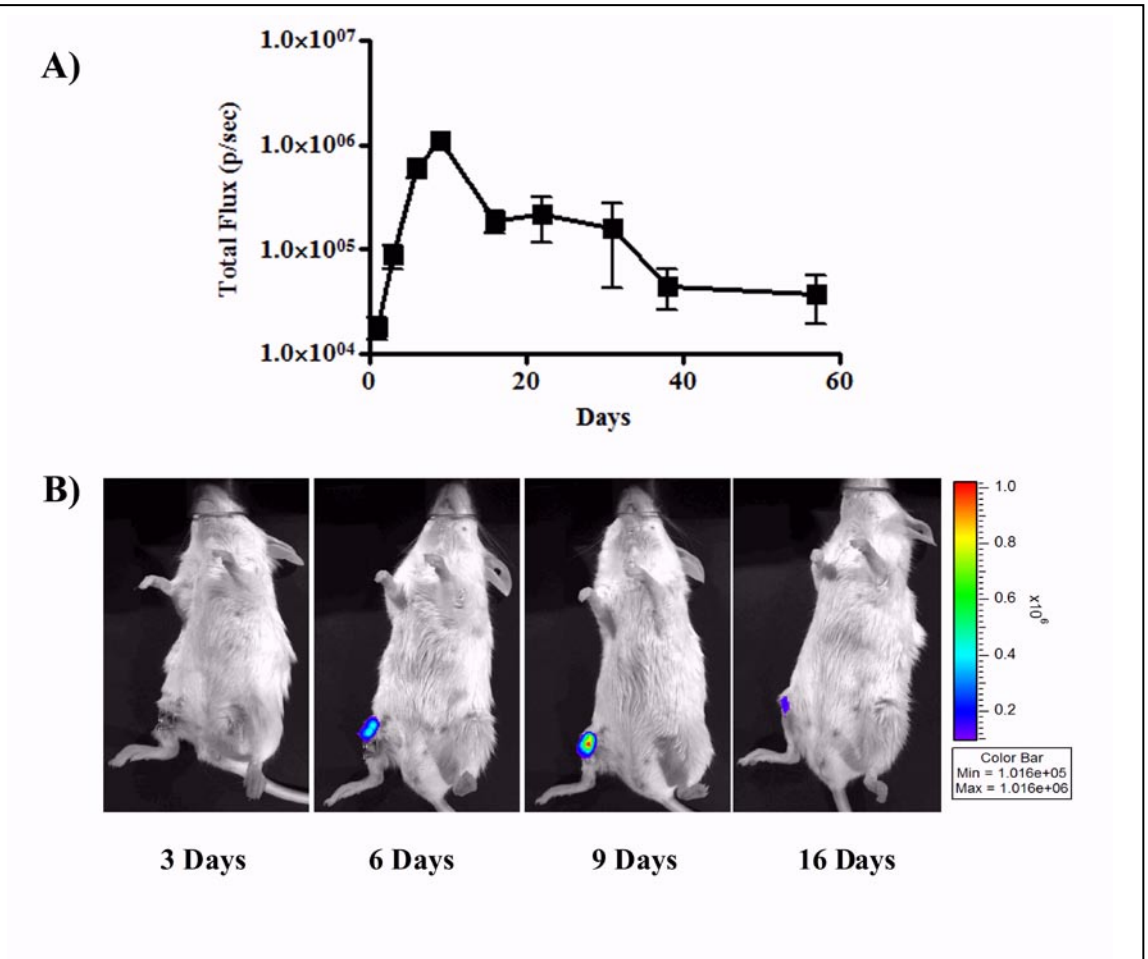

Figure 2.2: *Development of localized osteomyelitis in the mouse.* Mice (n=5 per group) were challenged with luciferase-transfected *Staphylococcus* biofilm-coated suture material introduced into the medullary cavity of the tibia, and imaged using a Xenogen In Vivo Imaging System to track the intensity and localization of the infection. A) Time course analysis of infection intensity of osteomyelitis with means (± SEM) calculated. B) Serial images of a representative mouse compared using a standardized scale show changes in intensity over time without dissemination.

Tumor Growth is inhibited in Mice with Bacterial Osteomyelitis

To determine whether bacterial osteomyelitis could inhibit tumor growth, *Staphylococcus aureus* infected suture was placed in the tibia of C3H-HeN mice (n=5 per group). Three days later, mice were injected with syngeneic DLM8 tumor cells on the contralateral rear flank. Tumor growth was assessed every 2-3 days using calipers. We found that mice with bacterial osteomyelitis had a significant decrease ($p<0.02$) in tumor growth compared to uninfected control mice **(Figure 2.3)**. However, in mice with tumors established 3 days before infection, we did not observe significant tumor growth inhibition **(Figure 2.3)**. Mice that were sham-infected with control suture did not have a significantly altered tumor growth compared to untreated mice with tumors only **(Figure 2.3)**. In addition, survival times, using time to reach 10 mm as the event, were significantly increased ($p<0.01$) in mice with osteomyelitis compared to control mice **(Figure 2.3)**. These results indicated that localized bacterial infection significantly inhibited the growth of tumors at distant sites. This finding was most consistent with the induction of a systemic antitumor response by the localized bone infection.

We next conducted experiments to determine whether the inhibition of tumor growth observed above was tumor type or mouse strain specific. To address these questions, we assessed the effects of osteomyelitis on tumor growth using the CT26 (colon carcinoma) model in BALB/c mice and the B16 (melanoma) model in C57BL/6 mice. In both of these models, we observed significant inhibition of tumor growth in animals with osteomyelitis compared to control animals **(Figure 2.4)**. Thus, we concluded that the tumor-inhibiting effects of localized osteomyelitis were not tumor type or mouse strain specific.

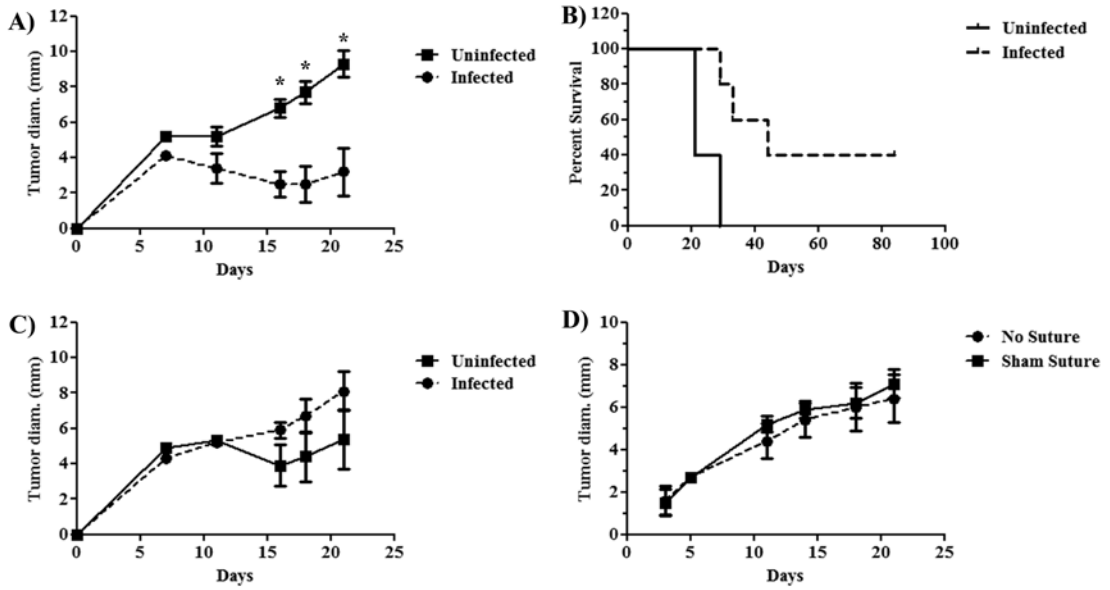

Figure 2.3: *Osteomyelitis is associated with tumor growth inhibition.* Mice (n=5 per group) were infected with *S. aureus* containing biofilms three days before or after tumor implantation. Growth kinetics were assessed by two-dimensional measurements and the longest diameter reported as the mean (± SEM) and statistically analyzed by repeated measures two-way ANOVA. A) Mice receiving infections prior to tumor challenge with syngeneic DLM8 osteosarcoma cells have a significantly inhibited tumor growth (A, $p<0.02$; *$p<0.001$) and increased survival (B, $p<0.01$), as described as time for tumor to reach 10 mm longest diameter, compared to uninfected mice. C) Mice infected after tumor challenge did not have a significant growth inhibition ($p>0.05$). D) Mice challenged with a sham segment of suture did not have a significantly different ($p>0.05$) alteration in tumor growth compared to mice only challenged with tumors. All experiments are representative of two independent experiments.

The preceding results indicated that chronic staphylococcal infection was capable of eliciting broad antitumor activity. However, it was possible that the antitumor activity was specific to staphylococcal infection. Therefore, osteomyelitis was induced in mice using *Pseudomonas aeruginosa* instead of *Staphylococcus aureus* and the effects on tumor growth were assessed. As with the staphylococcal osteomyelitis model, we observed a significant ($p<0.01$) inhibition of tumor growth in mice infected with *P. aeruginosa* derived osteomyelitis (**Figure 2.5**). These results suggested that inhibition of

tumor growth was not specific to staphylococcus, but could in fact be elicited by both gram-negative and gram-positive bacteria. Furthermore, tumor growth inhibition was independent of the effects of bacterial lipopolysaccharide (LPS), since significant inhibition was observed following staphylococcal infection.

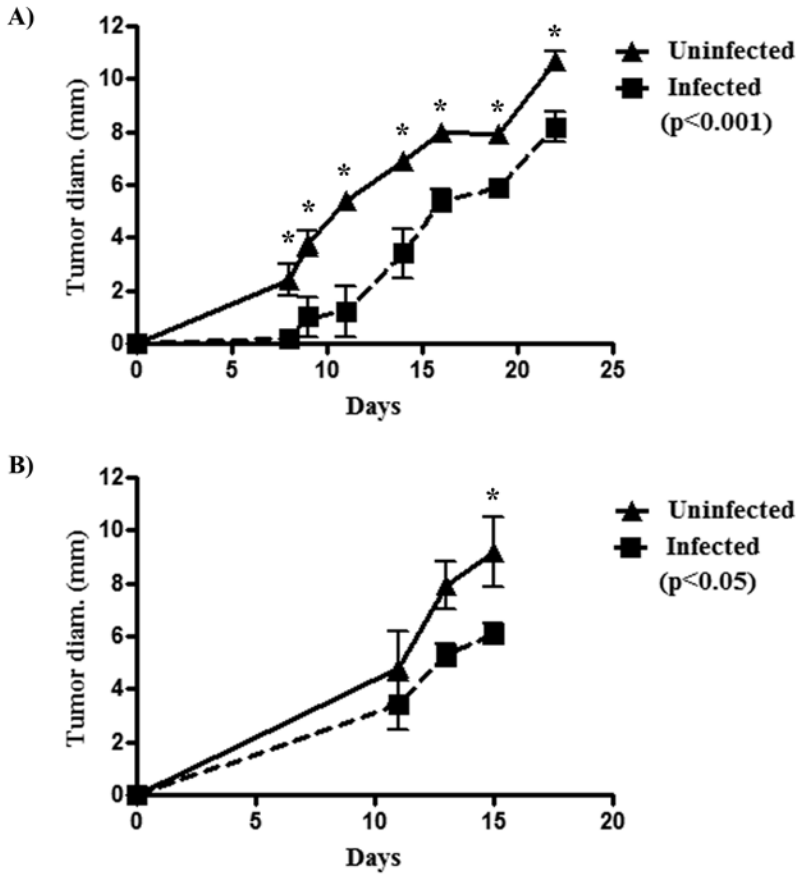

Figure 2.4: *Osteomyelitis inhibits tumor growth in multiple murine tumor models.* Mice were infected with *S. aureus* containing biofilms three days before tumor challenge. Growth kinetics were assessed by two-dimensional measurements and the longest diameter is reported as the mean (± SEM) calculated for each group. A) Infected BALB/c mice (n=5 per group) challenged with syngeneic CT26 colon carcinoma cells have a significantly ($p<0.001$; $*p<0.05$) inhibited tumor growth compared to uninfected mice as assessed by repeated measures two-way ANOVA. B) Infected C57BL/6 mice challenged with syngeneic B16 melanoma cells were followed as previously described. Infected mice had a significantly ($p<0.05$; $*p<0.05$) inhibited tumor growth compared to uninfected mice as assessed by repeated measures two-way ANOVA.

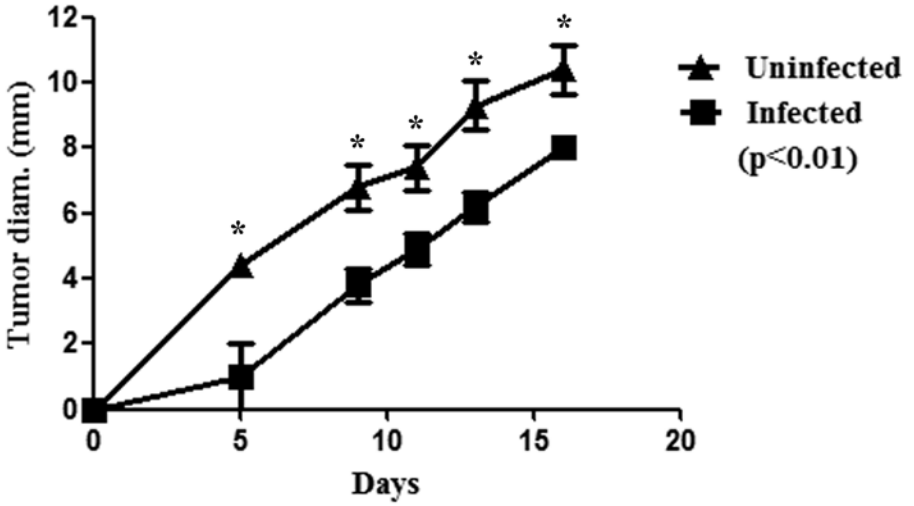

Figure 2.5: *Osteomyelitis associated tumor growth inhibition is not pathogen specific. Pseudomonas aeruginosa* biofilm-impregnated suture was implanted three days prior to challenge with DLM8 cells. Tumor growth was assessed as previously described and is reported as mean ± SEM. Infected mice had a significantly ($p<0.01$; $*p<0.01$) decreased tumor growth compared to uninfected mice as assessed by repeated measures two-way ANOVA.

Infection is Associated with Inhibition of Tumor Angiogenesis

Previous studies have shown that tumor angiogenesis was inhibited following infection with *Toxoplasma gondii* [29]. To determine whether localized bacterial osteomyelitis was capable of inhibiting tumor angiogenesis, we assessed tumor microvessel density (MVD) in tumors from infected and control mice. Tumor sections from control and *Staphylococcus* infected mice (n=5 per group) were analyzed using CD31 immunohistochemistry (**Figure 2.6**). We observed a significant decrease ($p<0.01$) in tumor MVD in infected mice compared to the uninfected mice. A similar decrease in tumor $CD31^+$ endothelial cells was also noted when collagenase-digested tumor tissues were analyzed by flow cytometry (data not shown).

Recent studies indicate that tumor angiogenesis results from two independent processes; local sprouting of tumor vessels and the seeding of tumor tissues by circulating endothelial progenitor cells [30, 31]. Therefore, we used flow cytometry to assess the effects of osteomyelitis on circulating endothelial cells (CEC) in tumor-bearing mice. Using multicolor flow cytometry, we evaluated $CD45^{-}CD11b^{-}CD3^{-}CD31^{+}LDS\text{-}751^{+}$ CEC in the blood of tumor bearing infected and control mice (**Figure 2.6**). We found no significant difference ($p>0.05$) in CEC in infected mice with tumors compared to control mice with tumors. These results suggested that the inhibition of angiogenesis elicited by localized osteomyelitis was not mediated by suppressing the number of CEC.

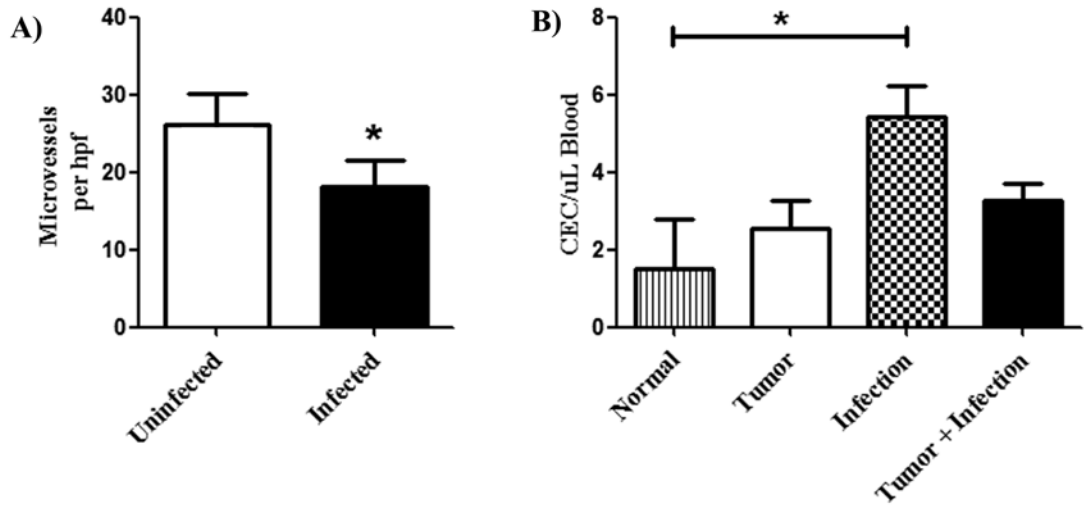

Figure 2.6: *Osteomyelitis inhibits tumor angiogenesis by a CEC independent mechanism.* A) Tumors from infected and uninfected mice were flash frozen in OCT and cut into 4 μm sections for CD31 immunohistochemistry. Microvessel density was significantly (*$p<0.01$) decreased in the infected mice compared to the uninfected mice by t-test. Results are representative of two independent experiments. B) CECs were assessed by flow cytometry as described in the Methods. The mean (± SEM) number of CEC per μL of blood was calculated and compared by one-way ANOVA with Bonferroni post-test. CECs from infected-tumor-bearing mice were not significantly ($p>0.05$) different from those of tumor bearing mice. An increase in CECs was observed in mice only challenged with infections (*$p<0.05$), but not significantly compared to tumor only or infected mice with tumors ($p>0.05$).

Suppression of Tumor Growth by Infection is Dependent in Part on NK Cells

Prior studies have shown that IFN-γ plays a prominent role in the control of tumor growth elicited by infectious agents [3, 4, 29, 32]. Since conventional NK cells are a major source of IFN-γ following bacterial and protozoal infection, we investigated the role that NK cells played in mediating tumor growth inhibition in the bacterial osteomyelitis model. To address this question, we depleted NK cells *in vivo* using a depleting antibody (anti-asialo GM1), as previously reported [23]. Mice with chronic osteomyelitis were depleted of NK cells three days post injection of tumor cells and weekly thereafter. This treatment resulted in 73% depletion of $NKG2D^{+}CD3^{-}$ cells in the spleens of treated mice **(Figure 2.7)**. Tumor growth rates were compared to untreated mice with infection and uninfected tumor bearing mice **(Figure 2.7)**. NK cell depletion in infected mice with tumors led to a significant increase in tumor growth compared to untreated infected mice with tumors. However, NK depletion did significantly alter the intensity of *Staphyloccus* infection at a single day **(Figure 2.7)**. Thus, NK depletion reversed the tumor inhibitory effects of chronic bone infection. In contrast, NK depletion of uninfected mice did not significantly alter tumor growth **(Figure 2.7)**. These results indicated that NK cells were major mediators of the inhibition of tumor growth observed following bone infection.

Chronic Osteomyelitis Increases Inflammatory Monocytes in Infected Mice

The preceding experiments indicated that chronic osteomyelitis activated innate immunity, as evidenced by the anti-tumor activity conferred by NK cells. Therefore, we next investigated the effects of osteomyelitis on the mobilization of monocytes in tumor-

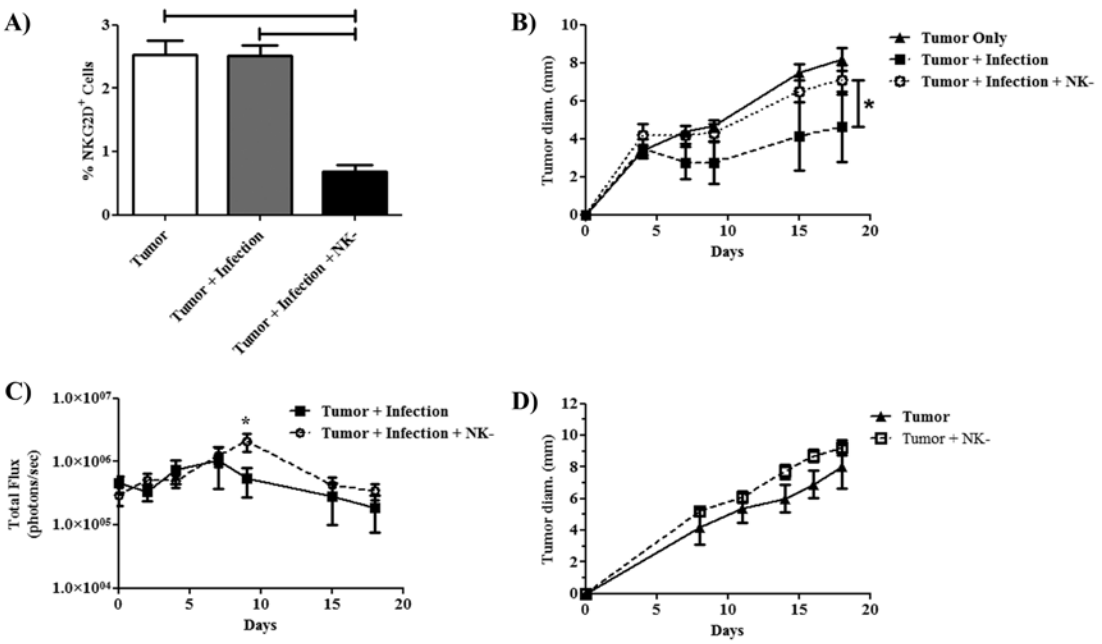

Figure 2.7: *NK depletion reverses tumor growth inhibition conferred by osteomyelitis.* Mice (n=5 per group) were challenged with infections and tumors as previously described. Three days after tumor challenge, mice were treated with an anti-asialo GM1 antibody administered IP to deplete NK cells, and treatment was repeated weekly. A) There was a significant decrease in NKG2D+ cells by flow cytometric analysis of spleens at sacrifice ($p<0.05$). B) There was significant ($*p<0.05$) growth inhibition in the infected tumor bearing mice when compared to the uninfected mice and the NK-depleted infected mice as measure by repeated measures one-way ANOVA with Bonferroni post-test. C) There was a significant difference in infection intensity between infected mice receiving the anti-asialo GM1 antibody and those not ($p<0.05$) at a single point as measured by repeated measures two-way ANOVA. D) NK-depleted infected mice did not have a significantly ($p>0.05$) different tumor growth when compared to tumor only mice. Results are representative of two independent experiments.

bearing mice. Previous studies have reported conflicting results for the role of monocytes in tumor growth, suggesting that they may either suppress or promote tumor growth [33, 34]. Therefore, we assessed the effects of bone infection on circulating steady state and inflammatory monocytes in the blood and spleen of mice [34-37]. Steady state monocytes were identified as $Ly6G^{-}CD11b^{+}CD115^{+}Ly6C^{lo}$, while inflammatory monocytes were identified as $Ly6G^{-}CD11b^{+}CD115^{+}Ly6C^{hi}$. Inflammatory monocytes

have been found to be more likely to differentiate into activated macrophages with an anti-tumor phenotype than steady state monocytes [38, 39].

Total monocytes were significantly ($p<0.05$) increased in the circulation of mice with osteomyelitis compared to uninfected mice **(Figure 2.8)**. The numbers of steady state monocytes in the blood were not increased, whereas there was a significant ($p<0.001$) increase in inflammatory monocytes in infected animals **(Figure 2.8)**. A significant ($p<0.05$) increase in the number of inflammatory monocytes in the spleen was also observed **(Figure 2.8)**. These data are most consistent with the idea that osteomyelitis induces an inflammatory response that results in the mobilization of circulating inflammatory monocytes. This is important because these inflammatory monocytes may serve as a source of macrophages in tumor tissues.

Tumor Associated Macrophages are increased in Mice with Chronic Osteomyelitis

Next, we examined numbers of tumor associated macrophages (TAM) and how chronic osteomyelitis affected their numbers. Although TAM are generally thought to promote tumor growth, there are situations where activated TAM may suppress tumor growth [40-42]. We used both flow cytometry and immunohistochemistry to assess numbers of TAM in tumor tissues. Mice (n=5 per group) with osteomyelitis had twice as many $CD11b^{+}F4/80^{+}Gr\text{-}1^{-}$ cells in tumor tissues as uninfected mice by multi-color flow cytometry **(Figure 2.9)**. By immunohistochemistry, there was a significant ($p<0.02$) increase in $CD68^{+}$ TAM in mice with osteomyelitis compared to uninfected tumor-bearing mice **(Figure 2.9)**. These findings suggested that accumulation of TAM in this chronic infection model was associated with inhibition of tumor growth.

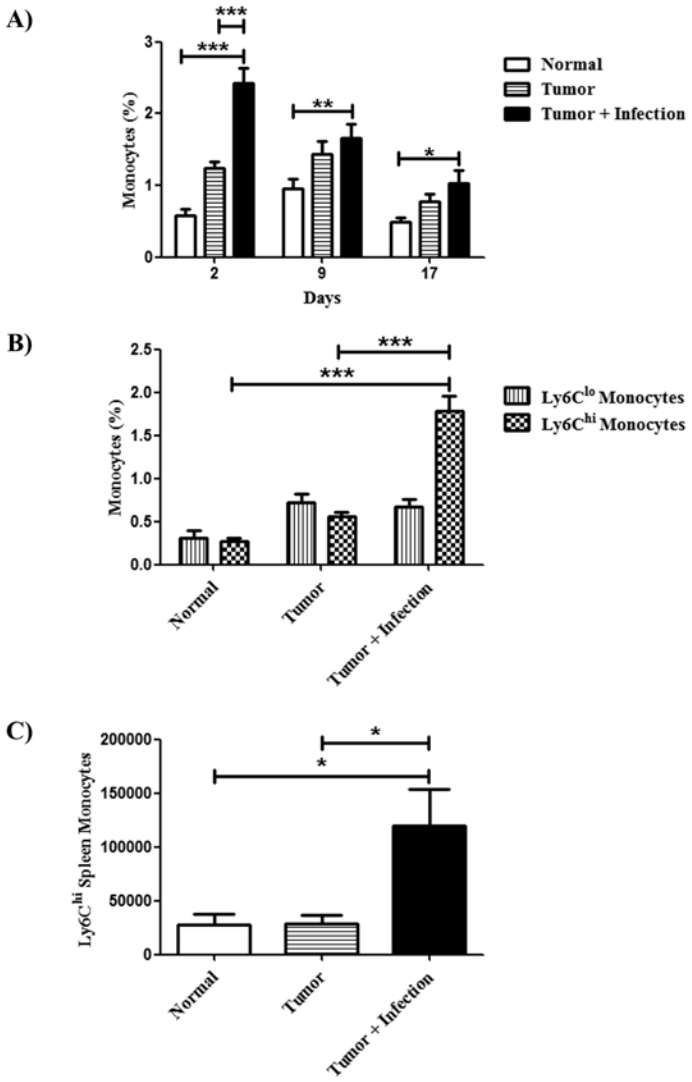

Figure 2.8: *Localized osteomyelitis induces monocytes in the blood and spleen.* Mice (n=5 per group) were challenged with *S. aureus* osteomyelitis and tumors as previously described. A) Mice were tail bled on days 2, 9, and 17 post-tumor challenge. Samples were processed and analyzed by multi-color flow cytometry as described in the Methods and means (± SEM) calculated for each group. There were significant (***$p<0.001$, **$p<0.01$, *$p<0.05$) increases in total blood monocytes at the bracketed measures by two-way ANOVA with Bonferroni post-test. B) Analyses from the day two bleed are further characterized showing a significant increase (***$p<0.001$) in $Ly6C^{hi}$ monocytes in infected mice with no difference ($p>0.05$) in $Ly6C^{lo}$ monocytes between groups. C) Mice were euthanized on day 21 and spleens were analyzed by flow cytometry as described in the Methods. There was a significant increase (*$p<0.05$) in the number of $Ly6C^{hi}$ monocytes present in the spleen measured by one-way ANOVA with Bonferroni post-test. Results are representative of three independent experiments.

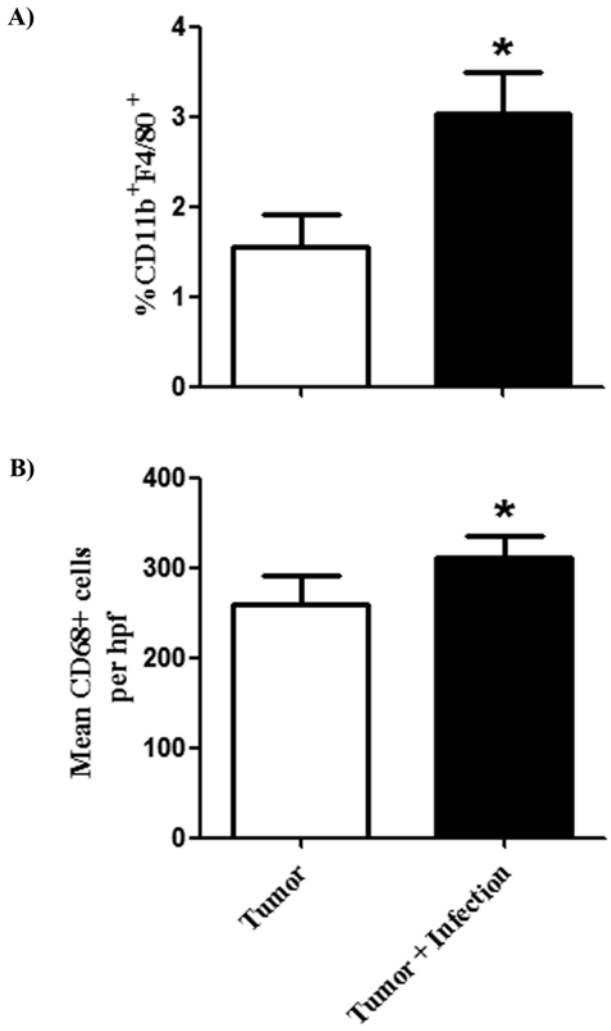

Figure 2.9: *Macrophages are induced in infected, tumor bearing mice.* Tumor-bearing mice (n=5 per group) were sacrificed when the first control mouse reached a maximal tumor diameter of 10mm. Tumors were removed and homogenized for analysis by flow cytometry or prepared for IHC as described in the Methods. A) There was a significant increase (*$p<0.05$) in the number of $CD11b^{+}F4/80^{+}$ macrophages in the tumors of infected mice compared to uninfected mice. B) There was a significant increase in the number of $CD68^{+}$ cells per 20X high power field (hpf) in the tumor tissue of infected mice compared to the uninfected mice (*$p<0.02$). Results are representative of two independent experiments and two-tailed t-tests were used to determine significance.

Tumor Associated Macrophages Are Not Directly Cytolytic to Tumor Cells

To assess the cytotoxic capabilities of TAM, the TAM were sorted using flow cytometry from tumors of uninfected and infected mice (n=5 per group) and used in an *in vitro* cytotoxicity assay, with DLM8 tumor cells as targets. We did not observe spontaneous cytotoxic activity from either population of TAM and there was no significant ($p > 0.05$) difference in cytotoxicity between the two groups of mice **(Figure 2.10)**. As a positive control, we did note however that significant anti-tumor cytotoxic activity was exerted by IFN-γ stimulated peritoneal exudate macrophages **(Figure 2.10)**. These results therefore indicate that infection induced cytotoxic activity of TAM was unlikely to account for the tumor growth inhibition observed in the infected mice with tumors.

Monocyte and Macrophage Depletion Reverses Tumor Inhibition by Chronic Osteomyelitis

Finally, to specifically address the antitumor role of increased inflammatory monocytes and TAM in infected mice, we conducted monocyte/macrophage depletion experiments. Depletion was accomplished by i.v. injection of liposomal clodronate (LC), which has been previously reported to efficiently deplete both monocytes and macrophages [24-26, 43]. Mice with established osteomyelitis and tumors were treated weekly with LC administered i.v. Treatment with LC resulted in a 71% depletion of blood monocytes as assessed by flow cytometry **(Figure 2.11)**.

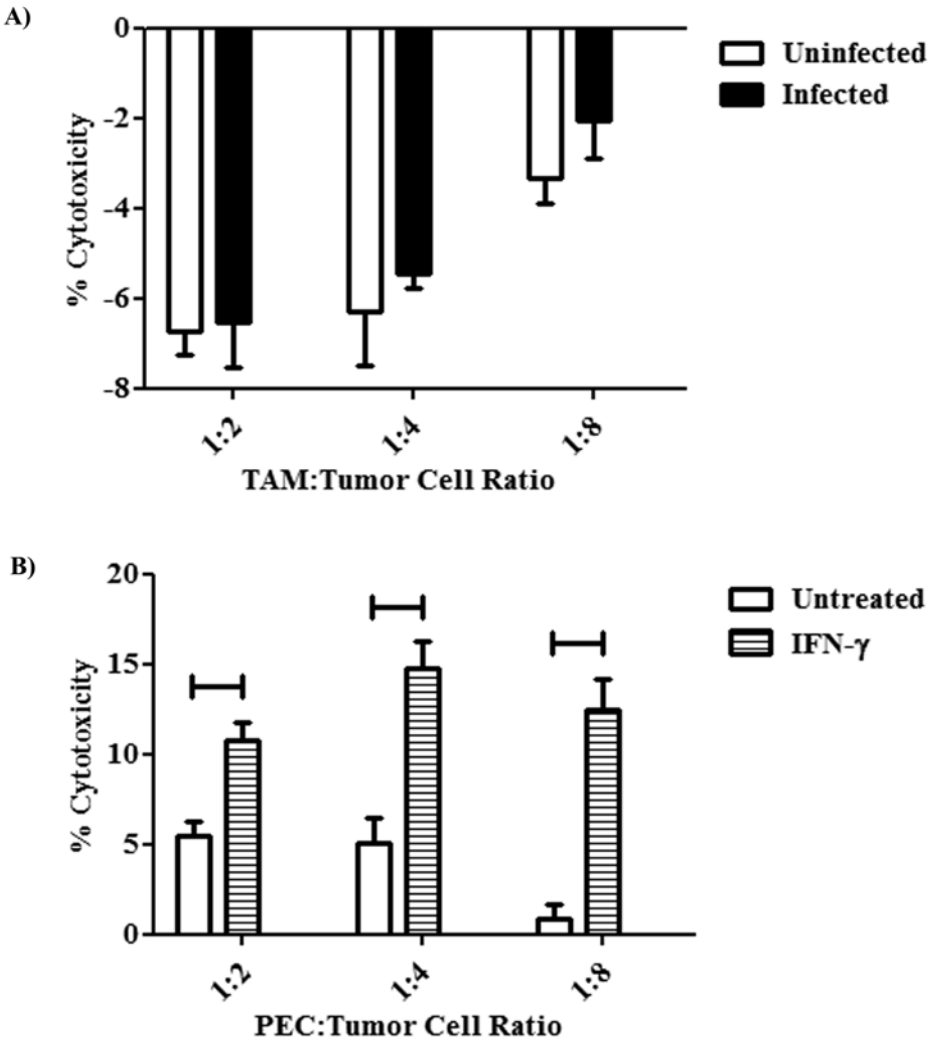

Figure 2.10: *TAM from infected mice are not cytotoxic to tumor cells.* A) Mice were infected and challenged with tumors as previously described. At sacrifice, tumors were digested and pooled within groups before TAM were sorted as described in the Methods. Sorted TAM were mixed with DLM8 tumor cells at various ratios and analysis of LDH release was performed. TAM from infected mice were not significantly ($p>0.05$) cytotoxic to tumor cells. B) Thioglycolate elicted PEC activated with IFN-γ (20 mg/mL) were significantly ($p<0.05$) cytotoxic at identical ratios as that of TAM.

We observed that treatment of infected mice (n=5 per group) with LC significantly increased tumor growth compared to tumors in infected mice not treated with LC **(Figure 2.11)**. Thus, monocyte/macrophage depletion by LC appeared to

reverse the antitumor effects of bone infection. In fact, tumor growth in infected mice treated with LC was similar to that of mice without infection. As a control, we found that treatment of infected tumor-bearing mice with control PBS liposomes did not have a significant impact on tumor growth compared to untreated mice with infections **(Figure 2.11)**. There was no significant alteration in infection intensity associated with LC treatment of infected mice **(Figure 2.11)**.

These results suggested that monocytes and macrophages elicited by chronic bone infection were in fact responsible for a significant degree of tumor growth inhibition. However, in a number of tumor models, we and others have observed that treatment of uninfected tumor-bearing mice with LC significantly inhibits tumor growth (Guth, AM; manuscript in preparation, [44-46]). Therefore, the results of LC depletion studies in our model suggested that substantial antitumor activity was elicited by activation of circulating inflammatory monocytes and their recruitment into tumor tissues.

Discussion

The ability of bacterial infections to suppress tumor growth was noted over a century ago by Dr. William Coley and provided the basis for modern day immunotherapy [1]. However, the role that systemic inflammation elicited by chronic bacterial infection plays in controlling tumor growth has yet to be well defined. Therefore, we developed a model of chronic bacterial osteomyelitis to investigate the reported ability of chronic bone infection to control the growth of osteosarcoma metastases in dogs and humans [18,

19]. Others have studied the role that localized tumor infection plays on tumor growth suppression; however, the role of systemic inflammation in mediating these tumor inhibitory effects has not been incompletely described [6, 8, 47, 48].

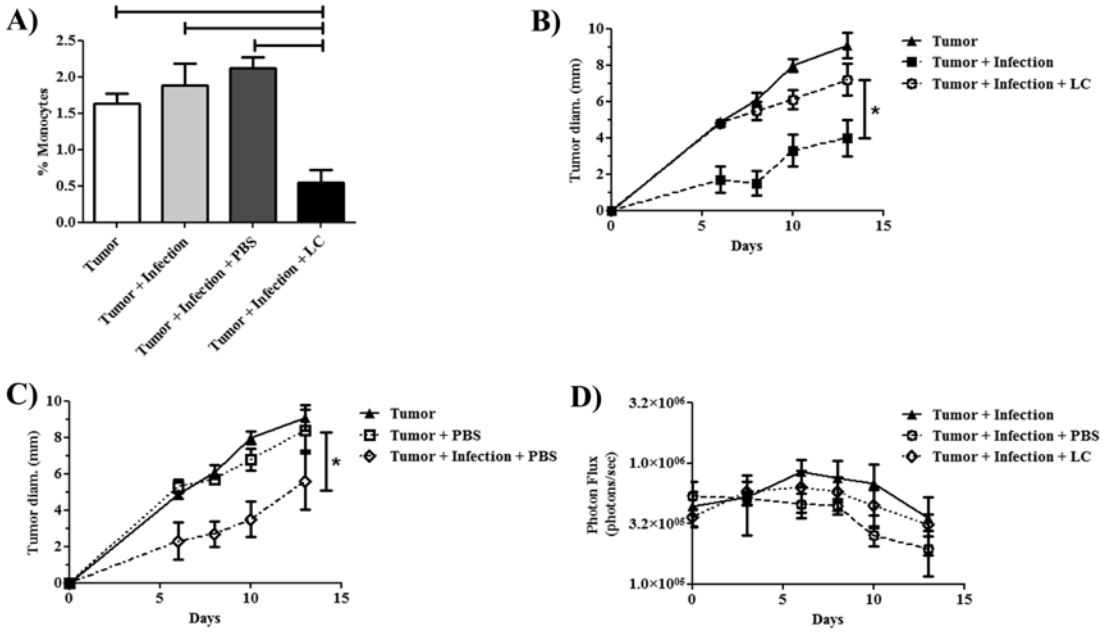

Figure 2.11: *Monocyte depletion abolishes the inhibitory effects of osteomyelitis on tumor growth.* C3H mice (n=5 per groups) were infected and challenged with tumors as described in the methods. Mice were administered liposomal clodronate (LC) or PBS control liposomes (PBS) IV 3 days after tumor challenge and then weekly thereafter. A) Flow cytometry was performed to determine the number of monocytes in circulation as described in the Methods. There was a significant ($p<0.05$) depletion of monocytes in the mice receiving LC compared to all other groups as determined by one-way ANOVA with Bonferroni post-test. B) Infected mice had significantly decreased tumor growth (*$p<0.05$) when compared to uninfected mice and infected mice receiving LC by repeated measures one-way ANOVA with Bonferroni post-test. There was no statistical difference ($p>0.05$) between infected mice treated with LC and uninfected tumor bearing mice. C) Infected mice receiving control PBS liposomes had a significant growth inhibition whereas uninfected mice did not. D) Administration of PBS or clodronate liposomes did not have a significant ($p>0.05$) effect on infection intensity.

In our infection model, we did not observe any bacterial dissemination from the site of infection, leading us to conclude that localized infection of the tumor tissues themselves did not occur. We also noted significant tumor growth inhibition in 3 different mouse strains, 3 different tumor types, and with 2 types of bacterial organisms, suggesting that the antitumor effect was non-specific and most likely due to systemic activation of innate immune responses. However, significant growth inhibition was not observed when the tumor challenge preceded the infection. This result might be explained by the rapid and aggressive growth of the tumor lines used in these studies and the amount of time required for infection to peak following initiation.

A previous study has found that systemic infection with the protozoan *Toxoplasma gondii* was capable of inhibiting tumor angiogenesis [29]. In our studies, we observed tumor inhibition and decreased tumor microvessel density following the development of *Staphylococcus* osteomyelitis, the most prominent bacterial species identified in dogs developing osteomyelitis following limb salvage surgery [18]. To better understand the nature of the anti-angiogenic effects of bone infection, we investigated the impact of infection on CEC. We observed that CEC were not decreased in tumor bearing mice with infections, leading us to conclude that CEC changes are not likely responsible for the inhibition of angiogenesis observed by conventional CD31 staining of tumor tissues.

Previous studies have found that IFN-γ suppresses angiogenesis, and is responsible for inhibition of tumor growth in the presence of *Toxoplasma* infection [4, 29, 32, 49, 50]. Since NK cells are the major innate immune source of IFN-γ production, we depleted NK cells to identify the role these cells play in tumor inhibition. We found

that depletion of NK cells reversed the protection associated with infection, indicating that NK cells were critical for inhibition of tumor growth. Others have shown that NK cell production of IFN-γ was the mechanism responsible for tumor growth control, rather than direct NK cell cytotoxicity [29]. We thus conclude that NK cells are important mediators of tumor growth control associated with osteomyelitis.

To better describe the systemic effects of infection, leukocyte changes in blood were assessed. A significant increase in inflammatory monocytes was observed in both the blood and spleen of mice with osteomyelitis. This result suggested a role for inflammatory monocytes in the inhibition of tumor growth, which the LC depletion studies confirmed. However, we cannot exclude a role for other cell types depleted by LC treatment, including macrophages and dendritic cells.

The phenotypic plasticity of monocytes and their derivatives allows them to have both pro and anti-tumor phenotypes dependent on the stimuli present in the tumor microenvironment [40-42, 51]. We propose that increasing the number of inflammatory monocytes in circulation inhibits tumor growth by repopulating the tumor with activated TAM [24, 52, 53]. Thus, activated TAM are more likely to inhibit tumor growth than to stimulate. Furthermore, we did not observe increased direct cytolysis of tumor cells by TAM sorted from tumors of infected mice, suggesting that tumor growth inhibition was likely mediated by other macrophage activities. Other groups have shown that NK cells and monocytes can activate each other, thus leading to an activated state which may be responsible for tumor inhibition [54-56].

In conclusion, we have shown here that bacterial osteomyelitis induces significant non-specific tumor growth inhibition. The tumor growth inhibition observed following

bone infection appeared to be mediated by NK cells, inflammatory monocytes, and TAM. Thus, systemic inflammation is a likely mechanism that explains the inhibition of tumor growth and metastasis observed in canine and human patients with osteosarcoma, and leads to the inhibition of metastasis and increased survival in this population. It is also plausible that sustained low-level inflammation may be an important mediator of tumor growth inhibition that could be applied to the clinical management of cancer.

Future Directions

In this chapter we have explored the mechanism by which osteomyelitis can systemically inhibit tumor growth. However, greater characterization of the monocyte/macrophage tumor infiltrate could be performed to greater understand the changes present in this environment. I hypothesize that these macrophages will be found to have what is considered a classical, or M1 type activation. We have shown that these macrophages are not cytotoxic, but were not able to further classify these cells. Preliminary experiments were performed to characterize these cells bys IHC, but the results of these tests were inconclusive. It would be plausible to sort cells as was done in this chapter and try to characterize these cells using RT-PCR, microarray, or *in vitro* cytokine release by CBA assay. These tests can investigate if the infiltrating macrophages have an anti-tumor disposition by being anti-angiogenic or immunostimulatory. The PCR and microarray can be used to determine if changes in these specific cells take on these characteristics, while the CBA assay may provide

evidence for factors released by these cells that may inhibit tumor growth but are not directly cytotoxic. These experiments will provide further description of the model and facilitate an understanding of the mechanism of action and provide information that may be duplicated in a therapeutic setting so as to illicit similar effects without an infectious agent.

The growth of literature concerning myeloid derived suppressor cells also provides ample evidence that these cells may have a modulatory effect in this model [57]. We hypothesize that MDSC will be decreased in infected mice compared to untreated tumor bearing mice. MDSC have been found to be increased in tumor bearing animals, and it is plausible that this effect may be reversed due to the systemic immune activation provided by the infection [57]. A decrease of MDSC may lead to decreased systemic and local immune inhibition and thus inhibited tumor growth. Induction of osteomyelitis and determination of changes in circulating and resident MDSC (spleen) can be performed. If differences are appreciated further characterization can be performed through cell sorting of these populations from the respective groups. Sorting of these cells and *in vitro* characterization of elicited cytokines would be of importance. Since MDSC are viewed as immature monocytes and neutrophils, it would be interesting to see if these cells preferentially differentiate based upon the presence of differing environment. For example, MDSC may be sorted from a naïve mouse and placed in culture. Serum from infected and non-infected tumor bearing mice could then be added to the isolated MDSC and characterization of the cells performed after incubation. Differences in maturation and differentiation may suggest an *in vivo* mechanism that could then be investigated.

The importance of MDSC was not investigated in this chapter, but the interactions of these cells are plausible mediators for the inhibition of tumor growth observed.

This chapter primarily focused on the innate immune regulation afforded by the infection, and did not investigate the role of the adaptive immune system. However, we hypothesize that T cells do not play a significant role in this model. During this study, we did not observe differences in T cell numbers in the tumor itself or the tumor draining lymph node using flow cytometry and IHC; this does not speak to the activity of these cells, but only the relative number. To test this hypothesis, nude mouse models could be used to better understand this phenomenon. Infected and uninfected nude mice of an identical background could be compared to infected and uninfected wild-type immune competent mice. If T cells play a role, then infected mice with tumors should have tumor growth similar to that of non-infected mice. If this is the case, further description will need to be performed by selectively depleting CD4 and CD8 T-cells using antibody mediated depletion or genetically altered mice. If T-cells are found to be involved, it would suggest that the infection could be a significant adjuvant in order to activate anti-tumor T cells. However, if T-cells are not found to play a role, it would suggest that the innate immune system and mechanism described herein is suitable to form an anti-tumor response on its own.

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Chapter Three

Induction of VEGF by Tepoxalin Does Not Lead to Increased Tumor Growth in a Canine Osteosarcoma Xenograft

Abstract

The purpose of this study was to determine the impact of the non-steroidal anti-inflammatory drug tepoxalin on canine tumor cell growth and describe the changes associated with tepoxalin treatment. *In vitro* experiments were performed to assess tepoxalin-associated alterations in tumor cell growth. Vascular endothelial growth factor (VEGF) production and hypoxia-inducible factor-1α (HIF-1α) expression were studied in the presence of tepoxalin. A canine osteosarcoma xenograft model was used to determine *in vivo* effects of tepoxalin on tumor growth and angiogenesis. Immunohistochemical analysis of the tumor infiltrate was performed to characterize changes in the tumor environment. Normal dogs were administered tepoxalin to assess effects on systemic VEGF production. Clinically achievable tepoxalin concentrations did not significantly alter tumor cell growth *in vitro*. Tepoxalin dose-dependently increased

VEGF production *in vitro* through an increase in HIF-1α. Despite increased VEGF *in vitro*, there was a significant growth delay associated with tepoxalin treatment of osteosarcoma xenografts. Tepoxalin treated normal dogs had variable increases in circulating VEGF. In conclusion, tepoxalin did not exacerbate tumor growth in a canine osteosarcoma xenograft, despite induction of VEGF *in vitro* by a HIF-1α dependent mechanism.

Introduction

Cancer is one of the primary causes of death of companion animals. The incidence of cancer in dogs increases with age, as do other illnesses such as osteoarthritis [1, 2]. Typically, older dogs may receive multiple medications, including non-steroidal anti-inflammatory drugs (NSAIDs). The NSAID tepoxalin (Zubrin®, Schering-Plough Animal Health) is approved in dogs for the treatment of osteoarthritis. Tepoxalin acts through the dual inhibition of cyclooxygenase-1/2 (COX-1/COX-2) with inhibition of 5-lipoxygenase (5-LOX) [3]. This multifocal block of leukotriene and prostanoid synthesis acts to inhibit inflammation and provide analgesia [3, 4]. Since many older dogs undergoing treatment for osteoarthritis are at risk for cancer, it was our goal to determine what the anti-inflammatory effects of tepoxalin were on tumor growth.

Inflammation is a complex process encompassing a number of pathways and interactions, each of which varies based upon the local tissue environment, cytokine milieu, and infiltrating cells. The cyclooxygenase (COX) enzymes, particularly COX-1 and COX-2, are regulators of inflammation through the production of leukotrienes and prostaglandins which can promote inflammation [3]. The induction of COX-2 has been seen as a possible target for a range of diseases including Alzheimer's disease, arthritis, and cancer [5-9]. Differential NSAID-associated side effects can occur due to the specific inhibition of COX-1 versus COX-2. [10, 11]. Leukotriene synthesis by 5-lipoxygenase (5-LOX) has also been characterized as a potential target for cancer since the inhibition of inflammatory mediators and suppressed cellular proliferation can lead to tumor growth inhibition [12-14].

Tepoxalin is an efficacious analgesic in dogs owing to its anti-inflammatory effects, and has a favorable gastrointestinal and renal toxicity profile when compared to other drugs [4, 15-20]. Tepoxalin's anti-inflammatory activity is through non-selective inhibition of COX-1/COX-2 and 5-LOX activity leading to an inhibition of NF-κB activity via inhibited degradation of IκBα, resulting in a decrease in prostaglandin and leukotriene synthesis [5, 21]. Tepoxalin has also been observed to inhibit lymphocyte proliferation due to its ability to chelate iron, which may be the mechanism leading to the inhibition of NF-κB [22, 23]. It is this inhibition of NF-κB, in addition to its COX-1 and COX-2 inhibitory activity, which may promote tepoxalin as a unique anti-tumor compound. The pharmacologic inhibition of COX-2 may inhibit diverse oncogenic functions including proliferation, angiogenesis, inhibition of apoptosis, and immune suppression [14, 24]. These COX-2 dependent effects in combination with inhibition of NF-κB may lead to suppression of tumor growth.

Folkman and others have described the necessity of functional vasculature as a requirement for tumor growth and progression [25-27]. The promotion of angiogenesis occurs due to the interactions of a variety of mediators, one of the most prominent and potent of which is vascular endothelial growth factor (VEGF) [28]. The production of VEGF is partially controlled by the transcription factor hypoxia inducible factor 1-alpha (HIF-1α), which is stabilized and thus more active in the presence of hypoxia [29, 30].

We show here that tepoxalin does not significantly alter canine tumor cell growth *in vitro* at clinically achievable concentrations. However, tepoxalin's unique structure, which allows it to chelate iron, leads to increased HIF-1α protein concentration and subsequent induction of VEGF in canine tumor cells and in normal dogs. However,

tepoxalin inhibited tumor growth in a canine osteosarcoma xenograft suggesting that anti-tumor effects not observed by *in vitro* growth inhibition dominate over potential pro-tumorigenic effects from increased VEGF production.

Materials and Methods

Cell Lines and Drugs

The canine cell lines utilized for these experiments are as follows: B cell lymphoma (1771), mast cell tumor (C2), melanoma (17CM98 and CML-6M), mammary carcinoma (CMT-12 and CMT-27), hemangiosarcoma (DEN-HSA and Fitz-HSA), bladder carcinoma (Bliley and K9TCC), and osteosarcoma (Abrams and D17). The murine macrophage cell line AMJ was also used. Cell lines were maintained in C/10 [minimal essential medium (MEM) (Lonza, Walkersville, MD) supplemented with 1X MEM vitamin solution (Cellgro, Henderson, VA), 2 mM L-glutamine (Cellgro), 1 mM sodium pyruvate (Cellgro), 1X non-essential amino acid solution (Cellgro), 1X antibiotic/antimycotic (Cellgro), 5% heat inactivated fetal bovine serum (FBS) (Atlas, Fort Collins, CO), and 5% heat inactivated newborn calf serum (Hyclone, Logan, UT)]. Once confluent, cells were washed with 1x phosphate-buffered saline and detached with 0.25% trypsin (Cellgro) supplemented with 0.5 mM EDTA.

Tepoxalin and its primary active metabolite, RWJ20142, were generously provided by Schering-Plough Animal Health (Kenilworth, NJ) [31].

Cell Growth Inhibition

Cell lines were plated in 96-well plates in C/10 at a density of 3,000 cells per well in triplicate and then incubated overnight at 37°C and 5% CO_2. Medium was aspirated and replaced with varying concentrations of tepoxalin and RWJ20142 spanning peak and trough plasma concentrations achievable in dogs following administration of pharmaceutical grade tepoxalin [31, 32][1]. For experiments evaluating the interactions of tepoxalin ± RWJ20142 and doxorubicin, all drugs were plated concurrently or doxorubicin was added 24 hours before or after treatment with tepoxalin ± RWJ20142. Doxorubicin was serially diluted by 4-fold dilutions in the presence of peak and trough concentrations of tepoxalin ± RWJ20142. For drugs that could not be directly dissolved in media, they were first dissolved in dimethyl sulfoxide (DMSO, Sigma), and then dissolved in media. For these experiments, DMSO at the highest concentration was used in the control wells. Cells were then incubated at 37°C and 5% CO_2 for 72 hours. At the end of this incubation period, relative viable cell number was determined using a bioreductive colorimetric assay (CellTiter Blue, Promega, WI) according to manufacturer directions, and absorbance determined using a Synergy HT plate reader (Bio-TEK, Winooski, VT). Relative viable cell number was then expressed as a percentage of control-treated cells. Each experiment was repeated a minimum of three times and mean (± SEM) calculated.

Western Blot Analysis

Cells were grown to near confluence before being treated with clinically relevant peak (600 ng/mL) and trough (100 ng/mL) plasma concentrations of tepoxalin in C/10,

[1] Further information available in package insert.

determined from prior pharmacokinetic experiments in normal dogs, or 100 μM desferrioxamine (DFO; Sigma-Aldrich, St. Louis, MO) as a positive control. Cells were incubated for 12 hours followed by whole cell lysis using an SDS based lysis buffer; 2% SDS combined with 1 complete mini tab protease inhibitor (Roche, Indianapolis, IN), 1 mM sodium orthovanadate, and 1 mM PMSF in 7 mL of M-PER (Thermo Scientific, Rockford, IL). Protein quantification was performed using a CBA kit (Thermo Scientific) with a NanoDrop 1000 microspectrophotometer (Thermo Scientific). Equal amounts of protein were loaded on a 10% TBE gel (Invitrogen, Eugene, OR) and electrophoresed in 1x MOPS/SDS running buffer (Teknova, Hollister, CA) for 1.25 hours at 100 mA. Bands were then transferred to a PVDF membrane by electrophoresis at 170 mA for 1 hour followed by 115 mA for 15 minutes. Blocking was performed using 5% instant milk in TBST (Tris-buffered saline, 1.37 M NaCl, 0.2 M Tris-base, and 8.96 mM Tween 20 in DI water) for one hour. Primary antibodies used were a rabbit polyclonal antibody to HIF-1α (Novus Biologicals, Littleton, CO) at a dilution of 1:500, and a rabbit polyclonal anti-β-actin (AbCam, Cottonwood, AZ) at a dilution of 1:2000. Primary antibodies were incubated overnight at 4°C. The membrane was rinsed in 1x TBST three times. A goat anti-rabbit secondary antibody (Thermo Scientific) was used against both primaries at a 1:1000 dilution, and incubated at room temperature for two hours. Detection was accomplished by exposing the membrane to ECL reagent (SuperSignal West Pico, Thermo Scientific) and development using radiographic film (Kodak, Rochester, NY).

In Vitro VEGF Production

Cells were plated in C/10 at a concentration of 250,000 per well in a 6-well plate and allowed to incubate overnight at 37°C and 5% CO_2. The supernatants were removed, and the cells treated with 100 or 600 ng/mL of tepoxalin in complete MEM with 0.1% FBS; cells were then incubated overnight. Supernatants were then collected from each of the treatment wells and stored at -20°C until measurement of VEGF using a canine-specific ELISA (R&D systems, Minneapolis, MN).

Pharmacokinetic Analysis

All animal studies were performed in an AALAC-approved facility, with approval of the Colorado State University Institutional Animal Care and Use Committee. 6-8 week old nu/nu mice were obtained from the National Cancer Institute (Bethesda, MD). After a 1-week acclimation period, tepoxalin was administered via oral gavage or subcutaneous injection at 40 mg/kg, and non-tumor bearing mice were terminally bled at specified time points following administration. Plasma was prepared for LC/MS/MS, and analyzed similarly to Burinsky et al. with minor changes [33]. Briefly, 5 ng of internal standard (trazadone) was added to mouse plasma. Tepoxalin and the active metabolite were isolated via organic extraction by the addition of 2 mL ethyl acetate. Samples were vortexed, centrifuged (3200 RCF for 10 min), and the organic layer collected and evaporated to dryness. The samples were then reconstituted in 60% of 10 mM ammonium acetate with 0.1% AcOH: 40% tetrahydrofuran (THF), transferred to HPLC vials and analyzed with an ABI-3200 triple quadrapole mass spectrometer (Applied Biosystems, Foster City, CA) with a TurboIonSpray source interfaced to an

Agilent 1200 Series Binary Pump SL (Agilent Technologies, Waldbronn, Germany). Samples were chromatographed using a 100 Å Phenomenex Luna C18 5 µm, 2 X 50 mm column maintained at 40°C with gradient elution at a flow rate of 200 µL/min as follows: 40% THF for 0.5 min; a linear increase to 70% THF from 0.5 to 6 min; THF held at 70% for 0.5 min; linearly adjusted from 70% to 40% from 6.5 min to 7 min; with a final equilibration at 40% THF for 1 min. Samples were quantified by internal standard reference in multiple reactions monitoring mode by monitoring the transitions *m/z* 386.0 → 339.0 for tepoxalin, 357.0 → 339.0 for RWJ20142 and 372.0 → 176.0 for the internal standard (trazodone).

<u>In Vivo</u> Tumor Growth

To determine the effects of tepoxalin on *in vivo* tumor growth, 6-8 week nu/nu mice (National Cancer Institute) were injected with Abrams osteosarcoma (OSA) cells ($2x10^6$) subcutaneously in the rear flank. Three days post-tumor challenge, mice (n=5 mice per group) were randomized to 1 of 3 treatment groups: tepoxalin dissolved in DMSO at a dose of 20 mg/kg subcutaneously, administered five consecutive days per week followed by a two-day drug holiday, continuously throughout the study; DMSO vehicle administered similarly; or no treatment. Bi-dimensional tumor measurements were obtained at least twice weekly using calipers. Tumor volume was calculated using the formula $V = (S^2 \cdot L)/2$, where 'L' is the longest dimension measured and 'S' is the perpendicular measurement. Mice were sacrificed when the first mouse in the control group reached a maximal tumor diameter of 15 mm. Throughout this time, peripheral blood was obtained via the lateral tail vein weekly to assess changes in neutrophils,

monocytes, and circulating endothelial cells (CEC). Plasma was prepared from blood and VEGF concentration was measured using murine and canine VEGF ELISAs (R&D Systems). At the time of sacrifice, tumors were placed into OCT embedding matrix (Sakura, Torrance, CA) for immunohistochemical analysis. Terminal phlebotomy was performed and spleens were processed for analysis by flow cytometry.

Flow Cytometry

Flow cytometry of peripheral blood was performed by lysis of blood using ACK lysis buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA in 1x PBS). Cells were then stained with primary antibodies in two panels to determine changes in CEC (CD31, CD45, and LDS-751, a nuclear stain) and neutrophils/monocytes (CD11b, CD115, F4/80, Ly6G, and Ly6C). CD31-FITC, CD45-PE, CD11b-PB, CD115-PE, and F4/80-APC were purchased from eBioscience (San Diego, CA). LDS-751 was purchased from Invitrogen. Ly6G-FITC and Ly6C-biot were purchased from BD Pharmingen (San Jose, CA). All primary antibodies were used at a 1:200 dilution from stock, and incubated with cells for 30 minutes at room temperature. A streptavidin-PE/Cy7 (eBioscience) was used to label the biotinylated antibody and was used at a 1:500 dilution. Relevant streptavidin conjugates were used to set instrument settings. Samples were processed using a CyAn MLE flow cytometer (Dako-cytomation, Fort Collins, CO) and analysis performed using Summit software (Dako-cytomation).

Immunohistochemistry

Samples (n=5 per group) stored in OCT embedding media were cut into 4 μm sections and placed on glass slides. Slides were air dried at room temperature before being fixed in acetone at -20°C and subsequently stored at -80°C prior to staining. Microvessel density assessment was performed by using a purified anti-mouse CD31 antibody (eBioscience) at a 1:100 dilution. To assess changes in infiltrating macrophages, a purified anti-mouse CD68 antibody (AbD Serotec, Raleigh, NC) was used. To detect primary antibodies, immunostaining with a biotinylated donkey anti-rat (Jackson ImmunoResearch) was used at a 1:400 dilution. Further detection was accomplished by use of a Vectastain ABC kit (Vector, PK-6100) and AEC peroxidase substrate (Vector, SK-4200). Slides were then counterstained with hematoxylin and mounted with crystal mount (Biomeda, Foster City, CA). Assessment of MVD was performed by manually counting the number microvessels per 20x high power field. Macrophage numbers were calculated using a Zeiss Axio 2 microscope and Carl Zeiss AxioVision Software v4.6 (Zeiss, Thornwood, NY). Samples were blinded and randomized for both of these analyses. Computerized determination of positively staining cells was performed using an isotype control antibody and characterizing positively staining cells in the formation of a protocol that was then applied to all of the slides. Five random sections from each tumor were evaluated, and the average number of vessels or macrophages was averaged before undergoing statistical comparison.

VEGF Induction in Normal Dogs

Clinically normal dogs owned by faculty and staff of various ages and breeds were given commercial tepoxalin tablets at a dose of 10 mg/kg/day for seven days. EDTA-anti-coagulated blood was collected from each dog at 4 hours, 24 hours, and 7 days post initial treatment. Plasma was isolated from each sample and stored at -20°C until analysis using the anti-canine VEGF ELISA as described above. All normal dog studies were performed with CSU-IACUC approval and informed consent.

Statistical Analysis

Statistical analysis was performed by using GraphPad Prism 5 (GraphPad Software, La Jolla, CA). Results are representative of the mean (± SEM) calculated for each group. Analysis was performed by using repeated measures one-way ANOVA in concordance with Tukey's HSD post-test, one-way ANOVA with Tukey's HSD post-test, and student's t-test where relevant. All tests had a significance cut-off of $p<0.05$.

Results

Growth Inhibition by Tepoxalin and its Active Metabolite (RWJ20142)

Given tepoxalin's unique inhibition of NF-κB and pan-COX/LOX inhibition, we sought to determine the effect of clinically achievable concentrations of tepoxalin and its major active metabolite on the growth of canine tumor cell lines *in vitro*. Twelve canine tumor cell lines of seven distinct histotypes were used to better understand the effects of

tepoxalin on a variety of tumor cell lines. The AMJ cell line was utilized to determine the growth inhibitory effects of tepoxalin on murine macrophages, which can be a large component of tumor stroma [34]. RWJ20142 was used at 5x the given tepoxalin concentration to mimic pharmacokinetic relationships observed in tepoxalin-treated dogs [31]. **Figure 3.1** depicts the relative viable cell number of all cell lines treated with tepoxalin, and a combination of tepoxalin with RWJ20142. It was determined that tepoxalin ± RWJ20142 did not cause significant growth inhibition in canine tumor cell lines over the range of clinically achievable concentrations, from 100 to 600 ng/mL. Furthermore, only one of the cell lines, CML-6M, reached an IC_{50} at the maximum concentration of tepoxalin tested (6,400 ng/mL) **(Figure 3.1)**. When RWJ20142 was added, CML-6M, CMT12, K9TCC, Abrams, and D17 reached an IC_{50} at the highest dose (6,400 ng/mL of tepoxalin and 32,000 ng/mL of RWJ20142). However, it is noteworthy that any IC_{50} reached only occurred at doses over ten times higher than the maximum plasma concentration achievable in the dog. Given the frequency with which OSA occurs in dogs and the common use of NSAIDs as part of its therapy, we chose to better characterize the effects of tepoxalin on an OSA cell line.

Synergism of Tepoxalin and Doxorubicin

To determine if tepoxalin may reverse the cytotoxic effects of a commonly used cytotoxic drug, doxorubicin, *in vitro* cellular viability tests were performed with three representative cell lines (DEN-HSA, D17, and K9TCC). Tepoxalin was used at its peak (T 600; 600 ng/mL) and trough (T 100; 100 ng/mL) concentrations and RWJ20142 was used at its peak (RWJ 1000; 1000 ng/mL) and trough (RWJ 500; 500 ng/mL)

concentrations. Concentrations of tepoxalin and RWJ20142 were held constant and a doxorubicin was diluted serially by 4-fold dilutions (30,000 – 29.3 ng/mL). **Figure 3.2** depicts the viable number of cells after 72-hour incubation with both compounds simultaneously. There was no significant ($p>0.05$) difference in viability due to the presence of tepoxalin ± RWJ20142. To determine if the timing of doxorubicin administration may play a role in altering cell viability, doxorubicin was incubated with cells for 24 hours before or after treatment with tepoxalin ± RWJ20142 before being washed out and tepoxalin ± RWJ20142 replaced as applicable. There was no significant

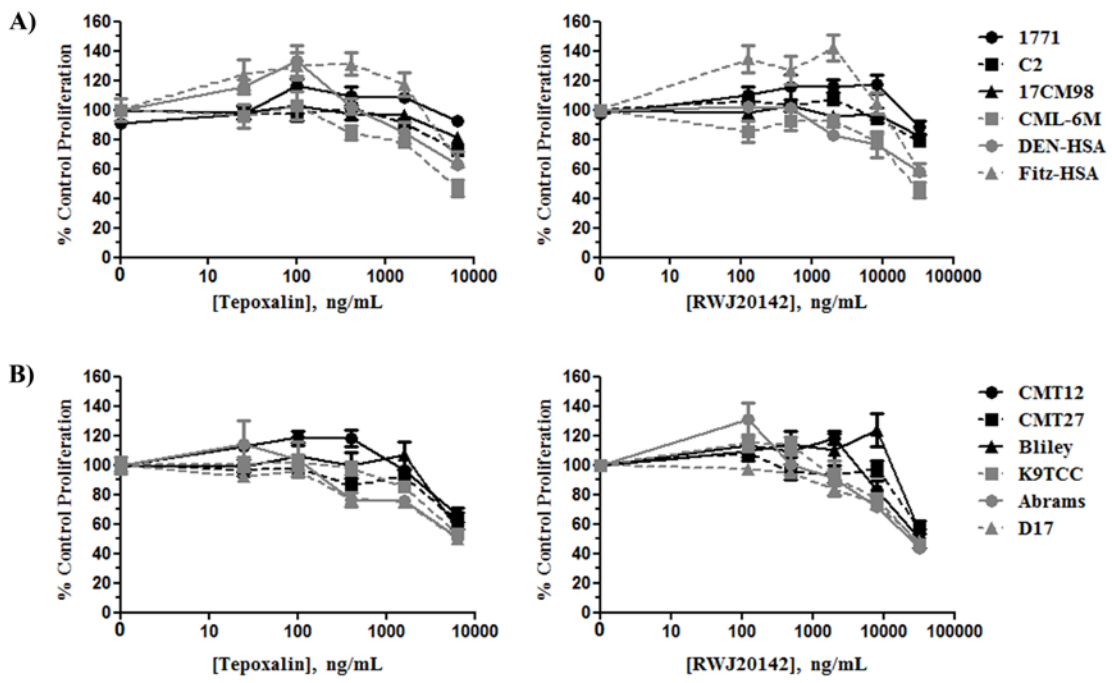


Figure 3.1: *Growth of tumor cell lines treated with tepoxalin and RWJ20142*. Tumor cell lines [**A**: B-cell lymphoma (1771), mast cell tumor (C2), melanoma (17CM98 and CML-6M), and hemangiosarcoma (DEN-HSA and Fitz-HSA); **B**: mammary carcinoma (CMT12 and CMT27), bladder carcinoma (Bliley and K9TCC), and osteosarcoma (Abrams and D17)] were treated with a range of tepoxalin ± RWJ20142 concentrations spanning the clinically achievable range of the drug for 72 hours. Analysis was performed by bioreductive colorimetric assay to determine relative viable cell number, and cell number expressed as a percentage of vehicle-treated cells with the tepoxalin concentration noted. Results shown are a compilation of three repeated experiments.

(p>0.05) change in viability when the timing of doxorubicin administration was altered (data not shown). Since there was no significant protection provided by tepoxalin ± RWJ20142 *in vitro* in the presence of a commonly used cytotoxic compound, we chose to further characterize the impact of tepoxalin on tumor cells.

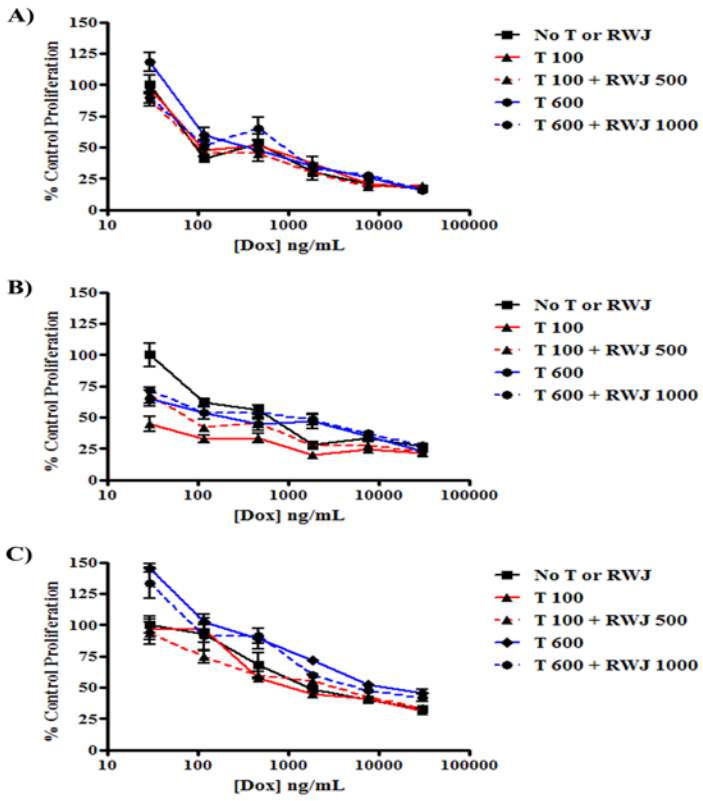


Figure 3.2: *Growth of tumor cells treated with doxorubicin in the presence of tepoxalin and RWJ20142*. Tumor cell lines [A) D17-Osteosarcoma; B) DEN-HSA-Hemangiosarcoma; and C) K9TCC-Transitional Cell Carcinoma] were treated with a range of doxorubicin concentrations in the presence of peak and trough concentrations of tepoxalin (T) and RWJ20142 (RWJ). Analysis was performed by bioreductive colorimetric assay to determine relative viable cell number, and cell number expressed as a percentage of vehicle-treated cells. There was no significant (p>0.05) growth inhibition potentiated by the addition of doxorubicin as measured by one-way ANOVA and Tukey's HSD post-test. Results shown are the mean (± SEM) and a compilation of two repeated experiments.

Induction of HIF-1α by Tepoxalin

Owing to reports showing that tepoxalin can act as an iron chelator and the demonstrated ability of other iron-chelating agents such as desferrioxamine (DFO) to stabilize HIF-1α [22, 35], we sought to determine if clinically achievable tepoxalin concentrations were capable of increasing HIF-1α concentrations in canine OSA cells *in vitro*. Tumor cells were exposed to tepoxalin concentrations corresponding to peak (600 ng/mL) and trough (100 ng/mL) plasma concentrations observed in dogs before undergoing whole cell lysis and western analysis for HIF-1α. Desferrioxamine was used as a positive control [35]. **Figure 3.3** demonstrates an increase in HIF-1α by 600 ng/mL tepoxalin treatment. Increased HIF-1α concentrations by physiologic processes, such as hypoxia, have been shown to alter a number of cellular processes, such as the production of VEGF [36]. Thus, we sought to determine if VEGF production was up-regulated by tepoxalin.

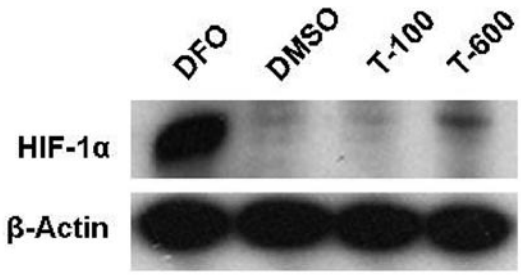
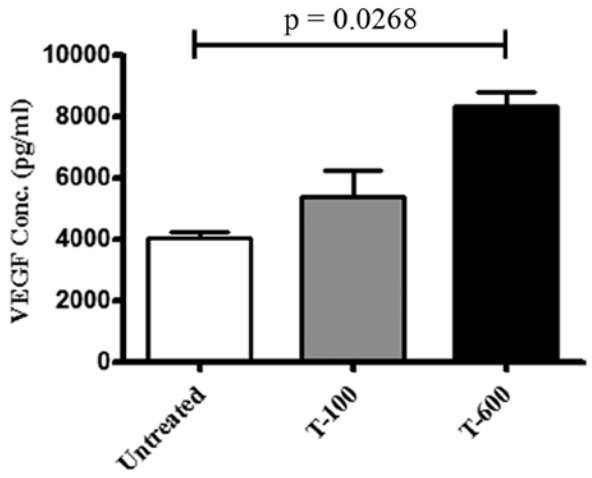


Figure 3.3: *Effect of tepoxalin on HIF-1α induction.* Abrams osteosarcoma cells were treated with peak (T-600) and trough (T-100) concentrations of tepoxalin for 12 hours before whole cell lysis. DFO served as a positive control for inducing HIF-1α. Western blot was used to detect HIF-1α and β-actin was used as a loading control.

<u>In Vitro</u> Induction of VEGF by Tepoxalin

Induction of HIF-1α is known to induce VEGF in order to stimulate angiogenesis in hypoxic tissues. Abrams cells were treated with peak and trough plasma concentrations of tepoxalin followed by determination of supernatant VEGF concentrations. **Figure 3.4** shows dose-dependent induction of VEGF from Abrams OSA cells by tepoxalin. Since VEGF is normally produced by tumors to stimulate angiogenesis, potentially enhancing primary tumor growth and metastasis *in vivo*, we sought to determine the *in vivo* effects of tepoxalin on canine OSA tumor growth.



<u>Figure 3.4:</u> *<u>In Vitro</u> VEGF induction following tepoxalin treatment*. Abrams cells were incubated with peak and trough concentrations of tepoxalin overnight prior to determination of VEGF concentration by ELISA. A significant (p=0.0268) increase in VEGF was observed at the 600 ng/mL concentration.

Pharmacokinetic Analysis of Tepoxalin in Mice

Pharmacokinetic experiments were performed to ensure that mice were exposed to clinically relevant concentrations of tepoxalin. Mice were first treated with 40 mg/kg of tepoxalin by oral gavage to determine plasma levels of drug. However, analysis showed that the peak tepoxalin concentration in mice was 0.71% of that observed in dogs

(Figure 3.5), with significantly increased concentrations of RWJ20142. We concluded that the rapid metabolism was occurring through first pass of the liver, leading to higher concentrations of the major metabolite in circulation rather than the parent drug, compared to those observed in dogs.

To bypass first-pass hepatic metabolism, tepoxalin was administered subcutaneously at 40 mg/kg to determine if the dose and route of administration would better approximate the dog pharmacokinetics. Peak tepoxalin concentrations in plasma (**Figure 3.5**) were approximately twice that observed in dogs. Due to the linear and dose-proportional pharmacokinetics of tepoxalin, we determined that 20 mg/kg would be sufficient to achieve peak tepoxalin concentrations approximating 600 ng/mL in mice.

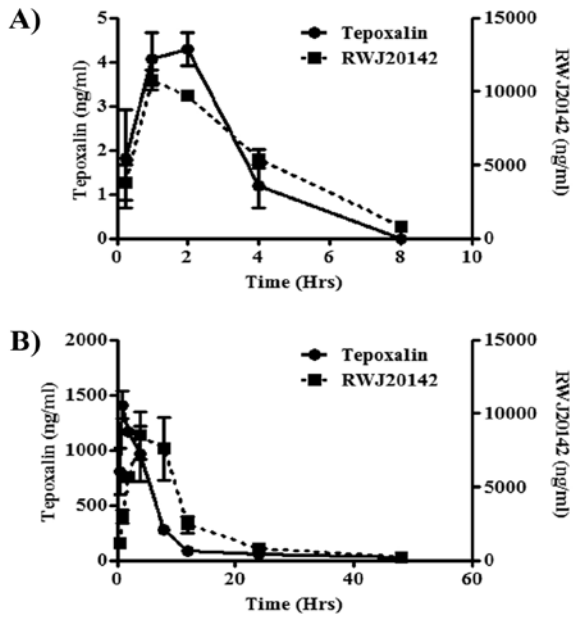


Figure 3.5: *Pharmacokinetic analysis of tepoxalin in mice.* A) Non-tumor bearing mice were administered 20 mg/kg of tepoxalin dissolved in DMSO by oral gavage. Mice were sacrificed and terminally bled at various time points to determine the concentrations of tepoxalin and RWJ20142. Due to the high levels of primary metabolite present and low levels of tepoxalin, it was concluded that first pass hepatic metabolism was rapidly degrading tepoxalin. B) A second group of mice was then administered 40 mg/kg of tepoxalin dissolved in DMSO subcutaneously. Due to the linear and dose-proportional pharmacokinetics of tepoxalin, we determined that 20 mg/kg would be sufficient to achieve clinically relevant tepoxalin exposure in mice.

<u>In Vivo</u> Effects of Tepoxalin on Canine Osteosarcoma-Bearing Mice

Due to an absence of growth inhibitory effects and the stimulation of VEGF production *in vitro* by tepoxalin, we sought to determine if tepoxalin treatment would result in an increase in tumor growth *in vivo* by stimulating angiogenesis. Athymic mice were challenged with Abrams canine OSA cells subcutaneously on the rear flank. Three days post-tumor challenge, mice began receiving tepoxalin or DMSO vehicle subcutaneously at a site distant from the tumor at a dose of 20 mg/kg. Mice were injected for five days and given two days as drug holidays, repeated weekly until sacrifice. Surprisingly, tumor growth was significantly decreased in mice receiving tepoxalin compared to all other groups, with no significant difference between vehicle-treated and untreated mice **(Figure 3.6)**. This suggests that, despite potential VEGF induction and a lack of growth inhibition *in vitro*, other mechanisms, potentially associated with modulation of the tumor microenvironment, contributed to tepoxalin's observed growth inhibitory effects.

Flow cytometry was performed on serially collected peripheral blood of treated mice to determine changes in circulating blood populations to better understand the inhibition of tumor growth by tepoxalin. No changes in neutrophils, monocytes, or CEC were noted in these experiments **(Figure 3.7)**. Analysis of spleens at sacrifice showed no significant difference in neutrophils and monocytes **(Figure 3.7)**. Interestingly, there was also no significant increase in canine or murine plasma VEGF concentrations between the treatment groups **(Figure 3.6)**; normalization by tumor volume did not alter these results **(Figure 3.6)**. The lack of systemic changes suggest that there was a mechanism present

in the tumor microenvironment leading to decreased tumor growth and inhibition of VEGF production that is contrary to the *in vitro* data and likely due to the presence of stromal components.

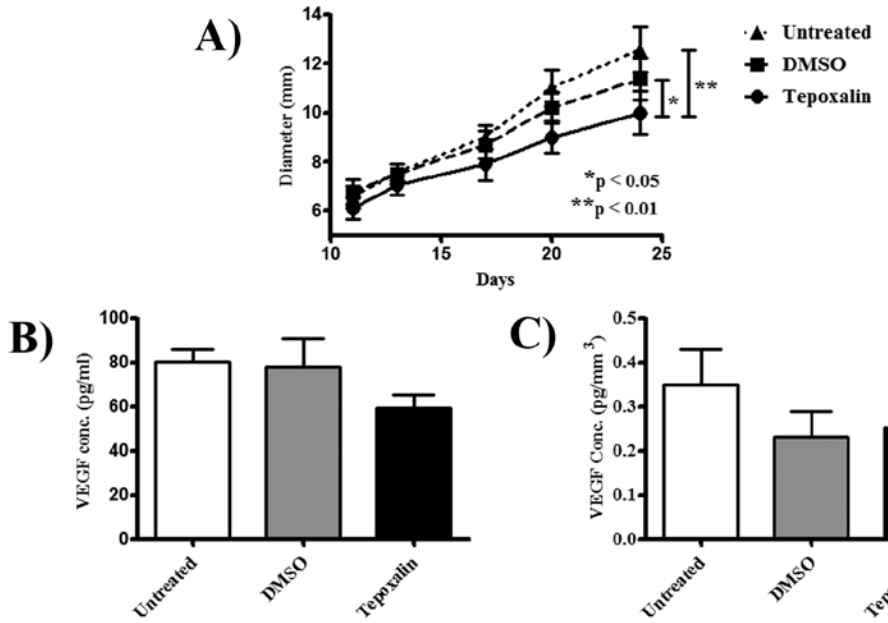


Figure 3.6: *In vivo effects of tepoxalin on tumor growth and plasma VEGF*. Mice were challenged with Abrams osteosarcoma subcutaneously and treated with tepoxalin (20 mg/kg) or vehicle beginning three days post tumor challenge. A) Growth kinetic measurements were performed using longest tumor diameter. Growth curves where concluded once the first control mouse was sacrificed. B) Plasma prepared from blood on day 24 was analyzed by ELISA for murine VEGF. C) Volume was determined from tumor measurements and VEGF concentrations were normalized to the tumor volume.

Immunohistochemical Analysis of the Tumor Environment

Immunohistochemistry was performed to determine if changes were induced in the tumor microenvironment following tepoxalin administration. Mice were challenged with Abrams OSA cells and administered tepoxalin as described above. All mice were euthanized when the first control mouse had a greatest tumor diameter of 15 mm to facilitate comparison between groups. Microvessel density (MVD) was assessed by

CD31 immunostaining and manual counting of the vessels present **(Figure 3.8)**. There was no significant difference between the groups, suggesting that an increase in angiogenesis did not occur in the tepoxalin treated group. Sections were also immunostained to determine changes in macrophage infiltrate by immunostaining with CD68 and computer aided analysis; no significant difference was observed **(Figure 3.8)**.

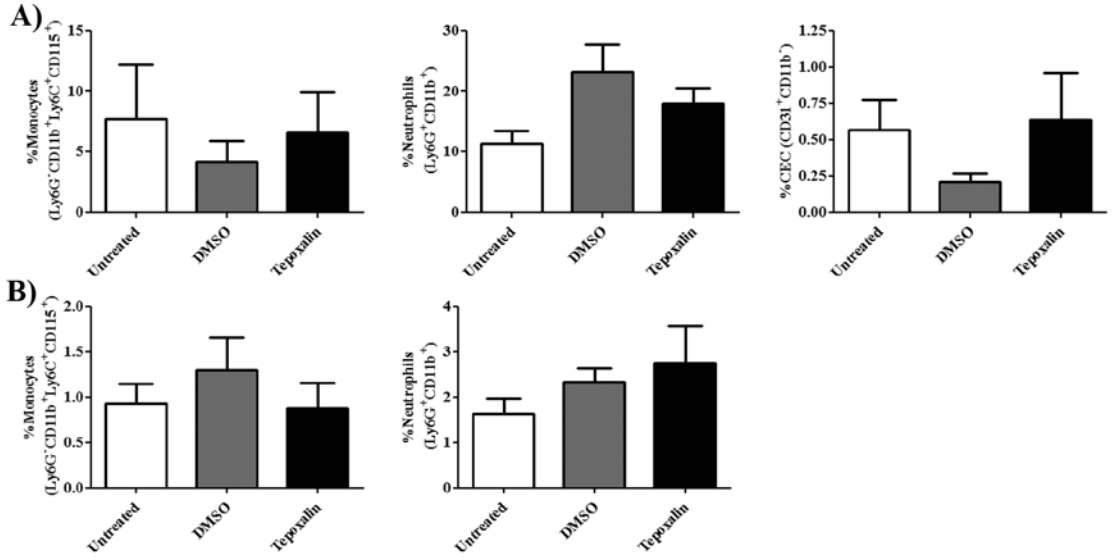


Figure 3.7: *Flow cytometric analysis of tepoxalin's effect on innate immune populations.* Mice were challenged with Abrams osteosarcoma subcutaneously and treated with tepoxalin (20 mg/kg) or vehicle control initiated three days after tumor challenge. Mice were sacrificed when the first mouse reached a maximal tumor diameter of 15 mm. Mice were terminally bled (A) and spleens (B) prepared as described in the Methods. Flow cytometric analysis of monocytes, neutrophils, and CEC in the blood revealed that neither vehicle control nor tepoxalin significantly altered either of these populations. Results are representative of two independent experiments and mean ±SEM are shown.

Tepoxalin Treatment of Normal Dogs to Assess Systemic Changes in VEGF

Five clinically normal dogs were treated with 10 mg/kg/day of tepoxalin daily to determine if changes in plasma VEGF occurred. There was a significant increase in plasma VEGF in three of five dogs at a single time point following tepoxalin administration when compared to pre-treatment concentrations. However, there was no

significant increase in plasma VEGF concentrations when the dogs were grouped for analysis **(Figure 3.9)**. The VEGF concentrations remained high while the dogs were continually administered tepoxalin, although not significant when compared to pre-treatment concentrations.

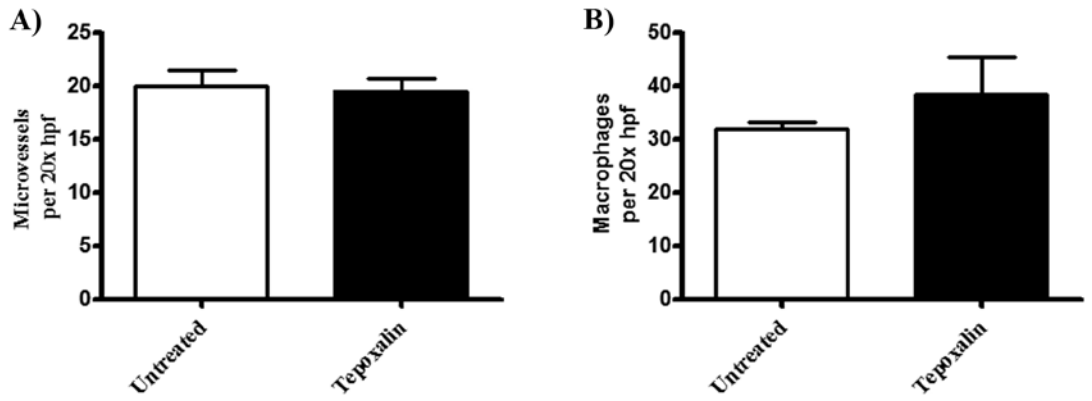


Figure 3.8: *Immunohistochemical characterization of the tumor environment.* Tumor sections were cryosectioned and immunostained for CD31 and CD68 to determine changes in microvessel density (A) and macrophages (B), respectively. Five random fields were selected from each tumor, and these values averaged before comparison between groups of mice. No significant difference was observed ($p > 0.05$).

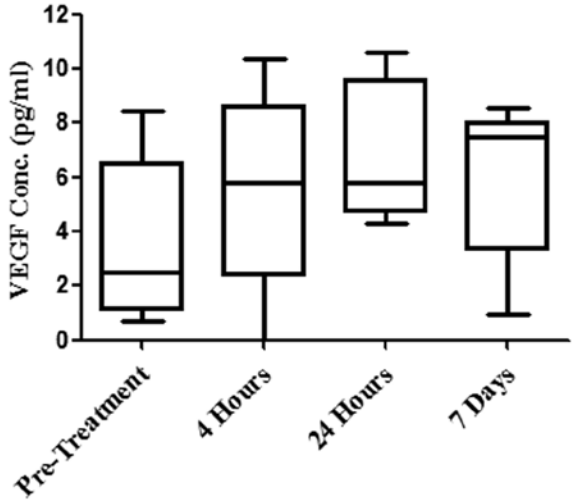


Figure 3.9: *Induction of VEGF in normal dogs treated with tepoxalin.* Normal dogs were administered tepoxalin orally at a dose of 10 mg/kg/day for seven days. Plasma VEGF was measured by ELISA. Three of five dogs had a significant individual increase in VEGF at single time point post treatment, although there is no overall significant increase in circulating VEGF.

Discussion

Due to the age related increase in cancer incidence, many animals with cancer may have a variety of chronic medical conditions requiring treatment. A better understanding of potential effects of these non-oncologic medications is needed. Thus, it was our goal to understand how an anti-inflammatory drug, tepoxalin, used in the management of osteoarthritis, would impact canine tumor growth. A number of studies have suggested that NSAIDs and specific COX-2 inhibitors may inhibit tumor growth and metastasis in various mouse models [37-39] and in dogs with cancer [9, 40, 41]. Treatment with COX inhibitors may lead to decreased cancer risk in some human populations as well [42, 43]. New evidence suggests combination of COX and LOX inhibitors may be more efficacious against tumors than single agent therapy, and tepoxalin is a dual inhibitor of these enzymes [44, 45].

We observed that clinically achievable concentrations of tepoxalin, ± its major active metabolite RWJ20142, do not exert an anti-proliferative effect against canine tumor cells *in vitro* at clinically achievable concentrations. Furthermore, treatment with doxorubicin, a common cytotoxic therapy for OSA, in the presence of tepoxalin ± RWJ20142 does not alter viability at therapeutic concentrations. However, tepoxalin dose-dependently increased the concentration of HIF-1α in canine OSA cells, leading to a dose-dependent stimulation of VEGF production. Increased VEGF production has been associated with a more aggressive tumor phenotype due to its initiation and activation of angiogenesis, and higher pre-treatment systemic VEGF concentrations have been associated with an inferior outcome in dogs and humans with OSA [46-48]. We believe

that tepoxalin's effect on VEGF production is unique, owing to its ability to chelate iron, simulating a hypoxic phenotype in the tumor cells. Modulation of HIF-1α has also been shown to modulate a number of genes besides VEGF, although a majority of these genes modulate the metabolism and activity of cells in a pro-tumorigenic manner [49]. Furthermore, new evidence suggests that other NSAIDs can be used to inhibit the stabilization of HIF-1α, thus decreasing tumor cell adaptation to hypoxic environments [50]. Crokart et al. have used this information to show that NSAIDs can increase the oxygenation of tumors, and may act as powerful radiation sensitizers [51]. Given the absence of *in vitro* growth inhibition of canine tumors and significant stimulation of VEGF production by tepoxalin, we sought to determine if tepoxalin treatment would lead to the formation of a more aggressive tumor and increased angiogenesis due to the promotion of VEGF production in canine OSA tumors *in vivo*.

In vivo evaluation was performed to determine how treatment with tepoxalin would alter tumor growth in Abrams OSA subcutaneous xenografts. Extensive effort was placed on attempting to achieve similar drug exposure in mice when compared to dogs dosed with tepoxalin, given the dose-dependent changes observed *in vitro*. Clinically relevant mouse dosing strengthens the relevance of the *in vivo* model and its conclusions, due to equivalent drug exposure in the experimental model and the clinical situation. Surprisingly, mice receiving tepoxalin had a significantly slower tumor growth compared to control and vehicle treated mice, suggesting that tepoxalin was able to inhibit tumor growth despite the pro-tumorigenic effects of induced VEGF. However, this effect is of questionable clinical significance, since mice still displayed progressive disease. There were no observed changes to circulating populations of monocytes,

neutrophils or CEC following tepoxalin treatment. It has been shown that tepoxalin inhibits extravasation of monocytes, but we did not note any significant changes in these populations [52-54]. Circulating endothelial cells were measured due to the induction of VEGF observed *in vitro*, which can lead to CEC outgrowth from the bone marrow and promotion of tumor angiogenesis [55, 56]. However, there was also no significant difference in plasma VEGF concentration between treatment groups. These data suggest that tepoxalin-associated tumor growth inhibition is likely due to stromal interactions that were absent in the *in vitro* experiments.

Due to the lack of changes identified in circulating cell populations, we sought to explain the decreased tumor growth by performing immunohistochemistry on tumor sections to characterize the tumor microenvironment. Nevertheless, we were unable to detect a significant difference in microvessel density or the number of tumor associated macrophages. Increasing evidence suggests that anti-tumor activity may be associated with the polarization of macrophages to a tumor suppressive phenotype rather than a change in their overall numbers [57, 58]. This effect is independent of T cells since our experiments were carried out in athymic mice.

To characterize the response of clinically normal dogs to tepoxalin, dogs were administered tepoxalin for one week and plasma collected for determination of VEGF concentration. A variable increase in VEGF was observed, with 3 of 5 dogs demonstrating a significant increase in systemic VEGF at a single time point tested. However, on average, there was no significant increase in systemic VEGF over the course of treatment. It is plausible that in a clinically relevant post-operative setting that an increase in systemic VEGF could lead to revascularization of the surgical site and

promote healing. However, in order to definitively determine the relevance of observed changes in plasma VEGF and tumor growth in spontaneous canine OSA, a randomized, placebo-controlled clinical trial would be necessary.

In conclusion, we have shown that, despite the presence of increased HIF-1α and subsequent stimulation of VEGF production by tumor cells *in vitro,* tepoxalin does not enhance the growth of OSA in mice as a single agent when given at a dose that recapitulates the pharmacokinetics observed in dogs. Tepoxalin was able to control tumor growth despite the pro-tumor effect of increased VEGF production. Therefore, tumor bearing animals may be administered tepoxalin for continued treatment of arthritis and analgesia while undergoing treatment for cancer.

Future Directions

In this study we have suggested that tepoxalin does not exacerbate tumor growth even though it elicits VEGF *in vitro* from a variety of tumor cell lines. However, to properly test this observation, a clinical trial would need to be performed to ensure tepoxalin will not stimulate tumor growth in patients. I hypothesize that tepoxalin will provide pain relief to tumor bearing dogs, but will not significantly prolong survival when combined with standard of care therapy. We have performed the preliminary investigation that shows that combining tepoxalin with doxorubicin, a standard of care therapy in canine OSA, does not significantly alter cellular viability. A double-blinded randomized trial will need to be performed with two-arms: patients receiving standard of

care (amputation and chemotherapy), and those receiving standard of care in addition to tepoxalin. Long-term follow-up will need to be performed and information regarding DFI and OS gathered to determine if tepoxalin impacts either of these factors. Due to the *in vitro* data, and progressive growth observed using *in vivo* mouse models, it possible that tepoxalin may numerically increase DFI and OS; however, the complexity of the clinical scenario (confounding factors) may not lead to a significant change in outcome.

It would also be interesting to investigate the combined effects of chemotherapy and NSAIDs in OSA. In this chapter we presented data showing that there was no combined effect when doxorubicin and tepoxalin were compared. However, it would be interesting to combine carboplatin or gemcitabine in combination with tepoxalin. There has been previous work performed that shows both of these agents are efficacious in OSA, and the combination may lead to a synergistic and more effective treatment of OSA [59-62]. Therefore, I hypothesize that combination of tepoxalin with carboplatin or gemcitabine will be synergistic. Investigation of these combinations will first need to be performed in *in vitro* as was previously performed with doxorubicin. If the effect is seen as additive or synergistic, further study may be warranted. Movement into a murine mouse model would be the most relevant model at this point, and use of the orthotopic model presented in chapter 5 would be an ideal method for testing these compounds. Using this model would also allow us to investigate the ability of tepoxalin to inhibit micrometastatic disease as a single agent, as this has not been previously described. If these experiments show an efficacious increase in DFI or OS, it would be suitable to create a clinical trial testing the efficacy of the combined tepoxalin/chemotherapy regimen.

Herein, we reported that treatment with tepoxalin leads to an inhibition of tumor growth, although we did not observe any changes in angiogenesis or macrophage infiltrate. Therefore, further analysis of the phenotypic changes in the tumor microenvironment would be necessary to further describe the mechanism of inhibition. I hypothesize that there are changes present in the TAM leading to an inhibition of tumor growth. As in chapter 2, IHC was attempted to characterize the macrophages, but was inconclusive. TAM are the most likely targets as they have previously been implicated in being modulated by COX-2 compounds, and provide a likely basis for controlling tumor growth [63]. As described in chapter 2, examination of these TAM by RT-PCR and CBA assay may be the best way to describe the phenotype of these cells. Description of the phenotype is crucial since an anti-tumor response may be independent of cell number and associated with the phenotype of the infiltrative cells.

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Chapter Four

Minimally Invasive Assessment of Tumor Angiogenesis by Fine Needle Aspiration and Flow Cytometry

Abstract

The development of a new, less invasive, and more rapidly implemented method of quantifying endothelial cell density in tumors could facilitate experimental and clinical studies of angiogenesis. Therefore, we evaluated the utility of tumor fine needle aspiration (FNA) coupled with flow cytometry for assessment of tumor angiogenesis. Samples were obtained from cutaneous tumors of mice using FNA, then immunostained and assessed by flow cytometry to determine the number of $CD31^+$ endothelial cells. Results of the FNA/flow cytometry technique were compared with quantification of tumor microvessel density using immunohistochemistry. The ability of the FNA/cytometry technique to quantify the effects of anti-angiogenic therapy and to monitor changes in tumor angiogenesis over time in individual tumors was also

determined. We found that endothelial cell percentages determined in tumor tissue aspirates by flow cytometry correlated well with the percentages of endothelial cells determined in whole tumor digests by flow cytometry and with tumor microvessel density measurements by immunohistochemistry. Moreover, we found that repeated FNA sampling of tumors did not induce endothelial cell changes. Interestingly, by employing repeated FNA sampling of the same tumors we were able to observe a sudden and marked decline in tumor angiogenesis triggered when tumors reached a certain size. Thus, we conclude that the FNA/flow cytometry technique is an efficient, reproducible, and relatively non-invasive method of rapidly assessing tumor angiogenesis, which could be readily applied to evaluation of tumor angiogenesis in clinical settings in humans.

Introduction

Angiogenesis is a critical process for tumor development, thus making measurement of tumor angiogenesis a valuable biomarker for evaluating tumor biology and assessing responses to anti-angiogenic therapy [1-3]. Currently, tumor angiogenesis is most often measured by CD31 immunostaining of tumor biopsies to quantify microvessel density (MVD) [4, 5]. However, determination of tumor MVD in mice requires that the animals be euthanized, while in humans, tissue biopsy is required. Thus, other non-invasive approaches to measurement of tumor angiogenesis have been explored, including imaging techniques such as X-ray angiography, positron emission tomography, and doppler ultrasound [6-8]. However, these modalities are often expensive and labor intensive techniques that are not feasible for animal studies and are often not realistic for repeated measurements in humans or mice [4].

Therefore, we sought to determine whether an alternative, relatively non-invasive, but easily applied technique could be used for routine, direct assessment of tumor angiogenesis. Previous studies have shown that flow cytometry could be used to assess angiogenesis when frozen and embedded tumor sections were used as the sample source [9]. However, this technique also requires relatively large amounts of tumor tissue for analysis. Fine needle aspirates (FNA) of tumors are routinely used to identify tumors by cytologic criteria, based on staining of cell samples prepared on glass slides. However, the FNA technique typically also collects infiltrating leukocytes and mesenchymal cells in addition to tumor cells in the sample. Therefore, we hypothesized that sufficient

numbers of endothelial cells could be obtained in the FNA specimen to allow evaluation and quantification by flow cytometry.

To address this question, we developed an FNA approach to collect small samples of tumor tissue from cutaneous tumors. The technique was optimized to allow reproducible samples to be collected from small tumors (i.e., tumors with a diameter of 5 mm or greater). Sample preparation was optimized to provide suitable single cell suspensions for immunostaining and analysis by flow cytometry. We then conducted studies to determine whether the results of tumor endothelial cell analysis using FNA/flow cytometry were comparable to results obtained with conventional MVD analysis or whole tumor flow cytometry. The FNA/flow cytometry technique was applied to evaluate the effects of anti-angiogenic drug treatment and to assess changes in tumor angiogenesis over time in individual tumors, using serial FNA samples. Based on the results of these studies, we concluded that the FNA/flow cytometry technique could be used to accurately measure tumor angiogenesis in mice with cutaneous tumors.

Materials and Methods

Cell Lines

The mouse tumor cell lines 4T1 (mammary carcinoma) and MCA-205 (fibrosarcoma) were maintained in C/5/5 MEM medium [MEM (Lonza, Walkersville, MD) supplemented with 1X MEM vitamin solution (Cellgro, Henderson, VA), 2 mM L-glutamine (Cellgro), 1 mM sodium pyruvate (Cellgro), 1X non-essential amino acid

solution (Cellgro), 1X antibiotic/antimycotic (Cellgro), 5% heat inactivated fetal bovine serum (FBS, Atlas, Fort Collins, CO), and 5% heat inactivated newborn calf serum (Hyclone, Logan, UT)]. Once confluent, cells were washed with 1x phosphate-buffered saline and detached with 0.25% trypsin (Cellgro) supplemented with 0.5 mM EDTA.

Animals

All animal studies were performed in an AALAC-approved facility and were approved by the Colorado State University Institutional Animal Care and Use Committee. Female mice 6-8 weeks of age were used in all experiments and were purchased from Harlan Sprague Dawley (Indianapolis, IN) or Jackson Laboratories (Bar Harbor, ME). The 4T1 tumor cell line was injected in the mammary fat pad of BALB/c mice using $5x10^5$ cells in 100 μL of PBS. MCA-205 tumor cells ($2.5x10^5$ cells per mouse) were injected subcutaneously into the rear flank of C57BL/6 mice.

FNA Technique

Once tumors reached a minimal size of 5 mm in diameter, they were subjected to sampling using a 23 gauge needle. Mice were anesthetized using isoflurane (Minrad, Bethlehem, PA) and a 23 gauge needle (Beckton Dickinson, Franklin Lakes, NJ) (without syringe attached) was inserted into the tumor using a rotating motion. After insertion into the tumor, the needle was removed and the tissue sample was flushed from the needle by attaching a syringe filled with tissue culture medium and expelling the contents into a sterile eppendorf tube. This process was repeated twice more, each time inserting the needle at roughly perpendicular angles to the previous insertion, for a total of 3 samples

collected from each tumor. The 3 collected tumor samples were then pooled and processed using collagenase digestion [collagenase (5 mg/ml final concentration; Sigma-Aldrich, St. Louis, MO) dissolved in MEM (Lonza) with 0.0125 mg/ml DNase (bovine deoxyribonuclease I; Sigma-Aldrich), and 0.25 mg/ml trypsin inhibitor (Sigma-Aldrich)] at 37°C for 20 min in a water bath. The samples were then triturated using a 23 gauge needle and 1 ml syringe to assure that any tissue clumps were disaggregated. Single cell suspensions obtained in this manner were washed once using FACS buffer (1x PBS with 2% FBS and 0.1% sodium azide) and then counted and re-suspended in FACS buffer and kept on ice prior to immunostaining.

Immunostaining and Flow Cytometry

Single cell suspensions from tumor aspirates were placed in individual wells of 96-well round bottom plates at a concentration of $5x10^5$ to $1x10^6$ cells per well. Prior to immunostaining, non-specific staining was blocked using 10 μL unlabelled anti-mouse Fcr III antibody (CD16/32; clone 93; eBioscience, San Diego, CA) diluted in normal mouse serum (Jackson Immunoresearch). Immunostaining was performed at room temperature for 30 minutes using an anti-mouse CD31 antibody (FITC-conjugated; clone 390; eBioscience), anti-mouse CD11b antibody (Pacific blue-conjugated; clone M1/70; eBioscience, anti-mouse VEGFR2 antibody (PE-conjugated; clone Avas 12α1; BD Pharmingen, Franklin Lakes, NJ), and anti-mouse CD133 (APC-conjugated; clone 13A4; eBioscience). Samples were then washed twice and analyzed after addition of propidium iodide (5 μg/sample) for live-dead cell discrimination. Samples were analyzed using a CyAn ADP flow cytometer (Beckman-Coulter, Fullerton, CA) and analysis was

performed using FlowJo software (Tree Star, Inc., Ashland, OR). Endothelial cells were identified as $CD31^{+}CD11b^{-}CD45^{-}$.

Immunohistochemistry

Tumor tissues were embedded in OCT embedding medium (Sakura, Torrance, CA) and cryosectioned at a thickness of 4 μm onto Superfrost slides (VWR; West Chester, PA). Slides were rehydrated, non-specific binding blocked with appropriate serum, then incubated with anti-mouse purified CD31 antibody (clone 390; eBioscience) appropriately diluted primary antibody. After washing, sections were incubated with biotinylated donkey anti-rat (Jackson ImmunoResearch). A Vectastain ABC kit (Vector) and subsequent AEC peroxidase substrate kit (Vector) were used according to manufacturer's instructions. Slides were then counterstained with hematoxalin and crystal mount (Biomeda, Foster City, CA) applied.

Microvessel density (MVD) was determined by imaging five random 20x-high power CD31-stained fields using a Zeiss AxioVision 2 microscope and Carl Zeiss AxioVision Software v4.6 (Zeiss, Thornwood, NY). Computerized determination of positively staining cells was performed by blanking against sections stained with an irrelevant isotype control antibody. The average of positively stained areas for the 5 fields was determined for each tumor.

Tumor Growth Inhibition with ZD6474

The VEGF receptor-2 inhibitor ZD6474 was kindly provided by Dr. Dan Gustafson (Colorado State University) and AstraZeneca (Macclesfield, UK). Three days

after tumor cell injection in mice (n = 5 per group), treatment was initiated. One group of mice was treated with 25 mg/kg of ZD6474 dissolved in PBS with 1% Tween 80 vehicle, while a second group of mice was treated with diluent alone [10, 11]. Treatment was administered daily for the duration of the study. Tumor aspirates were obtained from all mice 14 days post-tumor challenge as described above immediately before animals were euthanized.

Statistical Analysis

Statistical analyses were performed using commercial software (Prism 5; GraphPad Software, La Jolla, CA). Statistical comparisons between two groups were performed using the Mann-Whitney two-tailed t-test. Comparisons between three groups were performed using one-way ANOVA, followed by the Bonferroni post-test. Tumor growth analysis was performed using repeated measures two-way ANOVA. Correlations were analyzed using a one-tailed Spearman correlation test. For all statistical analyses, p-values of less than 0.05 were considered statistically significant.

Results

Quantification of Endothelial Cells in Tumor Digests Using Flow Cytometry

In this study we identified tumor endothelial cells as $CD31^+$ cells that did not express the integrin CD11b or the hematopoietic marker CD45, as described previously [12-15]. Cells that expressed CD11b were excluded from the endothelial cell population

because some monocytes and macrophages are known to also express CD31 [15-17]. Propidium iodide staining was used to exclude dead cells from the analysis. Cells were first gated by FSC and SSC to exclude debris. Using this approach, we were able to routinely identify a distinct population of $CD31^{+}CD11b^{-}$ cells in tumor aspirates and tumor digests (**Figure 4.1**).

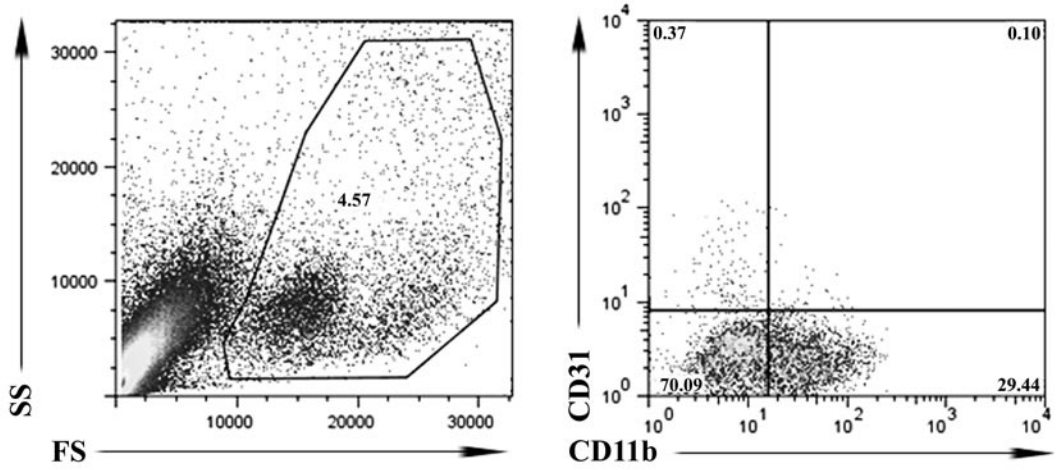

Figure 4.1: *Flow cytometric analysis of tumor endothelial cells.* Fine needle aspirates (FNA) of tumors were processed for immunostaining and analyzed by flow cytometry as described in Methods. Cells that were PI^{+} were first excluded to eliminate dead cells from analysis. Then, live cells were gated using forward and side scatter characteristics to eliminate cell debris, and subsequent analysis of CD31 and CD11b stained cells. Endothelial cells were classified as $CD31^{+}CD11b^{-}$.

We also assessed endothelial cell expression of other markers associated with tumor endothelium, such as CD133 and VEGFR2/Flk-1. However, these determinants were expressed on only a very small subset of $CD31^{+}$ cells and did not contribute substantially to endothelial cell identification and therefore not used further.

It should be noted that only tumors of a diameter of 5 mm or greater could be accurately aspirated using a 23 gauge needle. We experimented with smaller and larger gauge needles, but found that use of 23 gauge needles gave superior results in terms of

cell yield from small tumors. Moreover, it was possible to determine when only normal skin was aspirated, because FNA/flow cytometry of normal skin typically yielded fewer than 1,000 total cell events for analysis **(Figure 4.2)**.

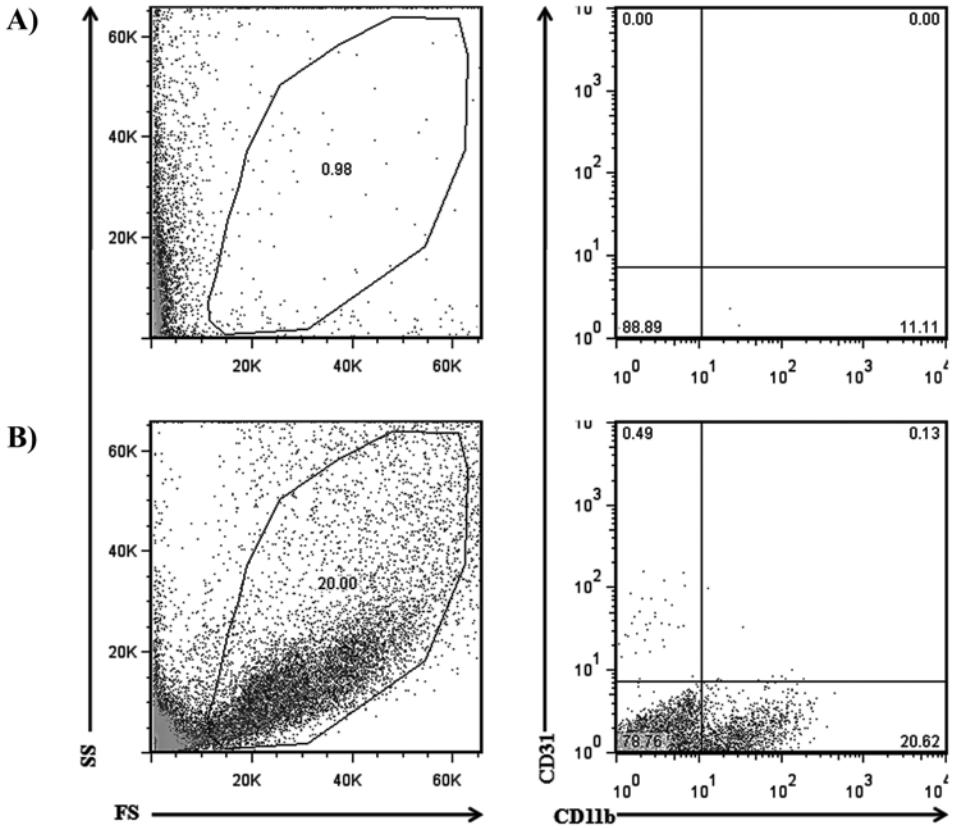

Figure 4.2: *Comparison of FNA from skin and tumor.* FNA were taken from mice with established tumors as previously described. At the same time, skin from the contralateral flank was also taken. FNA were processed and analyzed identically. FNA from normal skin (A) have a much lower yield of cells for analysis (<1000) compared to FNA from established tumors (B). Therefore, we can differentiate between FNA of tumors and that of normal skin.

Comparison of Endothelial Cell Percentages of FNA and Whole Tumor Digests by Flow Cytometry

Using the flow cytometry analysis scheme noted above, we first asked whether FNA/flow cytometry samples of tumors yielded similar results in terms of percentages of endothelial cells as samples obtained by whole tumor digestion and flow cytometry. To address this question, FNA samples were collected from tumors of anesthetized mice (n = 5 per group), then the mice were euthanized and whole tumor tissues were collected and digested enzymatically. Single cell suspensions obtained by both techniques were then immunostained and analyzed by flow cytometry, using the protocol noted in the Methods. Endothelial cell populations from two different tumor types were compared. We found that the percentages of endothelial cells obtained by FNA and whole tumor digests of 4T1 breast carcinomas were significantly correlated ($p<0.05$), as determined by Spearman correlation (**Figure 4.3**). In the case of the sarcoma cell line MCA-205, the two techniques also yielded results that were significantly correlated ($p<0.05$) **(Figure 4.3)**. Thus, we concluded that flow cytometric analysis of endothelial cell populations in tumors using samples collected by FNA yielded results similar to those obtained using whole tumor digests.

Correlation between FNA/Flow Cytometry and Tumor MVD Analysis

Next, we investigated the correlation between tumor angiogenesis assessed using FNA/flow cytometry and angiogenesis assessed using immunohistochemistry (IHC) and MVD analysis. As noted above, FNA specimens were collected from tumors before the tissues were collected for analysis by IHC. Results of endothelial cell analysis from

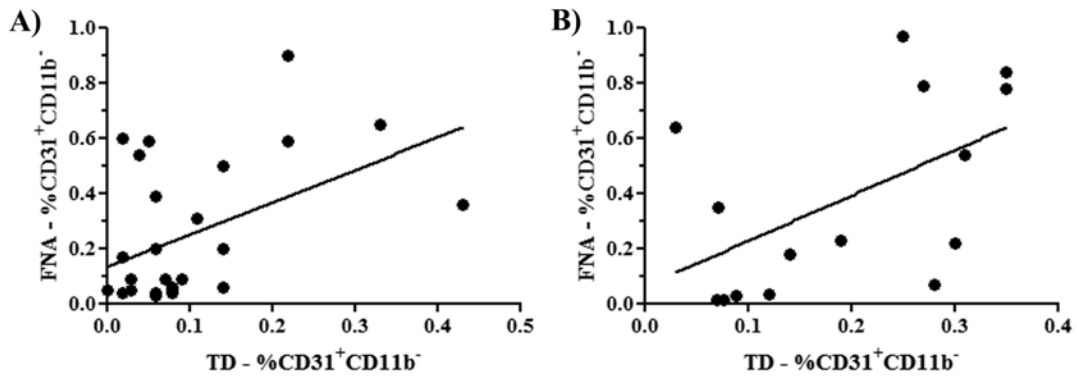

Figure 4.3: *Flow cytometric analysis of tumor FNA specimens and whole tumor digests yield similar estimates of tumor angiogenesis.* Tumor specimens were collected by FNA or tumor biopsy from mice with established 4T1 tumors (**A**) or MCA-205 tumors (**B**), and analyzed by flow cytometry to determine percentages of $CD31^+$ endothelial cells, as described in Methods. There was a significant ($p = 0.0402$) correlation between the percentage of $CD31^+$ cells in mice ($n = 27$) with 4T1 tumors (A) and the percentage of CD31+ cells in mice ($n = 15$) with MCA-205 tumors (B, $p = 0.0267$). Multiple experiments were pooled for each tumor type in this analysis.

FNA/flow cytometry samples were compared with MVD results from the same tumor sample. We observed a significant correlation ($p<0.05$) between endothelial cell percentages as determined by FNA/flow cytometry and tumor MVD for individual tumors in mice (**Figure 4.4**). In addition, we observed a significant correlation ($p<0.05$) between the results of whole tumor digest by flow cytometry and tumor MVD results **(Figure 4.4)**. This latter result is in agreement with a previous report correlating angiogenesis determination by flow cytometry on archived frozen tissues and tumor MVD analysis [9]. Thus, the estimates of tumor angiogenesis provided by the FNA/flow cytometry technique correlated well with tumor angiogenesis measurements determined by the conventional MVD assay.

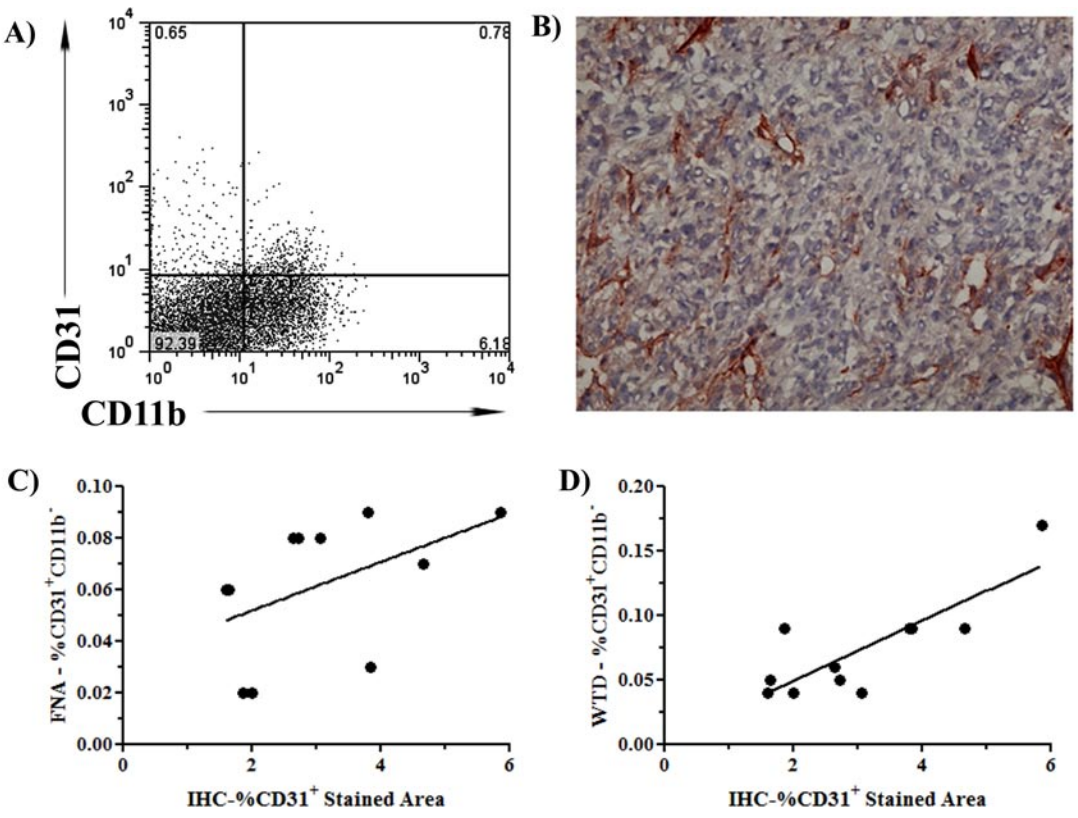

Figure 4.4: *Tumor angiogenesis assessed by FNA/flow cytometry correlates with angiogenesis measured by immunohistochemistry*. Fine needle aspirate samples were collected from mice (n= 10 per group) with cutaneous 4T1 tumors and numbers of $CD31^+$ endothelial cells were analyzed by flow cytometry (A), as described in Methods. Mice were euthanized, and tumors isolated and analyzed for MVD by IHC (B) as described in Methods. Correlation between MVD and percentage of $CD31^+$ cells as determined by FNA (C) and whole tumor digest (WTD; D) was determined using Spearman correlation, which demonstrated a significant ($p < 0.05$) correlation between the values. Results are representative of two independent experiments.

Repeated FNA Sampling of Tumors Does Not Alter Tumor Angiogenesis Measurements

To determine whether repeated sampling of tumors by FNA introduced artifacts into the endothelial cell analysis, we compared endothelial cell percentages determined after a single tumor aspirate to values obtained after 3 repeated tumor aspirates in mice with tumors established for equal periods of time. Mice (n = 5-6 per group) with

established 4T1 tumors were divided into two groups. In group 1, the tumor was only aspirated once, 7 days after tumor challenge. In group 2, tumors were aspirated on days 7, 10, and 13. Sixteen days after tumor implantation, FNA samples were collected from all mice in both groups and endothelial cell numbers compared by flow cytometry (**Figure 4.5**). The mean percentage of $CD31^+$ endothelial cells in tumors only aspirated once was 0.09%, while the percentage of endothelial cell in tumors aspirated 3 times was 0.11%, and these percentages were not significantly different (p=0.35). Therefore, we concluded that repeated sampling of tumors by FNA did not introduce significant artifacts in endothelial cell numbers.

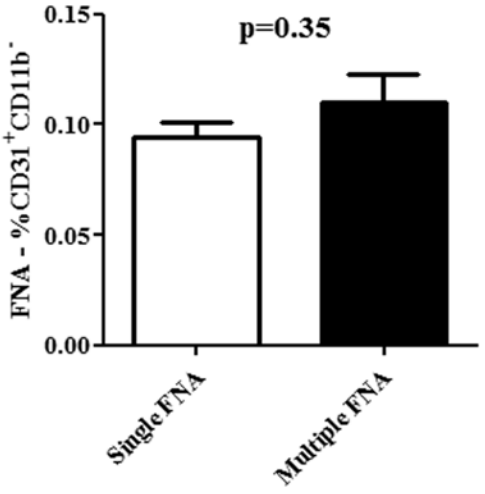

Figure 4.5: *Repeated FNA sampling of tumors does not lead to endothelial cell artifacts.* BALB/c mice with established 4T1 tumors were divided into two groups (n = 5 per group). In Group 1 mice, a single FNA was performed, while in Group 2 mice, 3 separate FNAs were performed, each 3 days apart. The percentage of $CD31^+$ cells (mean ± SEM) was compared between the two groups by Mann-Whitney test. Significant differences in endothelial cell percentages were not observed ($p < 0.05$). Results are representative of two independent experiments with 5 mice per group.

FNA/Flow Cytometry for the Assessment of Tumor Angiogenic Responses to Angiogenesis Inhibitors

The FNA/flow cytometry technique would be particularly useful clinically if it could be used to assess tumor responses to anti-angiogenic agents. Therefore, we assessed the ability of FNA/flow cytometry to detect changes in tumor angiogenesis in mice treated with the angiogenesis inhibitor ZD6474, a dual inhibitor of VEGFR2 and EGFR [18-20]. Mice with established 4T1 tumors were treated by daily oral gavage with ZD6474, which was initiated 3 days after tumor cells were injected. Control mice were treated by daily oral gavage of dilution buffer. Treatment with ZD6474 induced a significant ($p<0.0001$) delay in tumor growth compared to mice treated with dilution buffer (**Figure 4.6**). In addition, the percentage of endothelial cells was significantly reduced in tumors of ZD6474-treated mice compared to control mice, as assessed by FNA/flow cytometry (**Figure 4.6**). These results indicated that FNA/flow cytometry could realistically be used to monitor the efficacy of anti-angiogenic agents *in vivo*.

Repeated FNA Sampling to Assess Changes in Tumor Angiogenesis Over Time

Finally, we evaluated the potential for repeated FNA/flow cytometry analysis of individual tumors over time to provide insights into how tumor angiogenesis may change over time. Currently, to assess changes in tumor angiogenesis over time in mice, groups of mice with tumors must be serially sacrificed and tumor angiogenesis quantified by MVD analysis of each individual tumor tissue sample. The entire process is therefore costly in terms of mouse usage and analysis time. For clinical studies in humans, direct evaluation of changes in tumor angiogenesis over time is very difficult to accomplish

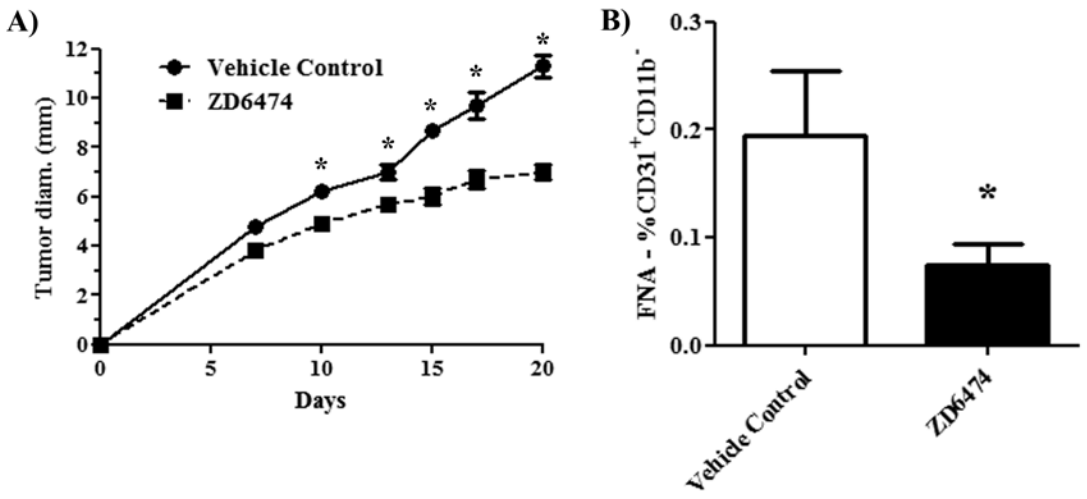

Figure 4.6: *FNA analysis can be used to assess the effects of anti-angiogenic therapy (ZD6474).* BALB/c mice (n=5 per group) with established mammary fat pad 4T1 tumors were treated daily with ZD6474 (25 mg/kg) or with control buffer by oral gavage. A) Tumor growth was assessed by measuring tumor diameter, and a significant ($p<0.0001$; $*p<0.05$) growth inhibition was observed in the presence of ZD6474 as assessed by repeated measures two-way ANOVA. B) Percentages of $CD31^+$ endothelial cells in treated and sham-treated tumors were determined (mean ± SEM) using FNA/flow cytometry, as described in Methods. The percentage of $CD31^+$ endothelial cells was significantly decreased ($p < 0.05$) in ZD6474-treated tumors, compared to control tumors, as assessed by Mann-Whitney test. Results are representative of two independent experiments.

because of the need for repeated tumor biopsies. Therefore, use of the FNA approach could offer substantial advantages over current technologies for repeated and relatively non-invasive assessment of tumor angiogenesis.

To address this question, we established cutaneous tumors (either 4T1 tumors in BALB/c mice or MCA-205 tumors in C57Bl/6 mice) in mice (n = 5 per group) and then serially evaluated tumor angiogenesis using tumor FNA samples collected from each individual tumor every 3 days using flow cytometry. We correlated tumor angiogenesis measurements with changes in tumor size. Results of the flow cytometric analysis showed that the percentage of $CD31^+$ endothelial cells in 4T1 tumors increased steadily

up until the point where the mean tumor diameter reached approximately 9 mm (Figure 6). At that point, the percentage of endothelial cells decreased dramatically, dropping from an average of 0.19% $CD31^+$ cells to 0.05% $CD31^+$ cells. A very similar phenomenon was observed in mice with MCA-205 tumors **(Figure 4.7)**. For example, in the MCA-205 tumor model, the average number of $CD31^+$ endothelial cells in tumors decreased from 0.83% to 0.21% once tumors reached a mean diameter of 7.6 mm. These results suggested that reaching a critical tumor size was associated with a rapid and pronounced reduction in endothelial cell density. It should be noted that these results were obtained using a total of 20 mice (groups of 5 mice for each tumor type, with one repeat each), whereas a similar experiment using serial sacrifice of groups of mice, with a repeat, would have required the use of 80 mice.

Discussion

Most standard assays of tumor angiogenesis require sufficient amounts of tumor tissue to section in order to perform IHC analysis. This generally requires obtaining tumor biopsies (humans) or removal of the entire tumor from euthanized animals (mice). Thus, techniques for quantifying tumor angiogenesis using much smaller specimens would greatly facilitate clinical and experimental studies. In this report, we provide results to support the idea that FNA/flow cytometry, using very small tumor specimens, is an effective alternative to conventional tumor biopsy and IHC for assessing tumor angiogenesis. The major findings from the current study were that the FNA/flow

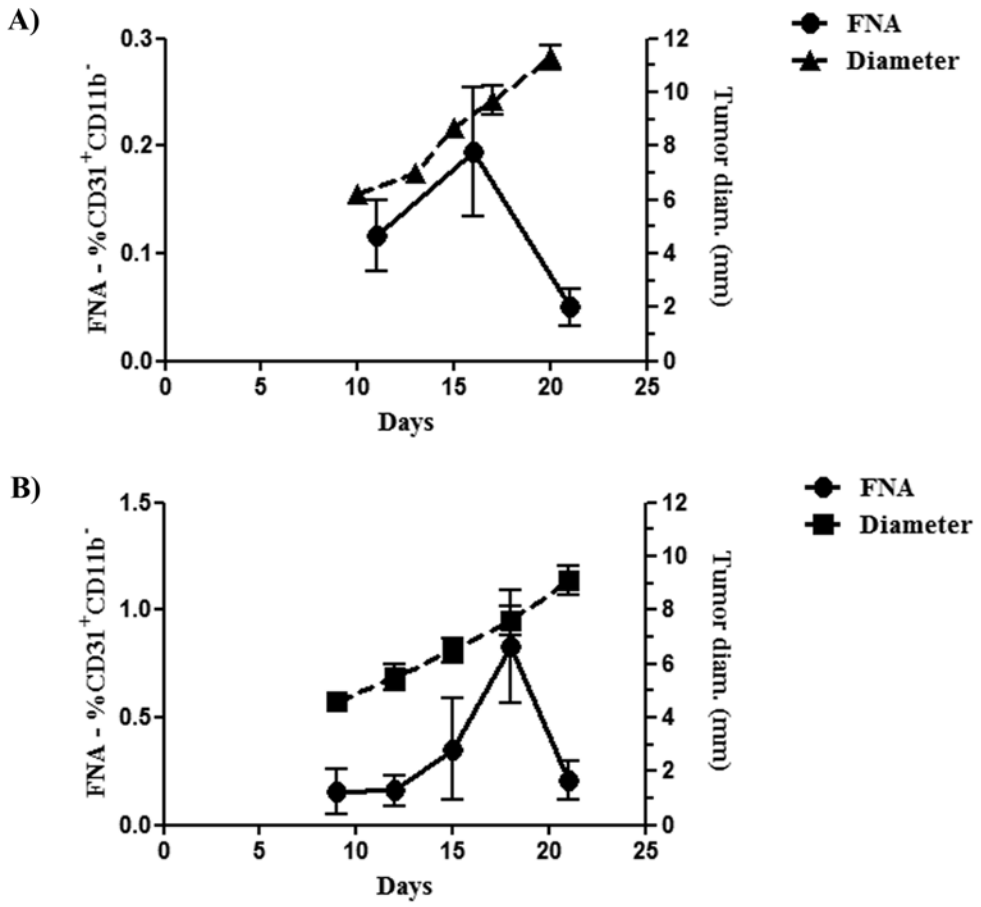

Figure 4.7: *Repeated measurement of tumors by FNA reveals angiogenic collapse of the tumor.* Syngeneic mice were challenged with 4T1 (A) or MCA-205 (B) tumors. Tumor measurements were initiated to coincide with the first FNA. FNA were taken every three days after the initial FNA until the first mouse had a maximal tumor diameter of 10 mm, at which time all mice were sacrificed. Tumors from mice challenged with 4T1 and MCA-205 tumors had a rapid decrease in the percentage of endothelial cells present suggestive of an angiogenic collapse. Results depict the mean (±SEM) of endothelial cells as determined by FNA and tumor growth as represented by maximal tumor diameter. Results are representative of two independent experiments.

cytometry technique could be adapted for use in mouse tumor studies, that the results of angiogenesis assays using FNA/flow cytometry correlated well with estimates of tumor angiogenesis provided by tumor MVD (analyzed by IHC) and whole tumor digest

samples (analyzed by flow cytometry), and that the FNA/flow cytometry approach could be used to repeatedly assess tumor angiogenesis over time.

The ability to assess angiogenesis relatively non-invasively and repeatedly using a technique such as FNA/flow cytometry represents an important advance, since it greatly reduces the number of mice required for animal experiments. Equally important, it is reasonable to assume that the same approach could also be used to assess angiogenesis in solid tumors of humans, so long as the tumor was accessible by needle for aspiration. Thus, the approach could be used for tumor staging and as a readily obtained biomarker for evaluation of anti-angiogenic treatments and new drugs.

Key variables for performing the FNA/flow cytometry technique were identified in the current study. Among these were tumor size, needle size, and sample digestion after procurement. For example, we found that tumors < 5 mm in diameter could not be reliably sampled. Thus, the technique could only be applied to analysis of more advanced tumors. Several different needle sizes were evaluated for their ability to obtain reliable tumor FNA specimens and we found that a 23 gauge needle gave the most reliable results. Larger needles were too large to use on mouse tumors, whereas smaller needles failed to yield sufficient cells for analysis. In addition, pooling aspirated specimens from a single tumor obtained by inserting the needle in 3 different orientations also improved the cell yield and served to decrease sample-to-sample variability. Finally, we found that a brief digestion step, using collagenase, increased the yield of cells overall and specifically endothelial cells from the FNA specimens.

A potential limitation to the FNA/flow cytometry technique was identified in the small number of events used for analysis. For example, the overall number of endothelial

cells detected in each sample was relatively small, thus limiting the assessment of complex subpopulations of endothelial cells. However, despite the small yield of endothelial cells, we still observed a relatively small and manageable variability in tumor-to-tumor endothelial cell numbers. In the case of human tumor samples, this limitation might be partially overcome by simply performing more needle aspirates of a tumor, which is reasonable given the much larger overall size of tumors in humans relative to mice. Regional variation in intra-tumoral angiogenesis did not appear to introduce significant problems with the FNA/flow cytometry technique, presumably because specimens were collected from several different regions of the tumor for each sample. Moreover, repeated sampling of the same tumor at 3-day intervals also did not appear to introduce significant angiogenic artifacts.

Use of the FNA/flow cytometry approach to repeatedly measure angiogenesis in individual tumors also provided us with a unique tool to assess changes in angiogenesis over time. Such information has not previously been reported in mouse or human tumor studies and generally can only be inferred by comparing levels of angiogenesis between tumors collected from carefully controlled groups of mice at different time points. Thus, previous studies assessing tumor angiogenesis may overlook rapidly occurring changes in angiogenesis [4]. In the present study, we noted a precipitous drop in the percentage of endothelial cells within tumors as tumor size reached a critical level of approximately 8-9 mm in diameter. This critical threshold of tumor size was detected in repeated experiments involving different types of tumors. Thus, the time and tumor size frame of such changes, such as this apparent vascular collapse phenomenon, can be documented using FNA/flow cytometry.

In summary, we describe here a new technique for simple, repeated and relatively non-invasive assessment of tumor angiogenesis in mice, which is also readily applicable to study of human tumors. Use of the FNA/flow cytometry technique should greatly reduce the number of mice required for experiments. Repeated, closely spaced sampling of angiogenesis in individual tumors using the technique may also provide unique insights into more rapidly occurring angiogenic processes.

Future Directions

Within this chapter we have created a technique to evaluate angiogenesis serially using a minimally invasive manner in mice. In this process, we have observed a unique phenomenon in that the percentage of endothelial cells present in a tumor rapidly change once the tumor reaches a size of almost a centimeter. This is an interesting observation that requires further study. Since the analysis used within is based on the percentage of endothelial cells measured, it would be important to better understand the changes occurring in other cellular populations to see how they relate to that of endothelial cells. Therefore, I hypothesize that the change in endothelial cells will be due to a rapid increase in tumor cell growth. It is plausible that a vascular network is developed in a growing tumor, and reaches a critical level at which time the tumor is able to rapidly increase in growth due to the presence of a richly oxygenated environment. At this time, the tumor cells rapidly divide due to the presence of favorable factors, thereby outgrowing their blood supply. The resulting decrease in endothelial cell percentage is

due as much to an increase in tumor cell growth as it is limited by the capacity of endothelial cells to sustain their own growth.

To determine if the above stated hypothesis is correct, serial sacrifice of mice will need to be performed and concurrent FNA taken. This data will show if FNA are related to the overall tumor burden at each point established, and remove any bias associated by taking a small sample. Samples can then be analyzed by IHC for Ki-67, CD31, CD11b, FSP (fibroblast surface protein) and measured for necrosis. Flow cytometry analysis may be performed to investigate PI, CD45, CD11b, and CD31. These analyses will allow for the study of the immune infiltrate, endothelial cells, necrotic tissue, and subtracted to analyze for tumor cells. There are not good markers for fibroblasts by flow cytometry, so these conclusions may have to be made from the IHC. Analysis of changes in each of these cellular types and how they relate to each other will be of importance in determining if and how endothelial cell numbers change in relation to the overall tumor burden. Serial sacrifice of mice will also help determine if the same observation of decreased endothelial cells can be made using a separate modality. These experiments should allow us to better understand the changes in angiogenesis observed by serial FNA, and better understanding of this underlying biology would be beneficial for investigating novel anti-angiogenic therapeutics.

Since FNA yields information about the dynamic nature of tumor vasculature, it would be beneficial if we could better characterize the endothelial cells within the tumor. I hypothesize that characterization of endothelial cells with markers such as CD133, CD34, CD117, and VEGFR1/2/3 may provide more information concerning the types of blood vessels formed during various points of tumor development. In this manuscript,

we noted that CD133 and VEGFR2 did not yield viable results for subpopulation analysis. However, pooling of FNA from multiple mice in a group may yield a sufficient number of cells for analysis, and thus allow us to make more concrete conclusions concerning the presence and phenotype of these cells. Pooling of groups of mice is still superior to using separate groups of mice since the pooled mice can be tracked over a time course. The pooling technique does not allow for extrapolation to individual mice; however, it does retain the repeated measures nature critical to the characterization of changes over time.

CD34, CD117, and VEGFR1/3 are other markers expressed by endothelial cells to various degrees based upon the maturation of endothelial cells, and could provide evidence for the types of endothelial present in a tumor. Many of these markers are developmental markers and characterization may be performed by multicolor flow cytometry. Specifically, VEGFR3 expression is thought to be limited to lymphatic endothelium; however, this receptor is present on a subpopulation of immature vascular endothelium [21]. Use of the VEGFR3 marker may also suggest differences in metastatic route. For example, an increase in VEGFR3 may allow for observation of differences in lymphatic infrastructure in tumors, and possibly suggest a bias towards lymphatic or hematogenous metastasis in cutaneous metastatic models. These experiments may also better describe differences between tumor and normal vasculature, and define how these changes occur over time. For example, if it is observed that tumors have predominantly immature endothelial cells whereas the other organs have more mature endothelium, this could act as a targeted site for anti-tumor therapy. Hypotheses about the immaturity of tumor endothelial cells have previously been formed, but an observation made during this

work has shown that ZD6474 decreased the percentage of endothelial cells in normal liver and spleen, suggesting that ZD6474 may also target more mature endothelium which maybe a unique effect of this compound. Endothelial cell markers such as CD31 and CD146 are not specific enough in this manner to make conclusions concerning the maturity of resultant endothelial cells. These experiments should not only provide more information as to the types of vasculature present in tumors, but the dynamic changes in tumor vasculature present.

One final experiment that could be performed along a common vein as the previous objective would be the characterization of pericytes in the tumor. Pericytes characterize mature vasculature, and are supportive cells of this vasculature. I hypothesize that tumor associate pericytes may be evaluated using the FNA technique developed herein. Since endothelial cells and immune infiltrate may be analyzed using the FNA technique described, it is also plausible that pericytes may be identified using this method. Pericytes could be defined by a variety of markers, but are thought to be $CD31^{-}Ang\text{-}1^{+}Thy\text{-}1^{+}Stro\text{-}1^{+}$ [22-24]. It would be plausible to use other pericyte markers, but anti-mouse antibodies for those previously listed are already commercially available for flow cytometry. By better characterizing the pericyte presence in tumors, it may alter the way in which tumor vasculature is viewed, and coalesce well with a better description of the maturity of the vasculature. It is plausible that changes in pericytes and there relation to blood vessels over time may enhance our understanding of angiogenesis and anti-angiogneic therapy.

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Chapter Five

An Orthotopic, Postsurgical Model of Luciferase Transfected Murine Osteosarcoma with Spontaneous Metastasis

Abstract

Osteosarcoma (OSA) is the most common bone tumor in humans. Newer, more clinically relevant models of OSA are required to investigate novel therapeutics. The ability to study spontaneous micrometastases independent of the primary tumor is important. Therefore, we have developed a novel model of murine OSA using the DLM8 cell line, which is syngeneic to C3H mice. We have engineered these cells to express firefly luciferase so the development of metastases can be followed serially and non-invasively. These cells form osteolytic/osteoproductive lesions and metastasize spontaneously after orthotopic implantation in the proximal tibia, and the development of soft-tissue metastasis can be followed serially following amputation. We have demonstrated a significant prolongation of disease-free and overall survival in the surgical adjuvant setting following treatment with doxorubicin or carboplatin, drugs

which form the mainstays of treatment for human OSA. In conclusion, we have developed a novel surgical adjuvant model of metastatic OSA in immunocompetent mice that closely recapitulates the clinical situation, allowing the evaluation of novel therapeutics in the context of minimal residual disease.

Introduction

Osteosarcoma (OSA) is the most common primary bone tumor in humans [1]. Tumor metastases are the most common cause of death from OSA [2]. For this reason, OSA patients typically receive systemic chemotherapy for their disease [3]. Patients who develop OS typically undergo surgery to remove the primary tumor; however, death due to metastasis is the primary concern for these patients [1, 3]. Despite the use of adjuvant chemotherapy, the 5-year survival rate is only 60% illustrating that new therapeutics are needed to treat OSA [3]. Therefore, new model systems of OSA are required in which to evaluate new therapeutics so as to best recapitulate the clinical scenario. Specifically, chemotherapy is typically employed in the surgical adjuvant setting for most highly metastatic human tumors but there are few models in existence that are useful for the study of therapy in this context. Consequently, we have created a luciferase transfected murine OSA model that postoperatively metastasizes from an orthotopic location in syngeneic mice.

Novel murine models that recapitulate the clinical scenario are appropriate and necessary as a screening tool for potential therapeutics. By not removing the primary tumor, treatment models aimed at treating metastatic disease will be influenced by the presence of the primary tumor, making it difficult to discern the effects of treating the metastases from that of solely treating the primary tumor. However, amputation of the tumor bearing limb in a murine model removes this as a source of variability in laboratory studies. Furthermore, the majority of OSA patients have micrometastases at the time of surgery. Therefore, targeting of metastatic disease supersedes the importance

of treating the primary tumor with regard to medical therapy. The development of a surgical adjuvant model, allowing for the direct treatment of micrometastatic disease will allow for trials of increased therapeutic efficacy.

There have been numerous murine tumor models of OSA developed recently [4-17]. These models include xenogeneic [4, 6-9, 13, 15] and syngeneic [10, 11, 14, 17] murine tumor models that have been used to better understand the biology of OSA. Syngeneic models are superior in that they retain immunocompetence and species dependent interactions between tumor and stromal cells [11, 17-19]. However, to our knowledge, none of these models allow for the study of metastatic disease in the orthotopic post-surgical model where the primary tumor can be removed to recapitulate the clinical scenario. However, a post-surgical model of subcutaneous excision is present [20]. Typically, mice are euthanized and organs removed for histologic analysis of metastatic disease. Serial sacrifice of mice requires large numbers of mice to be euthanized, and involves labor intensive analysis. Therefore, it is of great importance to define novel tumor models that better approximate the clinical scenario and allow for minimally invasive serial tracking of metastasis.

The evaluation of novel therapeutics in the metastatic setting is required since this is the primary cause of death in patients with OSA. Evaluation needs to not only include classical cytotoxic chemotherapeutic agents, but could also include novel therapies. Receptor tyrosine kinase inhibitors are currently of much interest today, and it is necessary to determine the effectiveness of these compounds in the treatment of metastatic disease [21-23]. Immunotherapeutics have also been of great interest, and testing of novel agents in the metastatic testing would be of greater relevance than

treatment of heterotopic tumor in a heterotopic site [24-28]. The ability to utilize syngeneic mice and retain the core components of the immune system strengthens the conclusions of the investigation. Maintaining the metastatic process is also of importance, as therapeutics that inhibit this process may be evaluated in a more relevant setting then that of heterotopic sites and intravenous injections as is the case in OSA.

The creation of a better model in which to test novel treatments, specifically in relation to metastases, is crucial for the advancement of OSA therapy. The ability to use immunocompetent mice and study a tumor that spontaneously metastasizes may therefore better approximate the clinical scenario. Furthermore, luciferase transfection of the tumor allows for serial noninvasive tracking of the dissemination of disease, and allows for the study and inhibition of metastases. Therefore, we have developed a murine model of OSA that is luciferase transfected and spontaneously metastasizes after being implanted orthotopically.

Materials and Methods

Cell Lines

The DLM8 cell line was generously provided by Dr. E. Kleinerman (M.D. Anderson Cancer Center) and transfected (described below) to produce the DLM8-luc-M1 cell line. All cell lines were maintained in C/10 [Dulbecco's minimal essential medium (DMEM, Lonza, Walkersville, MD) supplemented with 1X MEM vitamin solution (Cellgro, Henderson, VA), 2 mM L-glutamine (Cellgro), 1 mM sodium pyruvate

(Cellgro), 1X non-essential amino acid solution (Cellgro), 1X antibiotic/antimycotic (Cellgro), and 10% heat inactivated fetal bovine serum (FBS) (Atlas, Fort Collins, CO)]. Once confluent, cells were washed with 1x phosphate-buffered saline and detached with 0.25% trypsin (Cellgro) supplemented with 0.5 mM EDTA.

Animals

All animal studies were performed in an AALAC-approved facility, with approval of the Colorado State University Institutional Animal Care and Use Committee. 6-8 week old female C3H mice were purchased from Harlan-Sprague-Dawley (Indianapolis, IN). DLM8, DLM8-luc, and DLM8-luc-M1 were all injected into mice using $2x10^6$ cells per tumor challenge. Subcutaneous tumor challenge was accomplished by injecting cells subcutaneous into the rear flank. For orthotopic injection, mice were first anesthetized with isoflurane. The surgical site was prepared by first shaving and then cleaning with ethanol. A 23G needle was then used to drill a hole into the proximal tibia. Tumor cells ($2x10^6$) were re-suspended in PBS and injected into the proximal tibia in 50 µL to enhance focal tumor formation. Mice received 0.05 mg/kg of buprenorphine every 12 hours for 72 hours post-tumor challenge to manage pain associated with tumor challenge.

Luciferase Transfection, Imaging, and Selection

The DLM8 cell line was transfected by electroporation with RSV-pGL4.17, a plasmid containing the firefly luciferase gene and a neomycin selection cassette. RSVpGL4.17 was constructed by subcloning a Hind III fragment containing the Rous Sarcoma Virus 5' LTR (RSV) promoter into pGL4.17 (Promega Corporation, Madson,

WI). The resulting transfected cells were named DLM8-luc. Selection was accomplished by treating cells with G418 (500 μg/mL) for 2 weeks post transfection. Several luciferase positive clones were selected and mixed. Populations of the positive clones were expanded and frozen to retain low passage numbers.

To select for luciferase positive cells with metastatic potential to lung, DLM8-luc were passaged through mice a single time using a Fidler selection process [29, 30]. Mice were challenged orthotopically with DLM8-luc and followed by luciferase detection (described below) for luciferase positive metastases. For imaging, mice were injected intraperitoneally with 100 μL of 30 mg/mL luciferin (Regis Technologies, Inc., Morton Grove, IL) 10 minutes before being anesthetized with isoflurane. Imaging was performed using an IVIS 100 imaging system and Living Image 2.50.1 software (Xenogen). A one minute exposure time with high sensitivity binning was used to enhance quantification of the tumor cells. The minimum intensity was set at 10% of maximum and a contour ROI plot with default parameters (ROI edge value of 5%) chosen to increase objectivity of quantification. Total flux of the ROI was recorded as photons/sec for each animal/sample. Once luciferase positive clones were identified, mice were sacrificed and lung metastases were selected. Lung metastases were isolated and collagenase digested before being placed back into *in vitro* culture for further selection with G418 to remove any tumor cells that may have lost luciferase expression. This new line was named DLM8-luc-M1 to reflect the luciferase transfection and single Fidler selection.

Characterization of Luciferase Expression

To characterize the luciferase expression of cells in vitro, 250,000 cells were plated in a 24-well plate format in quadruplicate and serially diluted by 2-fold dilutions. Plates were incubated at 37ºC and 5% CO_2 for 24 hours. Luciferin was then added to each of the wells before imaging with the IVIS system as previously described. ROI were drawn around each well to measure the luciferase expression.

Imaging Modalities

To further characterize the orthotopic tumors, multiple imaging modalities were used. Mice were challenged orthotopically with DLM8-luc-M1 and primary tumors allowed to grow for two weeks. Mice were then imaged for luciferase expression as described above immediately before being euthanized and tissues collected. Tumor bearing and contralateral hind limbs were formalin fixed. Both limbs were then compared using microCT (ScanCo USA Inc., Southeastern, PA). After microCT, limbs were decalcified in dilute hydrochloric acid solution before paraffin embedding and sectioning for H&E staining.

Surgical Adjuvant Model

To more closely recapitulate the clinical situation, a surgical adjuvant model of therapy was created. Mice were orthotopically challenged with DLM8-luc-M1 tumors. At various times following injection, the tumor bearing limb was amputated by coxo-femoral dislocation. Briefly, mice were treated with 0.05 mg/kg of buprenorphine and anesthetized with isoflurane. An incision was made and muscle bellies bisected and

major vessels cauterized before coxo-femoral dislocation. The muscle and subcutaneous tissues were closed using 4-0 maxon suture (Syneture; Norwalk, CT) before closing the epidermis using surgical wound clips (Becton Dickinson; Sparks, MD). Mice were recovered on room air and received 0.05 mg/kg of buprenorphine every 12 hours for 72 hours post-surgery. Mice also received 100 µL of saline IP every 12 hours for 24 hours post-surgery. Weight was monitored as a surrogate for wellness, and mice with a greater than 10% weight loss postoperatively were euthanized.

To determine when metastases developed in the surgical adjuvant model, mice were challenged with DLM8-luc-M1 orthotopically. Beginning 7 days post tumor challenge, mice were amputated every three days as described above. Mice were then imaged for metastases twice a week to determine the time point at which 100% of mice developed luciferase positive metastases.

Characterization of Metastases

To further characterize the metastatic capabilities of DLM8-luc-M1, mice were challenged with orthotopic tumors and tumors allowed to grow for 16 days. The orthotopic tumors were then surgically resected by amputation of the tumor-bearing limb as previously described. Day 16 was selected as the time point after which 100% of mice developed metastases following tumor removal by amputation as evidenced from experiments above. Mice (n=5 per group) were euthanized on day 16 and every three days following. Following euthanasia, lungs, tumor draining lymph node, and ovaries were isolated, formalin fixed, and paraffin embedded. Organs were sectioned into 3 distinct and separate planes before undergoing H&E staining. Each section was then

reviewed by a single trained pathologist (EJE) for the presence of metastases. Images of representative metastases were taken at 100x.

Chemotherapy and Treatment Experiments

To determine if treatment responses could be measured in the surgical adjuvant model, mice were orthotopically challenged with DLM8-luc-M1, and then amputated 16 days post-tumor challenge as determined from previous experiments to ensure that all mice developed metastases. Treatment was then initiated 24 hours post-amputation. Mice (n=5-8 per group) were treated with cationic liposome DNA complexes (CLDC; 200 μL/mouse weekly IV), liposomal clodronate (LC; 200 μL/mouse weekly IV), metformin (250 mg/kg daily IP), carboplatin (60 mg/kg weekly IP), doxorubicin (5 mg/kg weekly IV), or 2-deoxyglucose (2DG; 500 mg/kg thrice weekly IP) for one month postoperatively. Mice were imaged twice a week after amputation to observe the formation of luciferase positive metastases. Mice were euthanized immediately upon showing signs of distress due to metastatic disease.

Statistical Analysis

Statistical analysis was performed using Prism 5 (GraphPad Software, La Jolla, CA). Time to metastasis data was analyzed using Kaplan-Meier log-rank analysis. For all analyses, a p-value of less than 0.05 was considered statistically significant.

Results

Characterization of Luciferase Transfected DLM8 Osteosarcoma Cells

DLM8 cells were transfected to incorporate the luciferase gene as described in the methods. To enhance for the metastatic phenotype, *in vitro* selection was performed and followed by orthotopically challenging C3H mice. Luciferase positive lung metastases were selected and underwent a single round of G418 treatment to further select for luciferase transfected DLM8 cells; resulting in the formation of the DLM8-luc-M1 cell line. Quantification of luciferase intensity of the DLM8-luc-M1 cell line was described by serially diluting cells *in vitro* and imaging using the IVIS system as described in the Methods **(Figure 5.1)**. Chemiluminescence above background levels was clearly detectable in each of the wells and increases in chemiluminescent signal correlated linearly (r^2=0.9639, $p<0.0001$) with increased cell number over this range. A significant (r^2=0.8060, $p<0.01$) correlation was also observed *in vivo* when luciferase intensity was compared to tumor size as measured by longest diameter **(Figure 5.1)**, cross-sectional area, and volume.

Characterization of the Orthotopic Tumor

To characterize tumor growth of the DLM8-luc-M1 cell line, cells were injected orthotopically into the tibias of syngeneic and immunocompetent mice as described in the Methods. Tumors were allowed to grow and could be visualized after intraperitoneal injection of luciferin **(Figure 5.2)**. After two weeks of tumor growth, mice were euthanized and the tumor-bearing and contralateral hind limbs were removed and

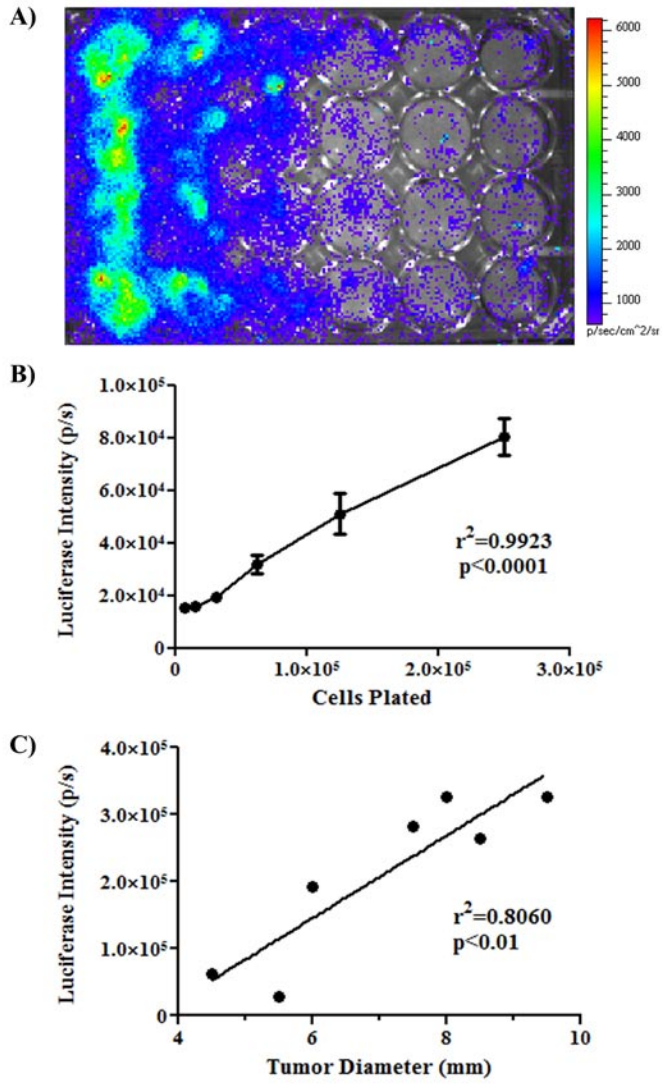

Figure 5.1: *Luciferase expression by DLM8-luc-M1*. A) Luciferase transfected DLM8 cells (DLM8-luc-M1) were plated in two-fold serial dilutions and imaged using the IVIS system as described in the methods. B) Quantification of the luciferase signal over this range of dilutions was observed to have a significant ($p<0.0001$) linear correlation. C) Quantification of the luciferase signal from subcutaneous DLM8-luc-M1 tumors was significantly correlated to tumor diameter ($p<0.01$).

formalin fixed before undergoing analysis by micro-computed tomography (microCT; **Figure 5.2)**. MicroCT revealed osteoproductive/osteolytic areas typical of OS growth when compared to normal bone. After imaging, limbs were decalcified and sectioned for H&E staining. Tumor invasion of the tibia was observed by histology compared to

normal bone **(Figure 5.2)**. Histopathology was characterized by sheets of plump neoplastic spindloid cells producing tumor-associated osteoid and causing significant effacement and osteolysis with residual embedded spicules of bone. The tumor had multifocal areas of necrosis. The tumor extended through bone cortices with a significant paraosteal soft tissue component.

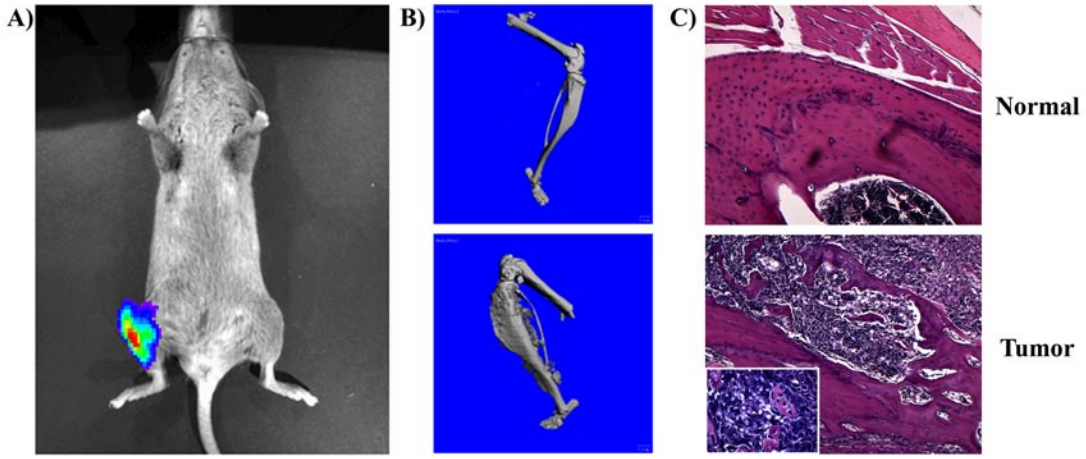

Figure 5.2: *Characterization of orthotopic DLM8-luc-M1 tumors.* Mice were challenged orthotopically with DLM8-luc-M1 tumors as described in the methods. Mice were imaged for luciferase expression (A) from the primary tumor immediately before sacrifice. Both the tumor bearing and contralateral hind limbs were formalin fixed and imaged using microCT (B) before being prepared for histology (C; 200x magnification). Tumor associated osteoid with atypical cells within lacunar like spaces (C inset image; 400x magnification).

Comparison of Orthotopic and Subcutaneous Tumor Metastasis

To compare the metastatic capabilities of the newly formed DLM8-luc-M1 cell line, groups of mice (n=7 mice per group) were challenged with the same number of cells orthotopically or subcutaneously. Mice were then followed for the development of luciferase positive metastases **(Figure 5.3)**. Metastatic disease was first apparent by luciferase imaging at Day 20 post orthotopic challenge and 100% of the mice implanted

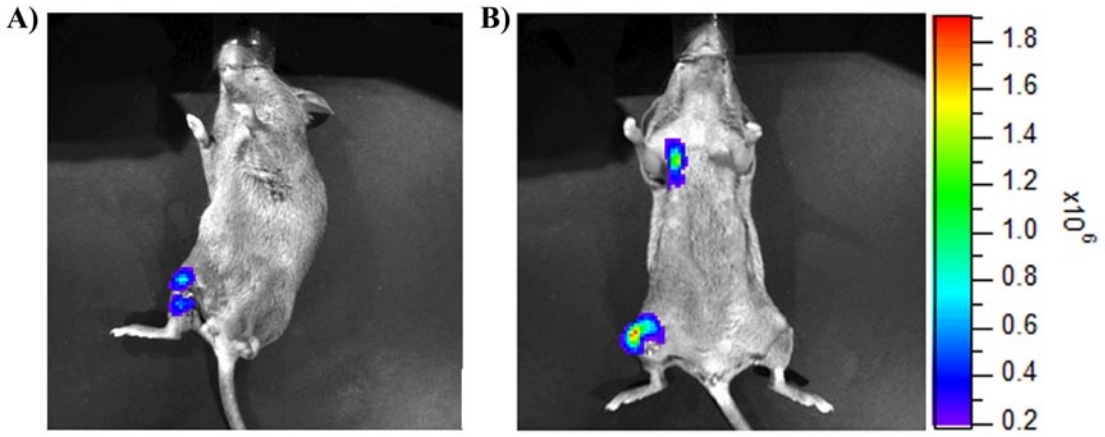

Figure 5.3: *Description of metastases to distant organs.* Mice were challenged orthotopically with DLM8-luc-M1 as previously described. Mice were serially imaged using luciferase to determine the progression of metastases. A) Mouse one week after tumor challenge with primary tumor. B) Mouse three weeks after tumor challenge with lung metastases and primary tumor.

orthotopically metastasized to visceral organs within 27 days after tumor challenge **(Figure 5.4)**. Surprisingly, none of the mice with DLM8-luc-M1 tumors implanted subcutaneously developed metastases before having to be euthanized due to the size (greatest diameter = 15 mm) of their primary tumors. These data confirm previous observations that tumor growth in an orthotopic environment can significantly alter metastatic formation [31, 32].

Characterization of Metastatic Progression in Target Tissues

Having validated the metastatic potential of the DLM8-luc-M1 line, we next determined the metastatic potential of the tumors in a surgical treatment setting. We asked how long the primary tumor needed to be in place prior to surgical treatment to guarantee that 100% of mice orthotopically challenged with DLM8-luc-M1 developed metastases. To accomplish this, a surgical amputation model was used by coxo-femoral

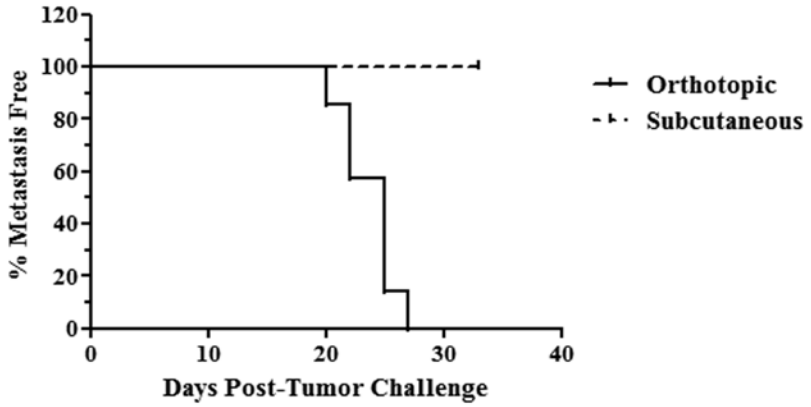

Figure 5.4: *Determination of metastatic capabilities of DLM8-luc-M1 from an orthotopic location.* Mice (n = 7 per group) were challenged orthotopically or subcutaneously with equal numbers of DLM8-luc-M1 tumor cells. Mice were then followed for the formation of luciferase positive metastases in visceral organs. All orthotopic mice had developed distant metastases by 27 days after challenge. However, all mice challenged subcutaneously were euthanized due to the large size of their primary tumors (15 mm greatest diameter), and none were found to have grossly visible metastases post-mortem.

dislocation and amputation of the tumor bearing limb as described in the Methods. Mice were challenged with orthotopic DLM8-luc-M1 tumors which were allowed to grow for 7 days. At this time, and every three days thereafter (days 7, 10, 13, and 16), groups of mice (n=5 per group) were amputated. Mice were then followed for two months to determine if they developed metastases. Mice amputated at 7 days did not develop metastases, suggesting that direct seeding of peripheral organs during tumor challenge did not occur, and that formation and maturation of the primary tumor is required. However, the earliest time point at which 100% of mice developed organ metastases was 16 days post-tumor challenge **(Figure 5.5)**. In mice amputated 16 days post-tumor challenge, detectable metastasis occurred at a median of 24 days postoperatively, and median survival time was 33 days. Gross metastatic disease after sacrifice was predominantly located in the lung, liver, and ovaries of mice succumbing to disease.

These data suggest that experiments can be performed in which all mice orthotopically challenged with DLM8-luc-M1 will develop metastases, thus eliminating variability in metastatic development.

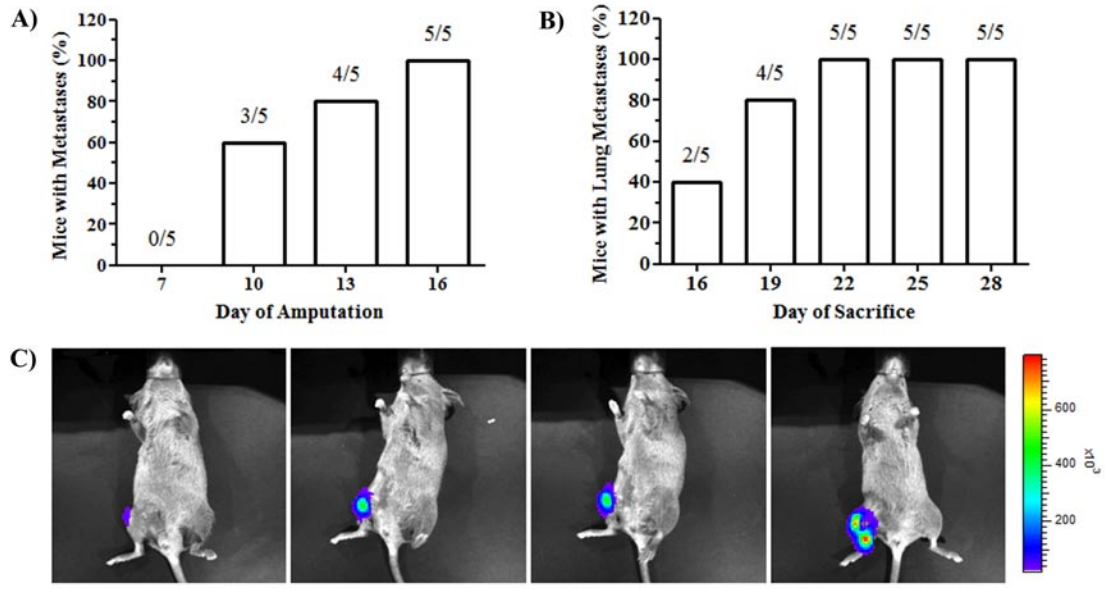

Figure 5.5: *Determination of time to micro-metastatic disease in DLM8-luc-M1 orthotopic model.* To determine the time at which 100% of mice whose primary tumor is amputated develop visceral metastases, mice (n = 5 per group) were orthotopically challenged with DLM8-luc-M1. Beginning 7 days after challenge, and every 3 days thereafter, one group of mice were amputated. Mice were followed to determine if metastases developed by luciferase imaging and gross inspection after sacrifice. The earliest time point at which 100% of mice developed visceral metastases was 16 days post-tumor challenge (A). Representative bioluminescent images of mice before amputation are depicted on a standardized scale (C). To determine the time at which 100% of mice develop histologic lung metastases in the post-surgical model, mice (n = 5 per group) were orthotopically challenged with DLM8-luc-M1, and tumor bearing limbs amputated 16 days after tumor challenge. Beginning 16 days after tumor challenge, and every 3 days thereafter, mice were sacrificed and lungs taken for histologic analysis of micro-metastatic disease. The earliest day at which 100% of mice develop histologic lung metastases was 22 days post-tumor challenge (B).

Characterization of Metastases

Mice were challenged with orthotopic DLM8-luc-M1 as previously described and tumor-bearing limbs amputated 16 days post-challenge. Mice were then euthanized on day 16 and every three days subsequently. Lungs and ovaries were harvested, formalin fixed, and sectioned for characterization of micrometastatic disease. A single board-certified pathologist reviewed the sections for the presence of micrometastases. One hundred percent (n=5 per group) of mice had evidence of micrometastatic disease in the lung by day 22 post-tumor challenge (6 days post-amputation; **Figure 5.5**). Tumor nests were present as early as 16 days post-tumor challenge **(Figure 5.6)**. The primary route of metastasis appeared to be hematogenous, owing to the presence of numerous intravascular tumor nests **(Figure 5.7)**. Tumor draining lymph nodes were also assessed, but there were no tumors present in these tissues. However, it is of interest that none of the mice developed large/gross lung metastases within this time period. Interestingly, a number of mice also developed metastases in the ovaries, and these organs became rapidly and completely effaced **(Figure 5.6)**.

Chemotherapy Delays Metastasis in the DLM8-luc-M1 Orthotopic Surgical Adjuvant Model

To determine if the presence of luciferase positive metastases could be used as an indicator of disease progression, we combined chemotherapy with the surgical adjuvant model previously described. The goal was to recreate the clinical scenario that accompanies treatment of OSA patients. Mice were challenged with orthotopic DLM8-luc-M1 tumors which were allowed to grow until seeding by micrometastases had

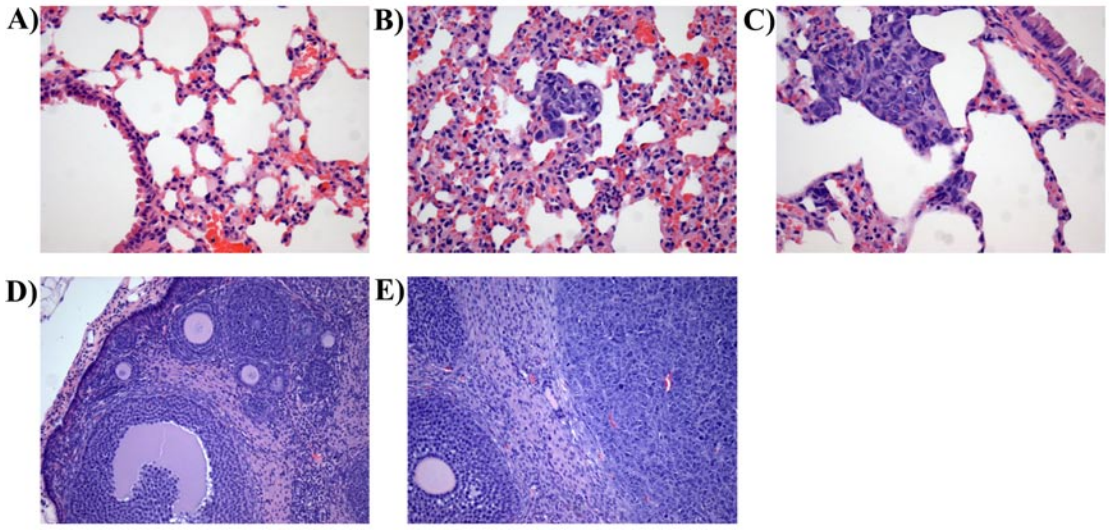

Figure 5.6: *Characterization of lung and ovary metastases in the post-surgical setting.* Mice were challenged orthotopically with DLM8-Luc-M1 tumors. Tumor bearing limbs were amputated 16 days post-challenge. Beginning on day 16 and continuing every three days thereafter, mice were sacrificed and lungs and ovaries were formalin fixed. All samples were evaluated for the progression of metastases and representative images shown at 100x magnification. A) Normal Lung. B) Small nest of cells in the lung. C) Large nest of pulmonary metastasis. D) Normal Ovary. E) Effaced ovary with normal follicle in field.

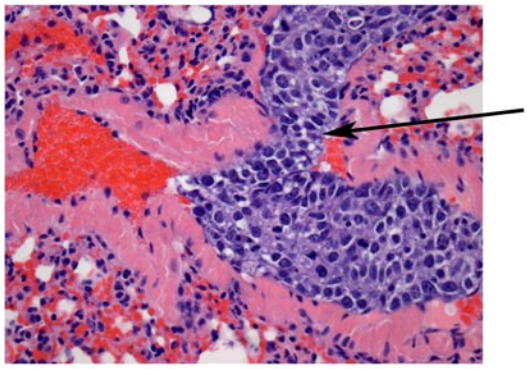

Figure 5.7: *Hematogenous metastasis as evidenced by intravascular nest in the lung.* Mice were challenged with DLM8-luc-M1 and amputated 16 days pos-tumor challenge as previously described. Lungs were placed in formalin as previously described. Presence of an intravascular nest with parenchymal invasion (arrow) is present suggesting the route of metastasis for lung metastases.

occurred (16 days post-tumor challenge) and mice were amputated as previously described. Twenty-four hours post-amputation, chemotherapy or immunotherapy were initiated as described in the Methods. Doxorubicin and carboplatin are considered standard of care in the adjuvant of OSA treatment, and were thus chosen for these experiments [33, 34]. 2DG is a glycolytic inhibitor which exploits metabolic changes in cells addicting them to glycolysis [35, 36]. CLDC and LC are immunotherapies previously described [25, 26]. Metformin is a mitochondrial inhibitor and acts as an inhibitor of gluconeogenesis [37, 38]. We found that treatment with doxorubicin ($p=0.007$), carboplatin ($p=0.004$), 2DG ($p=0.0246$), and a combination of doxorubicin and 2DG ($p=0.0029$), led to a significant delay in time to detection of luciferase positive metastases **(Figure 5.8)**. There was also a significant ($p<0.05$) increase in survival time in the treated mice when compared to control except mice treated with 2DG had a significant increase in DFI, but not overall survival ($p>0.05$) **(Figure 5.8)**. Metformin, CLDC, and LC failed to significantly ($p>0.05$) increase DFI or overall survival **(Figure 5.8)**. Therefore, we have shown that the orthotopic implantation of the DLM8-luc-M1 cell line followed by amputation of the tumor bearing limb after the production of micrometastases can be used to investigate the therapeutic efficacy of novel compounds for the inhibition of metastases. Thus, the surgical adjuvant model proposed herein in combination with chemotherapy may be used to further elucidate efficacious therapies and combinations of therapies in a syngeneic and immunocompetent model of OSA.

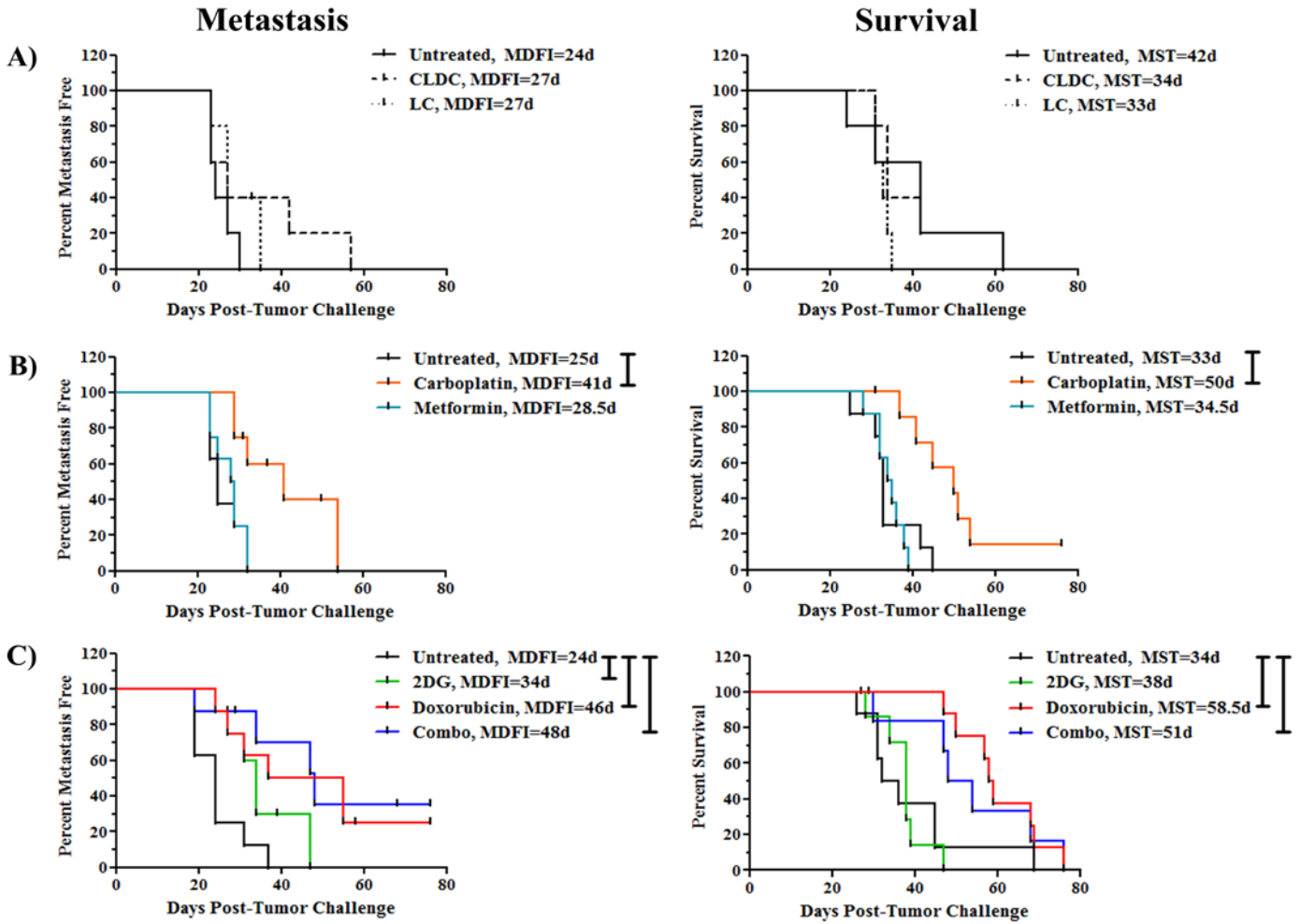

Figure 5.8: *Osteosarcoma metastases are inhibited by chemotherapy.* Mice (n=5-8 mice per group) were challenged orthotopically challenged with DLM8-luc-M1. 16 days later, mice were amputated. 24 hours post-amputation, treatment was initiated and DFI, as determined by luciferase positive metastases, and survival measured. A) Treatment with cationic liposome DNA complexes (CLDC) or liposomal clodronate (LC). B) Treatment with carboplatin or metformin. C) Treatment with doxorubicin, 2DG, or a combination thereof (combo). All significant comparisons ($p<0.05$) are denoted by bars between groups as determine by Mantel-Cox log rank analysis.

Discussion

Osteosarcoma is the most common primary bone tumor in humans and dogs [3, 33]. The primary cause of death in osteosarcoma is the development of metastatic disease. Consequentially, novel tumor models that are able to recapitulate the clinical scenario are required to screen new therapeutics for OSA. Therefore, we created a luciferase transfected murine OSA cell line that can be implanted orthotopically in syngeneic immunocompetent mice and spontaneously metastasize after removal of the tumor bearing limb. We have also identified a time point at which 100% of the mice develop micrometastases, further strengthening this model and decreasing inter-experiment variability. Furthermore, the ability to serially image these mice and define a time to luciferase positive metastases in a chemotherapy treatment model suggests that this model may be used to investigate novel therapeutics for the treatment of osteosarcoma metastases.

There are numerous models of OSA in mice currently, encompassing human xenografts [4, 6-9, 13, 15] and syngeneic murine tumors [10, 11, 14, 17]. These models can further be described by those that are luciferase transfected [14, 15], and those that may be implanted orthotopically [4, 6-8, 10, 11, 13-15]. However, to our knowledge, this is the first model that is luciferase transfected in immunocompetent mice that utilizes a surgical adjuvant approach.

Our goal was to define a model that closely resembled the clinical experience in which patients undergo surgery for removal of their primary disease so as to provide a more relevant model for investigating novel therapeutics. The tumor model developed

was based upon the DLM8 tumor model previously described [17]. The ability to transfect tumors with luciferase has allowed for the systemic tracking of metastasis formation [14, 15]; however, study of metastases independent of the primary tumor has not yet been evaluated. It has been shown that orthotopic tumor models better approximate the tumor microenvironment of naturally occurring tumors in humans, and thus serve as better models of disease [31, 32, 39]. It has also been suggested that the use of syngeneic tumors in immunocompetent mice is superior to that of human xenografts in nude mice [18, 19]. By using immunocompetent mice, natural interactions between the tumor and host microenvironment and immune system are preserved. The production of various factors from human tumor xenografts may not properly interact with their mouse counterparts as has been previously shown in various chemokines and cytokines [40]. The use of nude mice disrupts these interactions, and leads to the murine stromal and immune components interacting with human tumor cells [18, 19, 41, 42]. Therefore, we sought to create a model in syngeneic mice that could be implanted orthotopically and would metastasize spontaneously.

It has been shown that 20% of patients presenting with OSA will have clinically detectable metastases at diagnosis, although it is believed that a much higher percentage of patients have micrometastatic disease at this time [34, 43]. It is noted that 30-40% of patients with OSA will die from metastases, primarily to the lung, despite aggressive medical therapy [34, 43, 44]. Therefore, we defined a time at which 100% of mice challenged orthotopically with DLM8-luc-M1 have micrometastatic disease to decrease inter-experiment variability. Much like the clinical scenario, we are able to amputate the tumor bearing limb and remove the primary tumor while retaining the metastatic disease.

The ability to track metastases using a non-invasive modality such as luciferase imaging is a powerful tool that can be used to assess time to metastasis of novel OSA therapeutics.

We observed that a majority of mice developed histologically detectable micrometastatic lung disease within a week after amputation, with the presence of disease present in some mice on the day of amputation. The observation that mice develop pulmonary micrometastatic disease, but rarely succumb to these metastases upon gross inspection, suggests that these tumor cells are not able to form large tumor masses in the lung environment. It has been previously shown that numerous events are required for metastases to form in a peripheral organ [45-48]. For example, tumor cells must extravasate from circulation and colonize the foreign environment before expanding into larger metastases [45]. We have observed that metastasis of this model is by hematogenous spread, which mimics previous observations in human patients [49]. We have also demonstrated that tumor emboli that enter the lung invade into the vessel wall with extravasation into the adjacent parenchyma. It has previously been suggested that the transfection with GFP may alter OSA biology, and the transfection with luciferase may imply a similar caveat here leading to the altered behavior of lung metastases [50]. Furthermore, differences in the metastatic capabilities of various cell lines have been previously observed, and might be the cause for these observations [10, 12]. Even though mice do not succumb to gross lung metastases, these micrometastatic nodules may still be used to assess efficacy if novel treatments, since the lung can still be harvested and micrometastatic foci enumerated for comparison between treatment groups.

Previous studies have investigated the effects of a variety of therapeutics on primary orthotopic tumors [51, 52]. Therefore, the ability to evaluate novel therapies in relevant tumor models is crucial to discovering new treatment modalities. Interestingly, we observed that a novel agent (2DG) inhibited metastasis but did not significantly increase overall survival. It is plausible that the inhibition of the glycolytic pathway led to a decrease in cellular ATP, a known co-factor of luciferase, and diminished the luciferase signal so that metastases could not be visualized by this imaging technique although present. Further investigation into the phenomenon is currently being performed. However, conventional chemotherapeutic agents, carboplatin and doxorubicin, delayed metastatic formation and significantly increased survival. Combination of 2DG and doxorubicin was performed based on prior evidence in the literature suggesting this as a synergistic combination for the treatment of other tumors, although this does not appear to be true within this model [53].

In diseases such as OSA, where treatment of metastasis is of critical importance, it is important to have models where the clinical scenario is closely recapitulated. Treatment of micrometastases may be very different than treatment of primary tumors. In mouse models, many primary tumors are induced with large numbers of cells injected into a small focus, and typically in heterotopic sites. Our model allows the primary tumor to mature and metastasize, thus selecting for cells with a sufficient metastatic phenotype that must undergo intravasation, circulation, extravasation, and survival in distant tissues [45-48]. Indeed, study of these events and the role that various therapies may play in altering these pathways may be studied and targeted. The treatment of micrometastatic disease in animals is the most relevant model present, for it recapitulates

many of the pressures associated with the process of metastasis which cannot be studied *in vitro* or in primary tumors [18, 19]. By using a model that retains the metastatic phenotype, it may be possible to study the mechanism of metastasis as well as develop novel therapeutics to inhibit not only the metastatic foci present, but the process of metastasis as a biologic endpoint.

We have created a model of murine OSA that allows for the development of spontaneous micrometastases. The removal of the primary tumors is crucial because it differentiates treatment effects on the primary tumor from those indirect effects of tumor micrometastases. This model may thus be used to study the processes associated with metastasis and investigate novel therapeutics for the treatment of OSA. The transfection of luciferase into the DLM8 cell line allows for serial non-invasive imaging of metastases and acts as a positive indicator for the development of metastases in distant organs. Therefore, we believe that this model may allow for the study of novel OSA therapeutics and elucidation of mechanisms of metastasis.

Future Directions

In this chapter, we have explored the development of a novel model of osteosarcoma that spontaneously metastasizes in the post-surgical setting. The creation of this model will allow for the evaluation of the hypothesis made in chapter 2 that localized infection can systemically inhibit tumor growth. I hypothesize that localized infection can inhibit spontaneous and systemic micrometastatic disease in this model.

Preliminary data using a model in which mice are orthotopically challenged with tumors as described in this chapter and then infected with biofilms present on catheter segments (similarly to that presented in chapter 2) suggests that infection can inhibit systemic tumor growth and metastases **(Figure 5.S1)**. However, repeating these experiments is important to determine the validity of these findings, although preliminary observations are promising. It would also be interesting to combine infection with chemotherapy to best mimic the clinical scenario. Dogs developing infections also receive adjuvant chemotherapy, and incorporating this methodology into the mouse model would strengthen the conclusions made concerning inhibition of osteosarcoma by osteomyelitis/infection. Combination of this work with the proposed studies in chapter 2 investigating localized and systemic changes in infiltrating cells may better explain the phenomenon observed in the clinic.

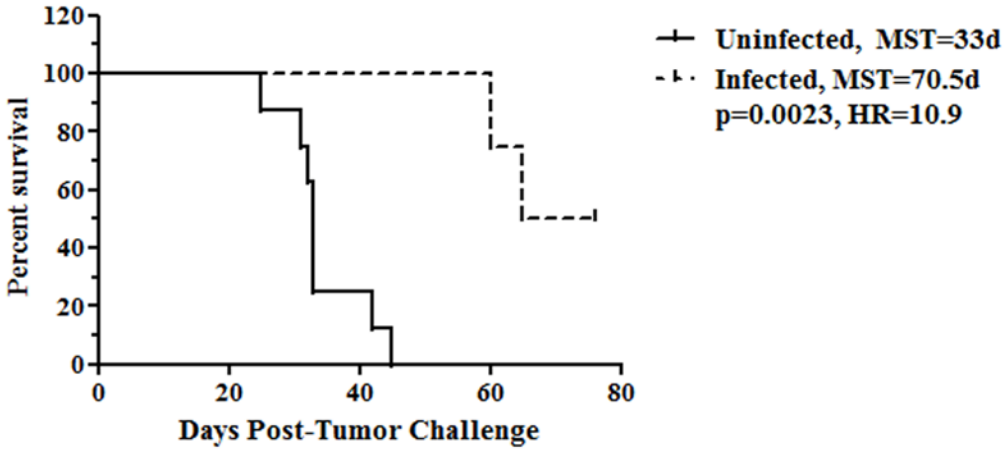

Figure 5.S1: *Infection increases survival in the post-surgical model.* Mice were challenged orthotopically with DLM8-luc-M1 and tumor bearing limb amputated 16 days post-challenge as previously described. Twenty-four hours after amputation, mice were challenged with infected catheter segments subcutaneously in the dorsum. Survival was significantly ($p<0.05$) increased for infected mice (n=4) compared to uninfected mice (n=8).

In this chapter, the observation was made that 100% of mice develop lung metastases in this model. However, few mice succumb to gross lung metastases, and rather succumb to disease localized to other organs. It would be of interest to further characterize these lung metastases, and describe differences between metastases of different mice. I hypothesize that mice that succumb to lung pathology have been selected for differently by the metastatic process and have a variant genetic expression profile compared to the primary tumor. To investigate this hypothesis, a group of mice would be challenged orthotopically before undergoing amputation as previously described. In order to circumvent death due to large ovarian metastases, male or ovectemized female mice may be used for study. At the time which the first mouse succumbs to disease (pulmonary or otherwise) lungs from both groups of mice would be taken and analyzed by microarray after being histologically described and graded. Comparisons would then be made between mice with microscopic lung pathology, and those with more extensive pathology. Genetic analysis would be performed to determine if clonal selection of tumor cells was a factor in enhancing the progression of lung metastases in a cohort of mice.

Analysis of the entire tumor burden is required since stromal and micro-environmental factors are likely playing a role in this phenotype and need to be included in the analysis. If genes of interest are discovered, analysis of the tumor cells themselves versus the stromal components in both the primary and metastatic lesions would be of interest. Differences in angiogenic factors, immune inhibition factors, pro-metastatic factors, and survival factors would likely be the basis of this analysis. PCR would then be performed to validate genes of interest by this technique. Furthermore, description of

the lungs and tumor microenvironments could also be performed by IHC to better characterize the lung environment to determine if changes in this location may have altered the progression of these metastases. For some reason there is a pre-disposition to some mice succumbing to lung metastases even though all mice develop small metastatic foci in the lungs. Similarly, it would be interesting to determine why this tumor invades the ovaries since this is not typically a location of metastasis in clinical disease. It is therefore important to understand these clonal variants and better describe them for they may speak to larger question in metastasis and OSA biology.

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Chapter Six

Increased Circulating Monocytes and Lymphocytes are Associated with Decreased Disease-Free Interval in Dogs with Osteosarcoma

Abstract

Identification of biomarkers that could better predict outcomes in dogs with osteosarcoma would be valuable to veterinarians and owners. Recent studies suggest that leukocyte numbers in peripheral blood may be associated with outcomes in some types of cancer in humans. We therefore examined whether pre-treatment numbers of monocytes and other leukocytes were associated with disease-free interval in dogs with appendicular osteosarcoma. Medical data from 313 dogs with osteosarcoma evaluated at Colorado State University over a 4-year period, including 69 dogs with appendicular osteosarcoma treated with amputation and chemotherapy that were selected for additional study. This was a retrospective study that evaluated possible associations between DFI and

leukogram values, tumor location, and serum alkaline phosphatase in osteosarcoma patients. Statistical associations were assessed using univariate and multivariate analysis. Higher pre-treatment numbers of circulating monocytes and lymphocytes were significantly associated with shorter DFI in dogs with osteosarcoma. Other parameters associated with poor outcomes were elevated alkaline phosphatase, primary tumor location, and age. These results indicated that pre-treatment evaluation of monocyte and lymphocyte counts provided prognostic information for dogs with appendicular osteosarcoma. Notably, most animals in this study had monocyte counts within the normal reference range, indicating that variations within the normal range of leukocyte values may also have prognostic significance.

Introduction

Osteosarcoma (OSA) is the most common primary bone tumor of dogs [1, 2]. The median survival time in dogs with OSA is approximately 1-year following surgery and adjuvant chemotherapy, with fewer than 30% becoming long term survivors [3]. Metastasis to the lungs is most common cause of death in dogs with OSA [3]. Given the fact that less than 50% of dogs with OSA will survive beyond a year, there is a need for biomarkers that might predict disease outcomes more effectively.

Many of the biomarker assays currently being investigated involve the use of gene expression profiling and proteomic approaches [4-6]. However, at this time, many of these techniques are time consuming and prohibitively expensive, particularly for veterinary patients [4]. Moreover, few of the gene profiling or proteomic approaches have been validated in dogs. To date, the serum alkaline phosphatase (SAP) concentration is the only biomarker shown to be associated with disease free interval (DFI) and overall survival (OS) in dogs with OSA [7-9]. Thus, there remains a strong need for reliable, easy-to-use, and cost effective assays for determining prognostic information for dogs with OSA.

Previous studies have shown that circulating monocytes can give rise to tumor promoting macrophages in mice, and are directed to the tumor by monocyte chemoattractant protein-1 (MCP-1) [10-14]. Recently, a great deal of attention has been focused on the role of macrophages in promoting tumor growth [14-16]. Moreover, several studies in human cancer have demonstrated a correlation between pre-treatment monocyte counts and tumor outcomes [17-21]. Furthermore, increased numbers of

macrophages in tumors have been associated with reduced survival times in humans with a variety of tumor types [22-25]. Therefore, it is plausible that increased monocytes may be prognostic in canine OSA.

Other studies have found that numbers of circulating lymphocytes also have prognostic relevance for human cancer patients [19-21, 26]. For example, increased numbers of total lymphocytes has been associated with increased survival in pancreatic and renal tumor patients [27, 28]. More recently, lymphopenia was found to be a negative prognostic finding in humans with various cancers [29]. Therefore, it is plausible that pre-treatment numbers of circulating lymphocytes may also be prognostic in dogs with OSA.

In the current study, we evaluated whether numbers of circulating leukocytes were associated with outcome (DFI) in dogs with OSA. The study retrospectively evaluated a relatively uniform population of dogs with appendicular OSA that underwent amputation followed by adjuvant chemotherapy. Other factors potentially associated with outcome, including age, primary tumor location, and SAP concentration were also evaluated. The results of these studies suggest that evaluation of pre-treatment leukogram values could yield important prognostic information for dogs with OSA.

Materials and Methods

Patient Selection

The study included a total of 313 dogs evaluated for OSA at the Animal Cancer Center at Colorado State University (CSU) between 2003 and 2006. Inclusion criteria included: 1) a diagnosis of appendicular OSA without metastases as determined by 3-view thoracic radiographs +/- ^{99}Tc scintigraphy; 2) treatment by amputation; 3) treatment with standard adjuvant doxorubicin (30 mg/m^2), carboplatin (300 mg/m^2), or an alternating combination of both therapies; 4) CBC and blood chemistry performed at CSU within one week prior to surgery. From the original 313 dogs that were screened, 69 were identified that met entry criteria. Disease free interval (DFI) was defined as the time between treatment initiation and detection of tumor metastases. It was recommended that patients undergo 3-view thoracic radiographs every 2-3 months following diagnosis to determine metastatic progression and thus DFI.

A second population of dogs with OSA that were treated at the Animal Cancer Center at CSU was evaluated using identical criteria to assess the reproducibility of results obtained from the first population. Dogs included in the original data set were excluded from this second population. A total of 21 dogs were identified in the second population (Population 2) and were compared to the 69 dogs in the first population (Population 1) and the 244 dogs excluded from population 1. Leukocyte values were also compared between dogs in Population 1 and Population 2.

MCP-1 Analysis

To determine if MCP-1 levels were associated with monocyte counts or DFI, serum from 31 of the dogs in population 1 archived at the Colorado State University Animal Cancer Center was obtained. Serum was analyzed using a commercially available ELISA for canine MCP-1 (R&D Systems, Minneapolis, MN). Analysis was then performed to investigate the association between MCP-1 and pre-treatment monocyte number, as well as the prognostic significance of pre-treatment MCP-1.

Statistical Analysis

Statistical analysis was performed using Prism 5 (GraphPad Software, La Jolla, CA) and SAS and PROC PHREG software (SAS v9.2, SAS Institute Incorporated, Cary, NC). Variables assessed for association with DFI included age (years), sex (male/female), breed, primary tumor location (humerus versus all other locations), serum alkaline phosphatase (SAP, normal (30-142 IU/L) or elevated (>142 IU/L), and leukocyte values obtained by CBC (segmented neutrophils, lymphocytes, monocytes, and eosinophils).

Population parameters between dogs included and excluded from the study were compared statistically using a two-tailed Mann-Whitney t-test since the variables were not normally distributed, as determined by the D'Agostino and Pearson normality test. Population parameters between dogs in Population 1 and Population 2 were also compared using Mann-Whitney t-test. Comparison of dichotomous variables (e.g. sex) between the groups was performed using a Fisher's exact test. Estimates of disease-free interval were generated using the Kaplan-Meier method. For univariate analysis,

categorical variables (e.g. leukogram values and age) were divided into two groups based upon the median (≤median and >median) value of all patients included in the study. Comparisons were then made using a Mantel-Cox (log-rank) test to determine significance.

A multivariable Cox's proportional hazard regression analysis was performed to determine the independent leukogram variables significantly associated with DFI. Variables and covariates such as: location, chemotherapy, SAP, age, sex, and breed were included in the model to account for their effect in the analysis. The groups determined by division of the median were also used for this analysis. PROC PHREG in SAS v9.2 allows inclusion of a 'CLASS' statement with reference specified for each categorical variable. Selection process of the significant variables was performed using a 'Stepwise backward elimination method' taking 'p to enter=0.25' and 'p to stay=0.1'. The variables that would impact the outcome (CBC variables in question) were forced into the model and other variables moved in and out of the model as per the selection criteria through the model building process.

The median and the inter-quartile range are depicted throughout all tables. All statistical tests had a significant cut-off of $p<0.05$.

Results

Patient Selection and Overview

Sixty-nine of the 313 dogs with OSA evaluated met the inclusion criteria and were included in the study **(Table 6.1)**. None of the included dogs received cytotoxic chemotherapy or surgery for their tumor prior to the time blood was collected for CBC and serum biochemical analysis. There were 244 dogs that did not meet inclusion criteria for the following reasons: presence of a second, unrelated tumor (n=3); incomplete evaluation for metastasis at the time of diagnosis (n=2); limb sparing surgery performed instead of amputation (n=69); axial tumor location (n=38); metastasis present at time of diagnosis (n=7); pre-treatment CBC result not in record (n=22); previous tumor diagnosed or treated (n=5); adjuvant chemotherapy not administered, or pursued at another institution and specifics of chemotherapy not detailed in the medical record (n=58); radiation therapy administered (n=10), surgery performed at a veterinary hospital other than CSU (n=11); or animal treated with non-standard chemotherapy (n=19). The medical records of dogs in the study were evaluated to obtain information on sex, breed, weight, age, primary tumor location, SAP, and CBC results, and DFI.

The signalment parameters of the 69 dogs included in the study were compared to signalment information from the 244 dogs excluded. We found no significant differences in age ($p=0.676$), body weight ($p=0.333$), or sex distribution ($p=0.785$) of dogs in the two populations.

Table 6.1: *Patient characteristics of 69 dogs in the primary population.*

Age (Yrs)	Median (Range)	8 (2-14)
Weight (Kg)	Median (Range)	37 (18-107)
Sex		
	Spayed Female	28
	Intact Female	1
	Castrated Male	38
	Intact Male	2
Breed		
	Mix	16
	Labrador retriever	11
	Greyhound	7
	Golden retriever	6
	Rottweiler	6
	Great Pyrenees	5
	Doberman Pinscher	3
	Mastiff	3
	English Setter	2
	Irish Setter	2
	Other (1 each)	8
Primary Tumor Location	Radius	22
	Humerus	21
	Tibia	12
	Femur	11
	Ulna	2
	Scapula	1

Univariate Analysis of 69 Dogs Included in the Study

Next, we sought to determine if leukogram values in the 69 dogs in the primary study population correlated with disease-free interval (DFI). In addition, we assessed whether primary tumor location, age, or SAP correlated with outcomes, based on results of previous studies [1, 7-9, 30, 31]. Twenty of the 69 dogs were censored in DFI analysis

because of loss to follow-up prior to an event (n=14) or euthanasia due to diseases other than cancer (n=6). The median duration of follow-up for the 20 censored dogs was 179 days. The median DFI for the entire population was 194 days, with 29% and 9% of dogs metastasis-free at 1 and 2 years, respectively.

When associations between leukogram values and DFI were assessed using univariate analysis, monocyte count (above or below the median value, 0.4 x $10^3/\mu L$) was the only leukogram variable significantly associated with DFI (p=0.015) **(Table 6.2** and **Figure 6.1)**. Notably, 59 of the 69 dogs in this study had monocyte counts within the normal range (0.1-1.0x10^3 monocytes/μL). This finding is important because prior studies in humans have only evaluated associations between increased monocyte counts (i.e., monocytosis) and outcomes and have not investigated outcome associations with monocyte counts in the normal range [17, 18, 20, 21]. In our study, only 5 dogs had increased monocyte counts (>1.0x$10^3/\mu L$) and 5 dogs had low monocyte counts (<0.1x$10^3/\mu L$). Interestingly, in the 5 dogs with true monocytosis (>1.0x10^3 monocytes/μL), the median DFI was significantly shorter (84 days) when compared with the overall DFI of the 59 dogs with normal monocyte counts (194 days; p<0.0001).

The only other variable associated with DFI that was detected using univariate analysis was location of the primary tumor. Dogs with primary tumors of the humerus had a significantly reduced DFI compared to dogs with primary tumors in other appendicular locations (p=0.002) (**Table 6.2**). All other primary tumor locations were evaluated and the humerus location was the only location significantly associated (p<0.05) with decreased DFI. There was no significant correlation between SAP concentration and DFI in this group of patients (p=0.092) **(Table 6.2)**. Furthermore,

there was no significant association between the type of chemotherapy administered (single agent doxorubicin, carboplatin, or a combination thereof) and DFI (p=0.6638).

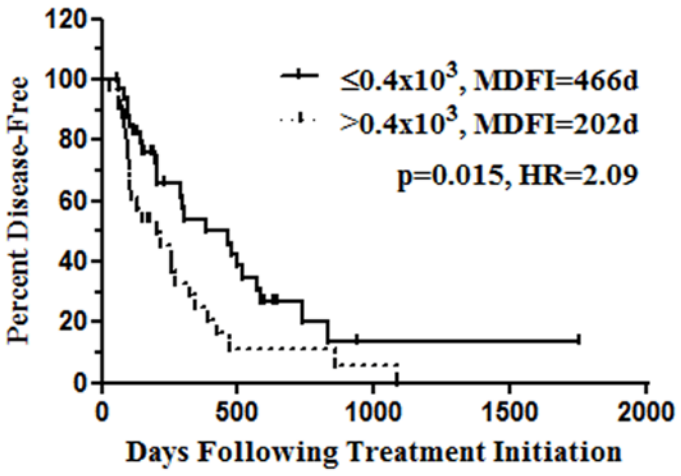

Figure 6.1: *Kaplan-Meier analysis of the relationship between DFI and monocyte counts in dogs with OSA.* Increased monocyte counts (>0.4x10^3 monocytes/μL) were significantly (p=0.015) associated with decreased DFI when compared to dogs with low monocyte counts (≤0.4x10^3 monocytes/μL).

Multivariate Analysis of 69 Dogs Included in the Study

Multivariate modeling was performed using the population of 69 dogs with OSA to identify variables that might be independently associated with DFI. When all variables were subjected to multivariate analysis, it was determined that age (p=0.0156), SAP (p=0.0065), primary tumor location (p=0.0087), monocyte number (p=0.03) and lymphocyte number (p=0.04) were each significantly and independently associated with DFI (**Table 6.3**). Specifically, higher monocyte and lymphocyte counts were associated with significantly reduced DFI, compared to dogs with lower monocyte and lymphocyte counts. It is important to note that treatment with adjuvant doxorubicin and/or platinum based therapy did not significantly (p>0.05) or independently affect DFI.

Table 6.2: *Univariate analysis of population one associations between leukogram and clinical variables and DFI in 69 dogs with appendicular osteosarcoma.*

Variable		Number of dogs	Median DFI (d)	p-value *a)*	HR *b)*	1 Yr *c)*	2 Yr *d)*
Monocyte Count ($x10^3$ cells/µL)							
	≤0.4	35	466	0.015	2.09	54%	27%
	>0.4	34	202			25%	11.03%
Lymphocyte Count ($x10^3$ cells/µL)							
	≤1	36	291	0.138	1.09	45%	15.63%
	>1	33	267			35%	7.81%
Location							
	Humerus	21	194	0.002	3.34	14%	0.00%
	Other	48	383			51%	21.13%
SAP							
	Normal	49	345	0.092	1.79	49%	21.64%
	High	20	149			19%	12.86%
Age							
	≤8	39	204	0.188	0.59	35%	15.63%
	>8	30	345			46%	23.70%

a) p-values were calculated using Log-rank (Mantel-Cox) Test; significance defined as $p < 0.05$.
b) HR = Hazard Ratio
c) 1 Yr = One-year disease free percentage
d) 2 Yr = Two-year disease free percentage

Table 6.3: *Multivariate analysis of associations between leukogram and clinical variables and DFI in dogs appendicular osteosarcoma.*

Variable	**Comparison**	**p-value**	**HR** *a)*	**95% CI** *b)*	
Lymphocytes	High vs. Low	0.041	2.076	1.03	4.18
Monocytes	High vs. Low	0.0311	1.984	1.06	3.70
Age group	>8 vs. ≤ 8yrs	0.0156	0.421	0.21	0.85
Location	Humerus vs. Other	0.0087	2.791	1.30	6.01
SAP	Elevated vs. Normal	0.0065	2.605	1.31	5.19

a) HR = Hazard Ratio
b) 95% CI = 95% Confidence Interval of the hazard ratio

Evaluation of Pre-Treatment MCP-1

Serum from 31 dogs in population 1 was analyzed by ELISA for MCP-1. We first sought to determine if there was an association between the circulating concentration of MCP-1 and pre-treatment monocytes since MCP-1 is one of the primary cytokines involved in eliciting monocytes from the bone marrow. There was no significant ($p>0.05$) relationship between pre-treatment MCP-1 and monocyte counts in these patients **(Figure 6.2)**. Next, we wanted to determine if prognostic information could be obtained from MCP-1 concentrations. Dogs were divided into two groups based upon the median MCP-1 value and compared by Kaplan-Meier log rank test. There was no significant ($p>0.05$) difference in outcome as measured by DFI based upon MCP-1 concentration **(Figure 6.2)**.

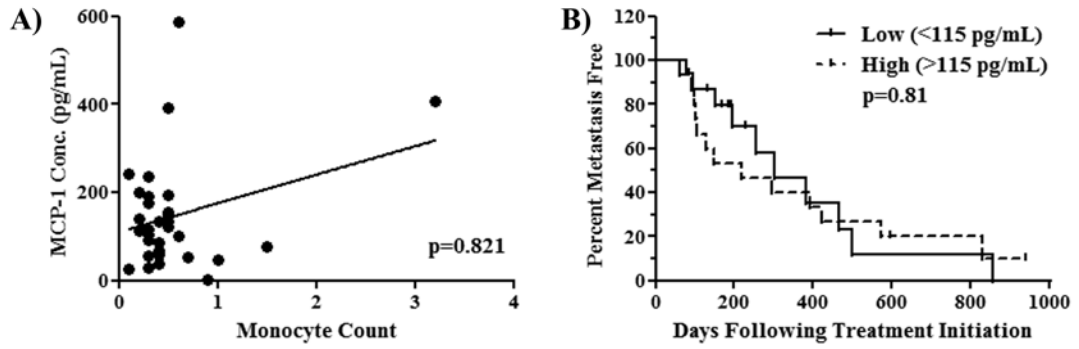

Figure 6.2: *MCP-1 concentration is not correlated with monocyte count or associated with DFI.* Serum from 31 dogs in population 1 were obtained from the CSU-ACC archive and analyzed by commercial ELISA. A) Monocytes and MCP-1 do not correlate by Spearman regression. B) Pre-treatment MCP-1 is not associated with DFI in dogs with appendicular osteosarcoma.

Evaluation of Values in a Second Independent Population of Dogs

Finally, we studied a second population of dogs (Population 2) with appendicular OSA that met the same inclusion criteria used for the original population to determine whether the associations detected in the first study population were reproducible in a second independent population of dogs with OSA. The second population of dogs was also treated at the Colorado State University Animal Cancer Center and 21 dogs met inclusion criteria; these dogs are described in **Table 6.4**.

The signalment parameters of the 21 dogs in Population 2 were compared to those of the 69 dogs included in Population 1 and to the 244 dogs excluded from Population 1. There was no significant difference ($p>0.05$) in age, sex, or weight when these comparisons were made. Furthermore, we also compared the leukocyte variables to those present in population 1 and also observed no significant difference ($p>0.05$) between the populations. Therefore, this population of 21 dogs was comparable, in all important respects, to the 69 dogs included in Population 1.

Table 6.4: *Patient characteristics of 21 dogs in the second population.*

Age (Yrs)	Median (Range)	9.2	(4.3-13.4)
Weight (Kg)	Median (Range)	37	(26-76)
Sex			
	Spayed Female	10	
	Intact Female	0	
	Castrated Male	9	
	Intact Male	2	
Breed			
	Mix	9	
	Rottweiler	4	
	Greyhound	2	
	Other (1 each)	6	
Primary Tumor Location	Radius	7	
	Humerus	5	
	Femur	4	
	Ulna	3	
	Tibia	1	
	Scapula	1	

Associations between monocyte and lymphocyte counts to DFI using identical values as the first population were evaluated by univariate analysis using Kaplan-Meier survival and Mantel-Cox (log-rank) test. Dogs with monocyte counts below the median value (median DFI=595 days) had a significantly ($p<0.002$) increased disease free interval compared to dogs with monocyte counts above the median (median DFI=200 days) **(Figure 6.3)**. Likewise, dogs with lymphocyte counts below the median value (median DFI=470) had a significantly increased ($p<0.03$) disease free interval compared to dogs with lymphocyte counts above the median (median DFI= 221 days) **(Figure 6.3)**. Primary tumor location was significantly associated with DFI ($p=0.0286$), although age ($p=0.6706$) and SAP ($p=0.1772$) were not.

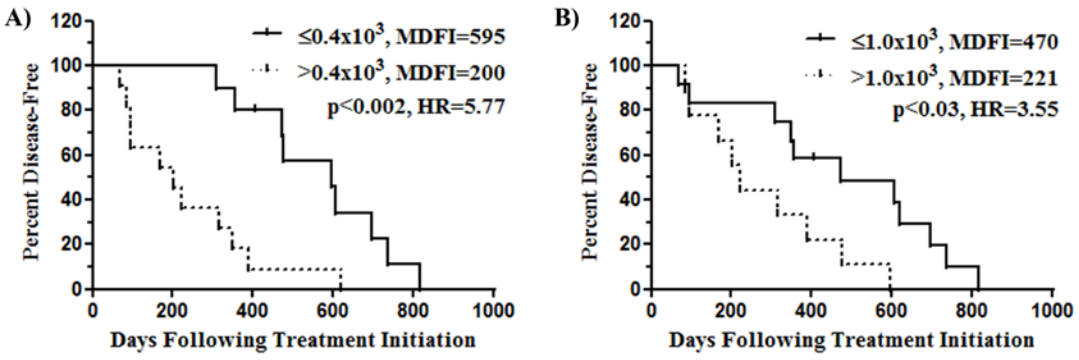

Figure 6.3: *Analysis of the association between monocyte and lymphocyte counts and DFI in a second population of dogs with OSA.* A second population of dogs with appendicular OSA, selected by identical methods, was analyzed to determine the reproducibility of the original analyses. Both monocyte (A) and lymphocyte (B) counts were significantly ($p<0.05$) associated with outcome.

Discussion

Osteosarcoma is the most common bone tumor in dogs, yet the only biomarker that is currently used to assess prognosis is SAP [7-9]. Thus, one goal of the current study was to determine if information from a routine CBC could be used to help assess prognosis in dogs with appendicular OSA. Notably, we found that the presence of increased numbers of monocytes and lymphocytes prior to treatment was significantly associated with decreased DFI in dogs with appendicular OSA. Moreover, multivariate analysis demonstrated that finding increased numbers of monocytes pre-treatment had the highest hazard ratio for association with decreased DFI of any variable evaluated except location of the primary tumor. This finding was also reproduced in a second,

independent population of dogs with appendicular OSA. However, MCP-1 concentration was not associated with monocyte counts or DFI.

The type of adjuvant chemotherapy administered (i.e. doxorubicin or carboplatin alone versus alternating doxorubicin and carboplatin) did not have a significant influence on outcome. The effectiveness of combined treatment with doxorubicin and carboplatin in dogs with osteosarcoma has been contradictory, and further study in a larger population of dogs would be necessary to conclusively determine whether combined treatment was more effective for preventing OSA metastasis [32, 33].

We also found that younger dogs (≤ 8yrs) had a shorter DFI compared to older dogs (> 8 yrs), which is in agreement with a previous report by Spodnick et al [1, 31]. We also observed that dogs with tumors of the proximal humerus had a significantly decreased DFI compared to dogs with tumors in all other appendicular locations. Others have suggested that tumors of the proximal humerus are relatively larger at the time of initial diagnosis, and this may be a plausible explanation for the decreased DFI observed within this study [1, 30, 34]. Interestingly, our univariate analysis did not find that SAP was significantly associated with DFI. However, it is likely that there was a confounding relationship present in the univariate analysis, since the multivariate analysis determined that SAP had an independent and significant relationship to DFI.

It is noteworthy that most dogs in the present study actually had monocyte counts that were within the normal range. Thus, dogs with monocyte counts in the upper end of the normal range, specifically above the median value of $0.4x10^3$ monocytes/μL, had a significantly decreased DFI when compared to dogs with monocyte values below the median. This effect was also observed in the second independent population of dogs that

we evaluated. This suggests that a value of $0.4x10^3$ monocytes/μL may be clinically useful as a cut-off for assessing prognosis in dogs with appendicular OSA. Furthermore, dogs (n=5) with overt monocytosis (i.e. $>1.0x10^3$ monocytes/μL) had a median DFI of only 84 days compared to an overall DFI of 194 days ($p<0.0001$).

One obvious question that arises from this study is why increased numbers of monocytes would be associated with decreased DFI, even in dogs with no obvious evidence of metastasis at the time of initial determination of monocyte count. We speculate that at least some of the monocytes detected on routine CBC may in fact be myeloid derived suppressor cells (MDSC). Recent studies have established that MDSC are comprised of a mixed population of immature monocytes and neutrophils [35-37]. Myeloid derived suppressor cells suppress antitumor immune responses and increased numbers of MDSC are found in mice and humans with cancer [35-39]. Therefore, we propose that the small increase in numbers of circulating monocytes above the median value may reflect increased numbers of MDSC that in turn may account for the decreased DFI observed in these dogs. This hypothesis would further suggest that tumor-specific factors could drive the expansion of MDSC. Such factors could include tumor production of growth factors associated with generation of MDSC, including CSF-1, GM-CSF, MCP-1 and other cytokines and growth factors [35, 36, 40, 41]. Since MDSC are directly involved in the pathogenesis of cancer, this makes them prime biomarkers for the evaluation of cancer progression and metastasis risk. However, confirmation of these hypotheses awaits the development of specific markers for identification of MDSC in dogs.

We also found that pre-treatment lymphocyte counts were associated with DFI in dogs with OSA in this study. Dogs with lymphocyte counts above the median value of $1.0x10^3$ lymphocytes/μL had significantly shorter DFI than dogs with lymphocyte counts below the median. In the present study, there were no dogs with lymphocytosis ($>4.8x10^3$ lymphocytes/μL), while there were 36 dogs with lymphopenia ($<1.0x10^3$ lymphocytes/μL). Dogs with lymphopenia had a significantly ($p<0.05$) longer DFI than dogs with normal lymphocyte counts. Thus, lymphopenia was associated with better outcome in dogs with appendicular OSA. This finding was unexpected and contrary to the results obtained in recent studies in human cancer patients, where lymphopenia was associated with poor prognosis [29, 42]. The explanations for this finding are unclear at present, but may be related to redistribution of lymphocytes from circulation and into tumor tissues in dogs with OSA micrometastases.

This study had several limitations. For one, the study did not assess overall survival times (OST) as an endpoint. Although survival time can be an important prognostic factor, the use of OST as an endpoint can be confounded by other medical problems that arise in older dogs with cancer. It is also possible that the study population assessed here may not be representative of all dogs with OSA. Since the CSU VTH is a referral institution, animals with OSA treated at CSU may represent a sub-group of the overall population of dogs with OSA. However, the length of time over which study patients were collected (4 years for the first population) may help eliminate some potential bias here. In addition, the CSU Animal Cancer Center receives patient referrals from over a wide geographic region, which should help eliminate possible geographical biases.

We observed that MCP-1 was not associated with monocyte count or DFI in this population of dogs. Recently, a study has shown that MCP-1 may be prognostic for colon cancer metastasis, although the authors did not investigate the role of moncytes in this study [43]. MCP-1 is an important mediator of release of monocytes from the bone marrow, and as a possible regulator of monocyte chemotaxis [13, 14]. It is plausible that concentrations of this cytokine in the local tumor microenvironment may have greater impact as a chemoattractant, and these associations were not observed due to sampling of systemic blood for analysis. It is also plausible that other cytokines, such as CSF, are mediating and maintaining the relatively increased numbers of monocytes present in the tumor bearing dogs [44-46].

In summary, we have shown here that routine evaluation of a pre-treatment CBC can provide important prognostic information for dogs with appendicular OSA. Investigations into the factors that regulate circulating monocyte and lymphocyte numbers in dogs with OSA, and how these factors may relate to tumor metastasis and response to chemotherapy will undoubtedly provide important insights into the immune regulation of cancer.

Future Directions

The ability to possibly prognosticate a disease such as OSA from a simple blood test is an exciting proposition. However, the human literature suggests that these observations are much more a function of cancer as a disease than any single tumor type.

Therefore, it would be interesting to determine if a CBC could be used as a prognostic tool in other forms of cancer, human and canine. I hypothesize that the effect would pertain across tumor types and not be directly related to any specific tumor type. For example, a study could be performed in canine soft tissue sarcoma or melanoma with a standard of care therapy implemented for each tumor type. I would also hypothesize that a similar effect would be observed in the study of human OSA due to the similarities present between canine and human disease. As performed previously, it is important to limit the impact that various treatments would have on a tumor so that conclusions would be correlated to the disease state rather than the type of treatment received. However, this conclusion could be controlled for in a multivariate analysis model so long as a sufficient number of patients are included in the study. After selection of dogs and relevant risk factors, analysis of the data would be performed in a synonymous manner to that performed in this chapter. It would be interesting to see what cell types are prognostic in these other tumors. Furthermore, it would be interesting to see what the cut-offs for a positive prognosis would be in these tumors. It is unlikely that the same cut-offs established above would be present, but if they were it may suggest larger ramifications to tumor biology in that there are finite divisions that are present across tumor types for these circulating leukocytes.

The first question may help provide prognostic information for other tumor types, but does not help define the biology of the interactions assessed in the above chapter. Therefore, it would be interesting to further characterize the phenotype of the circulating monocytes. As presented within this dissertation, the role of monocytes in tumor biology may be of significant importance to tumor growth. Therefore, characterization of these

cells may be of importance to understanding the differences in DFI observed herein. I hypothesize that dogs with increased monocytes will also have a greater number of steady state monocytes in circulation. Since steady state monocytes have been implicated in a pro-tumor phenotype, I suggest that they would compose a greater number of circulating leukocytes. However, to fully address this question, an antibody for canine Ly6C would have to be created or identified, which is not a trivial task. However, Ly6C is the murine homolog of human CD59, and thus commercially available anti-human CD59 antibodies might cross-react with canine cells [47]. If not, it would be plausible to prospectively follow dogs by a selection scheme similar to the one used in the study, and serially draw their blood to investigate changes in circulating monocyte populations.

If an association was observed between pre-treatment numbers of steady state monocytes and outcome (DFI/OS), it may suggest a biological explanation for the observations made herein. Preliminary evidence of this observation may be seen in the osteomyelitis model presented in this dissertation. In **Figure 2.8.a** of this dissertation, there is a significant ($p<0.05$) increase in total monocytes in uninfected mice with osteosarcoma compared to normal mice; this figure has been reproduced in part here **(Figure 6.S1)**. Interestingly, when this difference is broken down to investigate differences in steady state ($p<0.03$) and inflammatory monocytes ($p<0.01$), there is a significant difference in both of these groups; with a slight but non-significant ($p>0.05$), bias towards steady state monocytes. Indeed, this data suggests that changes in these subsets are present between tumor bearing mice and untreated controls. Therefore, it is possible that a similar phenomena may be occurring in dogs with OSA, and that these changes are biologically relevant. Furthermore, it would be interesting to see if serial

analysis of circulating monocytes reveals changes in monocytes were associated with outcome. For example, in the osteomyelitis model, an increase in overall monocytes (specifically Ly6C^{+} monocytes) lead to decreased tumor growth. Therefore, it would be interesting to see if chemotherapy or some other intervention could recapitulate this response in the dog model, and if so could alter survival. In other words, can a therapeutic intervention activate Ly6C^{+} monocytes to overcome a possible detriment established by high numbers of Ly6C^{-} monocytes at diagnosis.

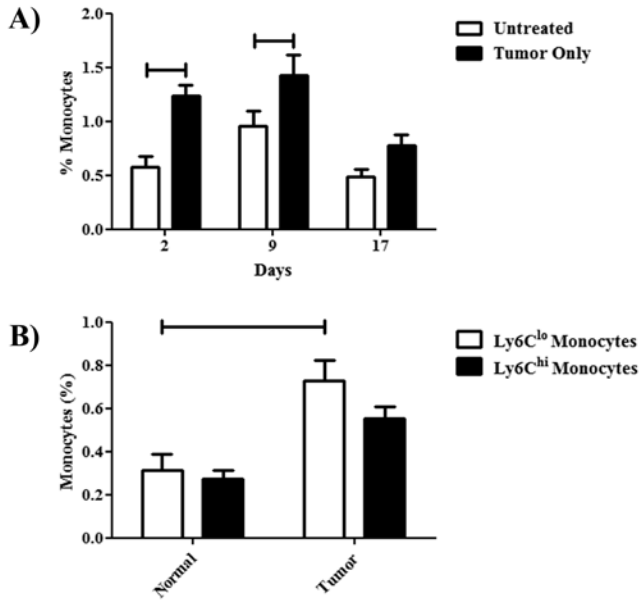

Figure 6.S1: *Increased total monocytes, and subsets, are increased in tumor bearing mice.* Mice (n=5 per group) were challenged with subcutaneous DLM8 tumors or none at all. Mice were tail bled and monocyte populations described by flow cytometry. A) Total monocytes are significantly ($p<0.05$) increased in tumor bearing mice. B) Tumor bearing mice have significantly ($p<0.05$) greater numbers of steady state and inflammatory monocytes. Results are representative of two independent experiments and analyzed by t-test with bars showing significant comparisons.

A similar question I would be interested in asking is akin to the characterization of monocytes. In the above chapter we propose that MDSC may be a component of the increased monocyte population observed, thereby leading to the decreased disease free-

interval observed. I hypothesize that MDSC will be increased in naïve patients with OSA, and that an increase in these cells will be associated with an abbreviated DFI. Flow cytometry and prospective analysis would need to be performed as suggested in the above experiment. Once again, patient selection and treatment should be monitored and incorporated into analysis to adjust for any significant impact these factors may have on outcome.

The final question I would ask would be if treatment with liposomal clodronate (LC), which has been shown to deplete circulating monocytes, would be a beneficial therapeutic for OSA. I hypothesize that LC treatment will be beneficial since it will deplete the inhibitory monocytes present in a naïve patient and allow for re-population with more activated monocytes in the presence of a stimulus, such as infection or possibly chemotherapy. I would further propose combining LC and conventional chemotherapeutics like doxorubicin and carboplatin since both of these therapeutics have inflammatory properties. Induction of tumor necrosis may be a potent inflammatory stimuli leading to activation of monocytes systemically leading to an activated phenotype of tumor infiltrating leukocytes. Therefore, I believe LC may be a possible therapeutic for OSA due to its modulation of monocytes and ability to inhibit tumor growth in mouse models of cancer. Even though the single agent LC results in the orthotopic model (chapter 5) suggest LC is not efficacious against OSA, it is plausible that combination therapy is required so as to repopulate the tumor with activated monocyte/macrophages subsequent to depletion by LC.

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Chapter Seven

General Conclusions

The work presented within this dissertation is intended to further the knowledge base concerning innate immune responses and angiogenesis in osteosarcoma (OSA) biology and metastasis. This research will help clarify novel mechanisms and models to study angiogenesis and OSA biology and metastasis. Furthermore, we are able to use this information to study clinical observations and novel compounds, and hope to eventually translate relevant hypotheses to the clinical setting. The purpose of this work is to expand the information available in the treatment of OSA, and identify novel therapeutics and mechanisms that can be exploited to increase survival.

Clinical observations in dogs and humans provided evidence that osteomyelitis may be a mechanism of increased survival in patients with OSA [1, 2]. We therefore sought to create a murine model of this phenomenon to more directly study the mechanism by which infection leads to systemic inhibition of metastases; and this information was presented in chapter 2. By utilizing a luciferase transfected strain of *S. aureus*, we were able to show that infection localized to the bone could inhibit tumor

growth at a distant site. Furthermore, we were able to show that this effect was not model specific, and was retained when multiple tumor types, mouse strains, and bacterial pathogens were studied. Depletion of NK cells and monocytes/macrophages was then performed, leading us to conclude that both of these cell types are required as mediators of the tumor inhibition in the presence of infection. Characterization of circulating monocytes also revealed that a differential stimulation of inflammatory monocytes was associated with decreased tumor growth in infected mice. Further examination of this mechanism may be performed by further characterization of the tumor associated macrophages and investigation of MDSC as a component of the systemic immune effect. These observations herein provide evidence for the mechanism of tumor inhibition, and provide insight into processes that may be exploited for therapeutic gain in OSA patients.

The duality of inflammation is of great interest, and creates a paradox for cancer research. How can inflammation be a promoter of tumor growth and progression, while also acting as a possible therapeutic as evidenced by the presence of infection? It is possible that the different environments under which these two forces come to bear may lead to differences in tumor growth. Interestingly, the effect may also be tumor type specific, as Coley had once postulated [3, 4]. Initiation of colon/gastric tumors due to an inflammatory stimulus is likely different from that concerning systemic activation of the immune system in response to a foreign pathogen [5]. Moreover, the initial acute phase inherent to the infection may provide priming and reorganization of the immune system which may then be prolonged by the chronic phase of inflammation. However, chronic infection after an acute phase reaction may in turn lead to prolonged effects of the acute response, thereby leading to tumor growth inhibition. For example, it may be necessary

to have a large stimulus to change the status quo adopted by the tumor, and a chronic phase of stimulation to maintain this altered anti-tumor phenotype. However, observations presented within this dissertation show the effect is not specific only to sarcomas, as growth inhibition was observed when mice challenged with melanoma or a colon carcinoma were infected with *S. aureus*. Further investigation into these questions is required to fully understand the complex processes of inflammation.

The duality of inflammation may also make it a possible target for therapy. The inhibition of inflammation that led to tumor promotion may lead to resolving tumor progression. This has been extensively demonstrated in the study of *H. pylori* and gastric tumors and that of the mucosal associated lymphoid tissue (MALT) [5-9]. Instead of providing a large acute inflammatory signal to reorganize the immune system, it is plausible that inhibition of the inflammation may be beneficial, and thus explains the efficacy associated with NSAID therapy for some tumors.

We sought to investigate the role of the NSAID tepoxalin on tumor growth using *in vitro* and *in vivo* analyses (chapter 3). Furthermore, we sought to understand these relationships using therapeutically achievable concentrations, as investigation outside of this range would not be clinically applicable. Interestingly, we observed that tepoxalin has a unique structure allowing it to chelate iron, thus leading to the simulation of a hypoxic phenotype in tumor cells. This hypoxic environment led to dose-dependent increases in HIF-1α and VEGF. We next investigated the role that tepoxalin therapy would play in a murine model utilizing a dose relevant to dogs, the target species for this drug. We observed an inhibition of tumor growth, but were unable to precisely describe the mechanism of action associated with this inhibition. Therefore, we proposed further

experiments to better describe the tumor microenvironment and investigate tepoxalin as an anti-metastatic compound in addition to standard of care chemotherapy in murine experiments culminating in canine clinical trials if promising results are observed.

In each of the first two chapters, we have investigated the role of various phenomena concerning the endpoint of angiogenesis. However, we were unable to investigate the changes in angiogenesis over time in single animals so as to determine a real-time assessment of these changes. Angiogenesis is a critical component of tumor development, but analysis of this component is time consuming and requires large numbers of animals [10]. Therefore, we proposed that fine needle aspiration (FNA) of tumors would provide a sufficient number of endothelial cells for analysis by flow cytometry (chapter 4).

Herein, we have provided evidence to show that FNA of tumor are representative of the overall tumor burden as compared by flow cytometry. Moreover, we are able to show that FNA/flow cytometry is comparable to the gold standard method of immunohistochemistry (IHC) for assessing angiogenesis. IHC is time consuming and requires large amounts of primary tissue, typically resulting in sacrifice of tumor bearing mice and invasive biopsies in humans. However, FNA is minimally invasive and allows for repeated analysis of single tumors over time without production of an artifact in relation to endothelial cells. Surprisingly, we observed a novel mechanism concerning the dynamic changes present in tumor angiogenesis, in which it appears that the percentage of endothelial cells rapidly declines once the tumor reaches a size of approximately 1 cm. This observation could only have been made by serially sampling mice or serially euthanizing large numbers of mice. Furthermore, the ability to

investigate changes in angiogenesis in response to anti-angiogeneic compounds further suggests that this technique may be used in investigational and clinical use. More work is required to characterize the dynamic changes of angiogenesis described herein, along with exploration of vascular supporting cells in the tumor and their role in angiogenesis and tumor biology.

Murine models are the primary means of *in vivo* testing of therapeutics and understanding mechanisms of action of metastasis. However, many murine models utilize tumor cells injected at heterotopic sites or intravenously, and thus do not recapitulate the full process of metastasis with integration of the tumor microenvironment in an orthotopic environment [11-15]. Further, no model currently exists that fully recapitulates the surgical options for patients with OSA. The use of a post-surgical model is important, because this is the arena in which many therapeutics will be utilized in the clinical setting, and provides the most appropriate testing site for these novel compounds. We therefore designed an orthotopic murine model of osteosarcoma in syngeneic mice that is luciferase transfected to allow for non-invasive analysis of metastatic progression (chapter 5).

The DLM8 tumor cell line is syngeneic to C3H mice and has been previously described. We luciferase transfected the DLM8 cell line and selected cells that retained the metastatic capabilities of the parental cell line so metastases could be non-invasively monitored. Characterization of the primary tumor and metastatic variation from orthotopic and heterotopic sites was also performed. A surgical model of amputation was created to recapitulate the clinical scenario in which the primary tumor burden is surgically excised. We then characterized the time to metastasis in the post-surgical

setting to define a model where metastases reproducibly developed in 100% of the mice challenged with tumors. Investigation into the characterization of these metastases was also performed. Lastly, we tested conventional and novel therapeutics for OSA in this model, and found that we could detect a significant inhibition in time to metastasis. It would be interesting to revisit and further define the characteristics of the metastatic process, especially the observation that all mice apparently develop micro-metastatic lung lesions, although few mice succumb to gross lung pathology. The overall purpose of development of this model was to create a more clinically relevant platform to test novel OSA therapeutics.

One of the focuses of this work has been the description of monocytes and other leukocytes in OSA. Monocytes have been implicated in murine tumor progression, and resultant macrophages can make up a significant proportion of the tumor burden [16-18]. Therefore, we hypothesized that pre-treatment monocytes may be prognostic in canine OSA. There is limited information in the human literature concerning monocytes as a prognostic marker, but OSA is not one of the tumors previously identified, possibly due to the relatively small number of cases in humans [19-23]. However, analysis of pre-treatment leukocytes as a predictor of outcome has not been performed in canine tumors to date. Therefore, we sought to determine if a pre-treatment CBC could be used as a prognostic in canine OSA (chapter 6). The need for cost-effective and easy-to-interpret diagnostics and biomarkers is of obvious importance to veterinary medicine, and the use of the ubiquitous CBC has met these criteria.

To decrease selection bias in the population, 313 dogs treated for OSA at the Colorado State University Veterinary Teaching Hospital between 2003 and 2006 were

evaluated. Dogs were then selected for study inclusion if they had appendicular disease, were treated with amputation and conventional chemotherapy, and did not have metastases present at diagnosis. Lack of metastases at diagnosis was necessary since we used disease-free interval as our measure of disease progression. Statistical analysis was performed on the 69 dogs included in the study for leukocyte and historical variables of importance in dogs with OSA. We observed that dogs with relatively higher monocytes and lymphocytes had a significantly decreased DFI compared to dogs with lower monocytes and lymphocytes. Interestingly, a majority of the dogs in the study had leukocyte counts within the normal reference range, and thus we discovered that significant prognostic information could still be obtained even though the leukocyte numbers were clinically normal. Furthermore, these observations were reproduced in a second and independent population of dogs. Further investigation is necessary to further phenotype the leukocyte populations in dogs to determine what the biological significance of these findings may be. For example, is there a population of expanded MDSC in patients with poor outcome? We concluded from these data that defined cut-offs can be applied to a CBC for prognostication of DFI in dogs with appendicular OSA.

Osteosarcoma is the most common primary bone tumor of humans and dogs, with a long term survival approximating 60% in humans and 20% in dogs. Understanding the metastatic biology of OSA is required to develop novel treatments for this disease since it is the primary cause of death from this disease. The work presented herein aims to better understand the role of immune responses and angiogenesis as they relate to OSA growth and metastasis. The development of novel models and techniques to study metastasis and angiogenesis is required to better understand this disease. The use of *in vivo* models are

required to understand observations made in the clinic that may provide a basis for future therapies, and as a platform for testing these therapies. Therefore, these findings suggest novel mechanisms by which to study OSA biology, and therapies for its treatment. We believe that the addition of these techniques and findings will add to the canon of knowledge concerning OSA biology; and the hope that therapeutic benefit will be gained through that addition.

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