A MURINE XENOGR AFT MODEL OF CANINE OSTEOSARCOMA: 
ESTABLISHMENT AND USE TO INVESTIGATE ANTI-TUMOR EFFECTS OF AN 
ANGIOGENESIS INHIBITOR AND COMBINATIONS OF RADIATION, 
CHEMOTHERAPY AND A VASCULAR TARGETING AGENT

By

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To my wife, Erin.
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STUDY OVERVIEW

**Chapter 2 - Phase I**

Objective: Establish tumor model and evaluate radiation response

Number of Mice: 
- Control $n = 9$
- 10 Gy $n = 9$
- 15 Gy $n = 9$

Duration of Study: 28 days

End-point: Tumor bearing limb $> 13$ mm in diameter

**Chapter 3 - Phase II**

Objective: Evaluate the local effect of bevacizumab-Avastin on xenografts

Number of Mice: 
- Control $n = 9$
- Avastin 2 mg/kg $n = 9$
- Avastin 4 mg/kg $n = 9$

Duration of Study: 21 days

End-point: Tumor bearing limb $> 13$ mm in diameter

**Chapter 4 - Phase III**

Objective: Evaluate the local single agent and radiopotentiating effects of combretastatin A-4 Phosphate and carboplatin on xenografts

Number of Mice: 
- Control $n = 9$
- 10 Gy $n = 9$
- CA4P 100 mg/kg $n = 9$
- Carboplatin 60 mg/kg $n = 9$
10 Gy + CA4P  
10 Gy + Carbo  
10 Gy + CA4P + Carbo 

Duration of Study: 23 days

End-point: Tumor bearing limb > 13 mm in diameter
Abstract of Thesis Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Master of Science

A MURINE XENOGRAFT MODEL OF CANINE OSTEOSARCOMA: ESTABLISHMENT AND USE TO INVESTIGATE ANTI-TUMOR EFFECTS OF AN ANGIOGENESIS INHIBITOR AND COMBINATIONS OF RADIATION, CHEMOTHERAPY AND A VASCULAR TARGETING AGENT

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Osteosarcoma (OSA) accounts for up to 98% of all canine primary bone tumors and 5% to 6% of all canine malignancies. Radiation therapy forms an integral part in both palliative and curative-intent therapies for canine appendicular OSA. Despite recent advances in radiation therapy protocols and improvement in local tumor response, amputation is considered the standard of care for appendicular OSA. Canine OSA serves as an excellent natural model for human Ewing’s sarcoma and osteosarcoma. As such, the objectives of this thesis were to review the literature regarding radiation therapy for canine appendicular OSA, develop a successful intramuscular murine xenograft model using highly metastasizing pulmonary osteosarcoma (HMPOS) cells, and to investigate the effect of novel anti-vascular therapies with traditional radiosensitizing agents on HMPOS xenografts.

HMPOS xenografts grew repeatably and predictably to a useable size in approximately 7 days. We documented approximately 8% tumor necrosis in untreated tumors, and significant tumor growth delay with single fraction radiation doses of 10Gy and 15Gy. Bevacizumab, a
humanized monoclonal antibody directed at vascular endothelial growth factor (VEGF),
inhibited vascular growth and delayed tumor growth at a dose of 4mg/kg, twice weekly.

Combretastatin-A4-phosphate is a tubulin binding vascular targeting agent that has been
shown to induce up to 90% tumor necrosis in other murine xenografts. In our HMPOS tumor
model, CA4P induced significant tumor necrosis (33%) compared to all other treatments and
control. As a single agent, CA4P had no effect on tumor growth, but when administered in
combination with the platinum chemotherapeutic agent carboplatin and radiation therapy (10Gy),
significant tumor growth delay was observed.
CHAPTER 1
RADIATION THERAPY FOR CANINE APPENDICULAR OSTEOSARCOMA

Introduction

Osteosarcoma (OSA) accounts for up to 98% of all canine primary bone tumors and 5% to 6% of all canine malignancies.\textsuperscript{1-3} Affected dogs typically present with progressive lameness, bony proliferation or swelling. Acute non-weight-bearing lameness is typically associated with the onset of pathologic fracture.\textsuperscript{1,2} Characteristic radiographic signs of appendicular OSA include bone lysis, periosteal proliferation, palisading cortical bone (sunburst effect), sub-periosteal bone formation (Codman's triangle), and soft tissue swelling, with calcification extending into surrounding soft tissues.\textsuperscript{1-3}

The management of OSA encompasses palliative or curative-intent strategies. Palliation can be achieved with combinations of radiation therapy, bisphosphonate treatment and/or analgesia.\textsuperscript{1} Curative-intent strategies include radiation therapy, amputation, or limb-sparing surgical techniques, each combined with chemotherapy.\textsuperscript{1,4} Limb amputation remains the current standard of care for local management of primary bone tumors, but some dogs are not considered suitable candidates for amputation because of concurrent severe orthopedic or neurologic conditions.\textsuperscript{4} Limb-sparing can be achieved with either surgical or radiation techniques, or both.\textsuperscript{5-16} Limb-sparing techniques in the dog have been described for treating tumors involving the humerus, radius, femur and tibia.\textsuperscript{5-17} While limb-sparing of the distal radius is largely successful despite post-operative complications (infection rate approaching 50%), surgical techniques have been less successful at other locations due to implant failure and poor limb function.\textsuperscript{3,5,15-17}

Alternatively, radiation therapy may be delivered with either curative- or palliative-intent.\textsuperscript{1,3,4,15,18-27}
Radiation therapy can be an effective method for the palliation of pain and some degree of local tumor control, especially when combined with chemotherapy. An earlier review by McEntee of radiation therapy and canine osteosarcoma provided much of the clinical recommendations still used today; however, recently published studies investigating both palliative (samarium and isolated limb perfusion) and curative-intent radiation therapy have prompted an up-dated review. The purpose of this report is to detail, compare and review the clinical applications of radiation therapy currently available for use in the management of canine osteosarcoma.

**Types of Radiation Therapy**

The radiation therapy (RT) modality most commonly used in veterinary oncology is teletherapy. Teletherapy is external beam RT, where a photon beam is produced from an orthovoltage or megavoltage (high-energy) radiation unit, and has several applications with canine osteosarcoma. Megavoltage irradiation involves the use of photons with energy of greater than 1 MV (million volts). The higher energy radiation beam penetrates further into tissue (than orthovoltage), allowing treatment of deeper seated tumors such as osteosarcoma. With megavoltage units, the maximum radiation dose is deposited some depth below the surface, resulting in a skin-sparing effect.

**Palliative Radiation Therapy**

The goal of palliative RT (PTRT) is to provide relief of specific clinical signs (decrease the pain and lameness associated with OSA) while resulting in minimal, if any, radiation-induced adverse-effects. This can generally be achieved by delivering several moderately large fractions (e.g. 8-10 Gy per fraction). Historically, PTRT has been used most commonly for pain relief in dogs affected with appendicular OSA patients that are not surgical candidates for amputation (due to concurrent disease), or if an owner has declined curative-intent
Treatment of OSA in these patients with PTRT can result in local reduction in inflammation, pain relief, slowed progression of metastatic lesions, and improved quality of life. While the exact molecular and cellular mechanisms through which radiation therapy reduces bone pain are still unknown, it has been shown that some effect results from acute disruption of inflammatory cells, decreased progression of tumor-induced osteolysis, and reduction in tumor size. The veterinary literature contains reports of PTRT for canine appendicular OSA delivering between 16 Gy and 32 Gy in two, three, or four fractions. The majority of these protocols achieve some level of analgesia within 7 to 14 days after the first dose of radiation, with clinical improvement lasting approximately 2 to 3 months. Recently, Boston et al reported longer survival (median survival time 130 days) in dogs with metastatic (stage III) appendicular OSA, treated with PTRT and chemotherapy although no details of treatment course were available. The reports of PTRT for canine appendicular OSA are detailed below and are stratified based on fractionation protocol.

**Two-fraction protocols.** All reports of two-fraction protocols deliver 16 Gy total doses split equally on day 0 and day 7. In 1999 Ramirez et al compared a traditional 0, 7, and 21 day protocol of 10 Gy per fraction of $^{60}$Co photon to a day 0 and 7 protocol of 8 Gy per fraction. This latter abbreviated protocol was intended for re-treatment following recurrence of clinical signs. Interestingly, no significant difference in the median time to onset of pain relief (11 days), rate of response (74%) or duration of response (73 days) was noted. These results support earlier work by McEntee detailing 70% response rate, lasting approximately 90 days with a two-fraction protocol of 8 Gy on days 0 and 7 day (16 Gy total dose).

**Three-fraction protocols.** Three-fraction RT protocols are the most widely reported variation of PTRT for canine appendicular OSA. Reported protocols deliver 8 to 10 Gy fractions,
typically on a 0, 7, 21 day schedule, for a total dose of 24 Gy to 30 Gy.\textsuperscript{18,21,24-27} These protocols have achieved a 50% and 83% response rate, with median onset of response occurring 14 - 21 days after the first fraction, and median duration of response lasting 53 - 180 days.\textsuperscript{18,21,24,25-27} Variations of the 0, 7, 21 day protocol have been recently described where two consecutive daily fractions of 8 Gy are administered (days 0 and 1), followed by additional 8 Gy fractions on a monthly basis or as required.\textsuperscript{1,20} To date, however, no response rates or durations of response have been reported for these protocols.\textsuperscript{1,20}

**Four-fraction protocols.** Because most three-fraction protocols specify two weeks between the second and third treatments, the possibility exists for repopulation of the tumor. In an effort to eliminate this two week gap, reduce the risk of tumor repopulation, and increase the duration of pain relief, Green \textit{et al} described a four-fraction (0, 7, 14, and 21 day) $^{60}$Co radiotherapy protocol, delivering 8 Gy per fraction for a total dose of 32 Gy.\textsuperscript{23} Compared to three-fraction protocols, this technique resulted in a higher response rate (93%), similar onset of response (14 days), and a shorter duration of response (95 days).\textsuperscript{23} No difference was evident between protocols for the rate of pathologic fracture (13%) or survival time (313 days).\textsuperscript{23} There was no apparent radiosensitization by platinum derivatives (cisplatin/carboplatin), although timing of administration of drugs was not consistent between cases.\textsuperscript{23} Interestingly, and unlike earlier two-fraction protocols described by Ramirez \textit{et al}, no significant difference in duration of response was noted for tumors that extended (radiographically) either < 42\% or > 42\% of bone length.\textsuperscript{23,24}

More recently, Mueller \textit{et al} compared three-fractions of 8 Gy (days 0, 7 and 21) and four fractions of 6 Gy (days 0, 7, 14, and 21) palliative protocols in 54 dogs, both with a total dose of 24 Gy. In this study, 45 dogs (83\%) experienced pain relief during or following treatment. The
median duration of effect was 53 days, with both protocols proving effective for palliation of clinical signs in dogs with appendicular OSA.\textsuperscript{21}

**Curative-Intent Radiation Therapy**

Osteosarcoma was previously thought to be a radiation-resistant tumor. Until recent years, no curative-intent radiation therapy strategies existed for dogs with appendicular OSA. Since 2004, curative intent RT for canine OSA has been described with either curative-intent full-course fractionated external beam protocol (CI-F), single mega-dose RT as part of an intraoperative extracorporeal irradiation (IORT) limb-sparing procedure, and as part of a stereotactic radiosurgery (SRS) protocol.\textsuperscript{3,5,15} With any of these curative-intent strategies, radiation therapy is delivered for local tumor control, while chemotherapy (either carboplatin or doxorubicin) must be given for metastatic disease.

**Curative-intent – fractionated external beam protocol (CI-F).** Fractionated curative-intent RT (CI-F) delivers a lower dose per fraction and a higher total dose of radiation in an attempt to offer long-term local tumor control, while minimizing the late effects of radiation that occur more frequently with large fraction RT protocols.\textsuperscript{29} Fractionated RT is commonly used in various applications in veterinary medicine, but has been reported only twice, with limited success, for canine OSA.\textsuperscript{3,32} Walter \textit{et al} report a response rate of 33% with a median local control time of 209 days and a median survival time of 209 days for appendicular OSA.\textsuperscript{3} These results were obtained from 9 dogs treated on a Monday-through-Friday schedule, with the majority of dogs receiving a total radiation dose of 57 Gy, with fractionated doses ranging from 3 - 5 Gy.\textsuperscript{3} These results did not show a substantial improvement over reported palliative protocols and although treatments were well tolerated, no substantial local disease control or survival benefit was evident in the findings. There is a similar paucity of published data
involving the use of radiosensitizing agents, such as cisplatin/carboplatin, which are often used in conjunction with definitive full-course RT.\textsuperscript{3,32,33}

The Comparative Oncology Lab (UF-CVM) has recently confirmed moderate radioresistance of canine OSA cell lines in vitro, with a relatively low alpha-to-beta ratio and relatively high survival fraction at 2 Gy. (Fitzpatrick personal communication) Such findings may explain why OSAs do not respond well to conventional fractionated radiotherapy protocols, and suggest that larger doses per fraction are needed to induce greater tumor cell kill. Thus, radiation treatment options that deliver large doses per fraction, while sparing normal surrounding tissue, may be more effective in achieving local tumor control.

**Intra-operative extracorporeal radiation and radiation in-situ.** Limb-sparing with extracorporeal IORT is a curative-intent, single fraction (CI-SF) protocol that involves isolation and exteriorization of the bone tumor segment so that the isolated bone segment can be irradiated with a single fraction of 70 Gy. Extraneous irradiated soft tissues are excised and the irradiated bone is reduced and stabilized with internal fixation.\textsuperscript{5,14} The biologic effect of a single dose of IORT is equivalent to 2-4 times the same dose of radiation delivered using fractionated external beam radiation protocols.\textsuperscript{34,35} Furthermore, 70 Gy IORT is tumoricidal to bone tumors and resulted in complete necrosis of OSA lesions.\textsuperscript{5,14} Experimental studies using single fraction, high-dose IORT have shown that peripheral nerves, muscle, and skin are particularly radiation sensitive. In contrast, bone matrix, ligaments, and articular cartilage are relatively resistant to high doses of radiation, which may allow preservation of normal joint and limb function.\textsuperscript{5,14,36} A principal advantage of extracorporeal IORT is that the radiation field can be focused on the target volume while sparing adjacent normal and radiosensitive soft-tissue structures.\textsuperscript{5} The clinical benefits of limb-sparing protocols using extracorporeal IORT include the maintenance of
autogenous bone with good anatomic fit, preservation of limb and joint function, and good local
tumor control. Specific surgical and radiation therapy techniques for extracorporeal IORT have
been reported for appendicular OSA in the distal radius, proximal humerus, distal tibia and
intercalary locations. Liptak et al reported 10/13 dogs (77%) were improved clinically after
IORT and assessed as having good to excellent limb function. Median local disease-free-
interval was 274 days and median survival time was 298 days. While IORT appears to have
comparable success to other definitive and palliative radiation therapy strategies, a large number
of dogs (69%) experienced post-operative complications, including deep infection, fracture of
irradiated bone, and implant failure, especially for distal lesions which have poor soft tissue
coverage.

Stereotactic radiosurgery. Conventional radiation therapy relies on the use of
fractionated protocols to minimize damage to surrounding healthy tissues. Conversely,
stereotactic radiosurgery (SRS) utilizes multiple, non-coplanar beams of radiation that are
stereotactically focused on the target to deliver the entire radiation dose in a single treatment.
Stereotactic radiosurgery minimizes damage to healthy surrounding tissues by relying on the
extreme accuracy of radiation delivery to a tumor and a steep dose gradient between the tumor
and surrounding normal tissues. The obvious benefits of this technique over fractionated
protocols include fewer anesthetic episodes and possibly a greater biologic effect on the tumor
tissue. Stereotactic radiosurgery has been widely reported in human and veterinary patients for
the treatment of intra-cranial abnormalities, but only one report exists describing the use of SRS
for the treatment of appendicular OSA. Farese et al describe a technique for SRS for
appendicular OSA in 11 dogs in which treatment plans were initially designed to ensure that the
peripheral border of the tumor was included within the 50% isodose shell (minimum dose of 20
Gy) and that the central portion of the tumor was included within the 75% isodose shell (30 Gy). Pre-treatment preparation involved placement of a targeting array and contrast-enhanced computed tomography images. In this report, two treatment protocols were documented. The first five dogs received SRS alone with lower doses of radiation. With the first protocol, tumor associated swelling and lameness were subjectively improved within 21 days and this effect continued for at least 3 months after treatment (median time to evidence of tumor progression, 105 days). The second group of six dogs received larger doses of radiation and was administered carboplatin (300 mg/m², IV) 30 minutes prior to irradiation. Interestingly, no local disease progression was noted in the follow-up period in the second group of dogs. Overall median survival time for these 11 dogs was 363 days. The SRS technique has subsequently been modified by attempting to now cover the entire tumor with a 30 – 35 Gy isodose line. Recently obtained (unpublished) histopathology from a dog with a proximal humeral OSA treated with this protocol, showed 100% necrosis of the lesion. Although such a treatment plan is usually possible in the proximal humeral location, the proximity of the skin to the outer cortical bone in the distal radius makes delivery of these doses possible only when the diseased tissue is mostly confined to the medullary cavity and endosteum (Farese, personal communication, 2007).

While the results of this study indicate that SRS is a promising non-surgical, limb-sparing treatment modality for canine appendicular OSA, the need for highly specialized equipment and personnel has currently limited its use to one veterinary academic institution (UF-VMC). In addition, lesions with advanced osteolysis and/or large tumor volumes (e.g. greater than 5 cm diameter) are not considered good candidates.

Radioisotopes

Radioisotopes offer a unique method of radiation delivery when combined with ligands. Ligands function as targeting agents which allow selective targeting of cancers for radiation dose
delivery by the radioisotope. Collectively these agents are then known as radiopharmaceuticals. One the most commonly reported therapeutic radiopharmaceutical is Samarium (Sm)-153-lexidronam (EDTMP).\textsuperscript{37} Samarium153 is the radioisotope which delivers a radiation dose to the target by beta decay (beta-emitter) and has the added property of gamma decay i.e. the distribution of the radioisotope can be monitored using a gamma camera. The ligand lexidronam (EDTMP) is an amino-bisphosphonate which, like the bone scan agent MDP (labeled with Tc-99m), localizes in areas of increased bone metabolism. This agent is not only selective for the cancer but will localize in areas with red marrow activity.

The first report of the radioisotope, Samarium-153-lexidronam (EDTMP), to treat canine bone tumors was by Latimer \textit{et al.}\textsuperscript{38,39} He treated forty dogs with naturally occurring osteosarcoma. Dogs were randomized to receive either a single dose of 37 MBq/kg or two doses a week apart. No significant differences were found between the two groups although early tumors and metastatic lesions appeared to show some response.

In a larger study Milner \textit{et al} treated 10 dogs with osteosarcoma. A single dog had a dramatic response to therapy, but all the others cases showed progression of the osteosarcoma.\textsuperscript{40} In 1999, Aas \textit{et al} treated fifteen dogs with 153Sm-EDTMP.\textsuperscript{41} Dogs were given between one and four doses of Sm-153-EDTMP at 36-57 MBq/kg. Their conclusions were that a favorable high tumor dose, was achieved in the tumor compared to surrounding tissue and that pain relief and in some cases, tumor growth was delayed. At necropsy a number were found to have metastases. No serious side effects were observed. Most recently, Barnard \textit{et al} reported 35 dogs receiving Sm-153-EDTMP.\textsuperscript{42} Of the 32 dogs with appendicular tumors, 20 (63\%) had an improvement in the severity of lameness 2 weeks after administration of the first dose of radioactive samarium, 8 (25\%) had no change in the severity of lameness, and 4 (12\%) had a worsening. Overall median
survival time was 100 days, with 3 dogs (8.6%) alive after 1 year. Median survival time for the 32 dogs with appendicular tumors was 93 days, with 3 (9.4%) alive after 1 year. This was not significantly different from the median survival time of 134 days for a historical cohort of 162 dogs with appendicular osteosarcoma that underwent amputation as the only treatment.

**Radiation-Induced Adverse-Effects**

Acute adverse effects are those which occur during a treatment course, while late effects may only manifest months or years following treatment. Acute effects of tumor irradiation in dogs with appendicular osteosarcoma may include moist desquamation, edema, ulceration and skin necrosis; while late effects include muscle fibrosis, alopecia, leukotrichia, bone necrosis and tumor induction.36,43

Time-dose-fractionation (TDF) is used to describe development of a radiation therapy protocol, considering the total dose of radiation given over a specific period of time (e.g. 10Gy in a single fraction versus 5 x 2Gy fractions per week). Large fraction TDF protocols (such as most palliative protocols for OSA in dogs) result in decreased acute adverse-effects but a greater incidence of late adverse-effects. The converse is true for conventional TDF protocols (more frequent acute adverse-effects with fewer late adverse-effects). In human patients, equal pain palliation is achieved with both single and multiple-fraction RT, but medical and societal costs are reduced with single fraction treatments.30 In dogs, palliative radiation therapy is not associated with significant acute adverse-effects, although alopecia, erythema and depigmentation have been reported, but these do not reduce quality of life.1 These adverse sequellae may be more common with high doses per fraction and high total cumulative radiation doses.1,15,20,25 A single fraction of > 4 to 5 Gy results in greater damage to late-responding healthy tissues than the same dose divided into smaller fractions.15,27 Many dogs with appendicular OSA treated with palliative-intent die or are euthanized before late adverse-effects
become apparent. Subsequently, PTRT protocols delivering 8 Gy to 10 Gy in each fraction are tolerated without clinically significant late adverse effects.$^{1,24,25}$

Definitive radiation therapy is associated with a higher complication rate, including alopecia (63%), bone resorption (31%), desquamation (27%), bone necrosis (23%), hyperpigmentation (18%), late muscle fibrosis (15%), leukotrichia (9%), fibrosis of the irradiated bone (8%), and acute skin necrosis (8%).$^{3,4,15}$ Pathologic fracture is reported in all definitive RT strategies (54% IORT, 36% SRS, 14% full-course external beam RT). The incidence of pathologic fracture may be reduced with judicious case selection, such as dogs with minimal cortical destruction and bony lysis.$^{15}$

In the event of radiation-induced adverse-effects, interventions such as stabilization of pathologic fractures and management of soft tissue wounds may be necessary to maintain quality of life. Desquamation and acute skin necrosis can be difficult to manage, due to the poor healing potential of irradiated soft tissues. A major advantage of SRS is that surrounding soft tissues remain well vascularized and therefore are available to be utilized as transposition flaps in the event of desquamation and focal skin necrosis.

**Conclusions**

Appendicular OSA is a relatively common tumor in large breed dogs. Radiation therapy has a role in both the palliative and curative-intent treatment of the local tumor in dogs with appendicular OSA. Palliative RT achieves analgesia with reliable response rates (50% - 93%) for approximately 100 days (range 53 – 180 days), at which time many dogs die or are euthanized due to disease progression. The major benefit of PTRT is that during the palliated period, quality of life is improved and RT-induced side effects are rare. Curative-intent RT can achieve good to excellent local tumor control, but is also associated with a moderate to high rate of radiation-induced complications, which may be ameliorated with appropriate case selection (small
intramedullary lesions for SRS and proximal extremity lesions with good soft tissue coverage for IORT). Curative intent – fractionated (CI-F) therapy is largely unsuccessful and while a large single fraction may be successful at controlling local disease, therapeutic success is reliant on the incidence and management of surgical complications (IORT) and appropriate patient selection (SRS).
CHAPTER 2
DEVELOPMENT OF AN INTRAMUSCULAR MURINE XENOGRAFT MODEL FOR CANINE OSTEOSARCOMA

Introduction

Both in-vitro and in-vivo experimental models for canine osteosarcoma (OSA) have been described. The use of in-vivo murine models offer the advantage over in-vitro models in that tumor biology more closely approximates spontaneously occurring disease. Murine models also allow rapid advances in research by generating large numbers of reproducible neoplasms in a short period of time, low cost, and accumulation of large volumes of data without the need for clinical cases. Many murine models have been reported for canine OSA, including interscapular subcutaneous (SQ), proximal hindlimb SQ, abdominal/flank SQ and orthotopic femoral xenotransplantation. These models, however, have been associated with various inadequacies including irregular tumor size and shape, recipient morbidity, and difficulty of ante-mortem tumor measurement.

External beam radiation therapy is currently utilized for curative- and palliative-intent therapies for canine appendicular OSA. In an effort to reduce radiation-associated side effects with in-vivo xenograft models, distal appendicular tumors are desirable. Similar intramuscular (IM) xenotransplants have been documented for human tumor models including Kaposi's sarcoma, breast cancer and melanoma. The purposes of this study were 1) to establish a reproducible distal appendicular IM murine xenograft model for canine OSA, 2) to establish tumor growth curves for select radiation doses, 3) to characterize tumor morphology and 4) to determine percent tumor necrosis resulting from select radiation doses. This model may serve as a translational model for human osteosarcoma, due to the biologic and histopathologic similarities between canine and human OSA (Table 2-1).
Table 2-1. Common features of osteosarcoma in humans and dogs

<table>
<thead>
<tr>
<th>Variable</th>
<th>Dog</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male:Female</td>
<td>1.5:1</td>
<td>1.5:1</td>
</tr>
<tr>
<td>Body Weight</td>
<td>99% &gt;20 kg</td>
<td>Obese</td>
</tr>
<tr>
<td>Site</td>
<td>77% long bones</td>
<td>90% long bones</td>
</tr>
<tr>
<td>Etiology</td>
<td>Generally unknown</td>
<td>Generally unknown</td>
</tr>
<tr>
<td>% Confined At Presentation</td>
<td>80-90%</td>
<td>80-90%</td>
</tr>
<tr>
<td>% High Grade</td>
<td>95%</td>
<td>85-90%</td>
</tr>
<tr>
<td>% Aneuploid</td>
<td>75%</td>
<td>75%</td>
</tr>
<tr>
<td>Metastatic Rate Without</td>
<td>90% before 1 year</td>
<td>80% before 2 years</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastatic Sites</td>
<td>Lung&gt;bone&gt;soft tissue</td>
<td>Lung&gt;bone&gt;soft tissue</td>
</tr>
<tr>
<td>Survival With</td>
<td>Significant</td>
<td>Significant</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genetic Changes</td>
<td>p53, Rb, c-myc, met</td>
<td></td>
</tr>
</tbody>
</table>

**Materials and Methods**

**Cell Culture**

Canine highly metastasizing parent osteosarcoma (HMPOS) cells were obtained from Dr. Tsuyoshi Kadosawa at the Laboratory of Veterinary Surgery, Hokkaido University, Sapporo, Japan. HMPOS growth medium was formulated by supplementing Hank’s (RPMI 1640) with 10% heat-inactivated fetal calf serum (FBS), 1% Pen-Strep, 1% L-glutamine, vitamin solution and non-essential amino acids. Cells were seeded (2 x 10^6) into 150-cm² flasks and maintained at 37°C under 5% CO₂ and 95% room air. Cells were grown to confluence (3 day passage time),

28
detached from their plates with 0.25% trypsin, washed with Hanks solution (pH 7.4), and counted with a hemacytometer. Cells were precipitated and re-suspended in phosphate buffered saline (PBS; pH 7.4) to a concentration of 5 x 10^5 cells/0.02ml (25 x 10^6 cells/ml), for intramuscular (IM) inoculation. This suspension was transported on ice to the animal housing facility.

**Induction and Measurement of Tumors**

All experiments were performed at the University of Florida under institutionally approved guidelines for animal welfare (IACUC). Mice were handled and restrained individually such that the left pelvic-limb of each mouse (recipient for xenograft) was immobilized over the handler’s left index finder, and the tail was immobilized between the handler’s left index and middle fingers (Figure 2-1).
Figure 2-1. Athymic nude mice were inoculated with $5 \times 10^5$ canine osteosarcoma cells (HMPOS) intramuscularly into the gastrocnemius muscle (A) and later assessed for tumor size by sliding tumor-bearing limbs through various sized metric circles (B).

HMPOS xenografts were initiated by injecting tumor cells intramuscularly into the left gastrocnemius-muscles of twenty-seven 5-week-old athymic BALB/c AnNCrl-nuBR mice (Charles River Laboratories, Wilmington, MA). Each mouse received $5 \times 10^5$ HMPOS cells suspended in PBS for a total injection volume of 0.02 ml. All mice were provided sterilized food and water *ad libitum* and housed in a specific pathogen free (SPF) barrier facility with 12-hour light and dark cycles. Mice were weighed at least every other day and observed daily for primary tumor growth at the site of inoculation, changes in behavior, and general appearance. Following initial appearance of the tumor, tumor bearing limbs the tumors were measured and examined daily for morphologic changes, such as skin ulceration. Maximum tumor diameter was determined using a metric circles template (R-2040 Template Metric Circle, Berol RAPIDESIGN, USA) by passing the tumor-bearing limb through the appropriate circle until
minimal skin-to-template contact was detected. Tumor diameter measurements were then
converted to tumor volume and weight using the formula: tumor volume = \( (1/6)\pi d^3 – 100 \), where
\( d \) is the diameter of the hole and 100 represents a volume correction factor for a mouse leg
without a tumor.\(^6\) Tumor volume in mm\(^3\) can then be used to approximate tumor weight, where
100 mm\(^3\) \( \approx \) 0.1 g. Daily data-points were computed to generate a tumor growth curve.

When tumor weight reached 0.2 g, mice were randomly assigned to one of three groups
and treatments were begun:

1. Control group: no treatment (\( n = 9 \))
2. XRT10: tumor bearing limbs received a single external beam radiation dose of 10 Gy (\( n = 9 \))
3. XRT15: tumor bearing limbs received a single external beam radiation dose of 15 Gy (\( n = 9 \))

**Radiation Therapy**

Conscious mice were restrained in customized plastic jigs and tumor irradiations were
performed using a 6-MV Clinac 600c linear accelerator (Varian Oncology Systems, Palo Alto, CA). The tumor-bearing limb was extended through an opening in the side of the jig, allowing
for local tumor irradiation at a rate of 4 Gy/min. 8 mm thick gelatin radiation boluses were
suspended above the tumor-bearing limb to ensure uniform tumor irradiation. (See Figure 2-2)
For radiation treatments, conscious mice were restrained in lucite jigs, to effectively isolate the tumor bearing limb for treatment, while sparing the remaining mouse from radiation associated adverse-effects. The tumor bearing limb and tail were taped with medical tape to prevent motion during treatment. Mice were comfortably restrained within these jigs for approximately 2 minutes while treatments were administered.

**Necropsy and Histopathological Examination**

All mice were euthanized (CO$_2$ + thoracotomy) when their tumor-bearing limb reached a maximum diameter of 13 mm at the level of the gastrocnemius muscle (tumor weight ~ 1.0 g), or earlier if a mouse’s quality of life was compromised.

After euthanasia, a complete necropsy was performed. The primary tumor was dissected with the limb in situ, transected at its greatest diameter, and fixed in 10% neutral-buffered formalin. The respiratory tract (larynx-to-lungs), heart and mediastinal fat were dissected free and the lungs were inflated with 10% neutral-buffered formalin. After fixing in formalin for 48 hours, all tissues were then transferred into 70% alcohol. A 5-um thick section of the primary tumor was embedded in paraffin and stained with hematoxylin and eosin (H&E) for microscopic examination.
**Tumor Parameters**

The behavior and development of the IM HMPOS tumor xenograft model was evaluated using the following parameters:

1. **Tumor Growth** \( (t^{0.2g}) \): the number of days required for the tumor-bearing-limbs to grow to 0.2 g was calculated for individuals.

2. **Tumor Growth** \( (t^{0.2 - 1.0g}) \): the number of days required for the tumor-bearing-limbs to grow from 0.2 – 1.0 g was calculated for individuals and groups.

3. **Tumor histomorphology**: H&E staining of the same tissue section was carried out in order to compare the distribution of tumor vasculature or necrosis with tumor architecture/structure.

4. **Tumor Necrosis**: during slide examination for tumor histomorphology, digital images of tumor sections (maximum tumor diameter) were captured with an Olympus BX41 microscope with attached Olympus DP70 10 megapixel camera and processed using DPController version 1.2.1.108 and DPManager version 1.2.2.107, Olympus Optical Co., Ltd. (2001-2003). Analysis was completed with Image Tool for Windows version 3.00, The University of Texas Health Science Center in San Antonio (1995-2002) and percent tumor necrosis was calculated with the following formula:

\[
\%TN = \frac{NTA}{TTA} \times \frac{100}{1}
\]

where \( NTA = \text{Necrotic Tumor Area} \) and \( TTA = \text{Total Tumor Area} \).

A single board certified veterinary pathologist (DT - Dr. David Taylor, BVSc, DipVetClinStudies, MVS, Diplomate ACVP) performed all immunohistochemistry, histomorphology and percent tumor necrosis interpretation and was blinded to individual identity and group assignment.
5. Morbidity: tumor-bearing mice were assessed for local and systemic morbidity (ulceration, bruising, dependent swelling, lameness, respiratory difficulty, death).

Statistical Analysis

Statistical calculations were performed using a computer software program (SigmaStat for Windows Version 3.0, SPSS Inc.). Data for tumor growth delay and percent tumor necrosis were computed using a Kruskal–Wallis one-way analysis of variance on ranks with a significance value of $P < 0.05$. Kruskal–Wallis analysis of variance on ranks was used because the data were not normally distributed. A pairwise multiple comparison procedure was performed using Tukey’s method.

Results

Tumor Growth $t^{0.2g}$

Macroscopic tumors developed in all mice. Post inoculation, a tumor was considered suitable (for treatment) when the inoculated area of the leg reached a maximum diameter of 7.5 – 8.5 mm (corresponding to a tumor weight of approximately 0.2g). This diameter was achieved in $7 \pm 0.18$ SEM days.

Tumor Growth $t^{0.2 – 1.0g}$

Tumors were allowed to develop until the maximum diameter of the inoculated area of the leg reached 13 mm (corresponding to a tumor weight of 1.0 g) and this was achieved in all mice. Duration for tumor growth from 0.2 – 1.0 g was significantly longer for both XRT15 (median 21 ± 1.68 SEM days) and XRT10 (median 11 ± 1.23 SEM days), compared to control mice (median 6 ± 0.70 SEM days, $p < 0.05$). No statistical difference was found between radiation therapy groups.
Figure 2-3: Graph of median tumor growth delay comparing control to 10 Gy and 15 Gy single-fraction irradiations. The solid bars connecting control to both 10 Gy and 15 Gy groups indicate a statistically significant difference between these groups (p < 0.05). No significant difference is observed between radiation therapy groups.

**Tumor Histomorphology**

All tumors were described as having infiltrative growth patterns. Tumor cells were either round or polygonal, with a moderate amount of cytoplasm and round-oval, chromatin stippled nuclei. All tumors contained both osteoid and bone, and no tumor cells were seen to be invading tumor vasculature (as evidence of hematogenous metastasis).
Tumor Percent Necrosis

Some degree of necrosis was observed in all groups. No significant difference was evident in percent tumor necrosis between groups (median Control 8.41 ± 2.98 SEM %, XRT10 7.92 ± 3.26 SEM %, XRT15 5.6 ± 4.59 SEM %; P=0.115). Necrosis was generally organized in linear, focal or individual areas and there was no specific consistent pattern of necrosis in any of the treatment groups.

![Median Tumor Percent Necrosis Graph](image)

Figure 2-4: Graph of median tumor percent necrosis, comparing control to 10 Gy and 15 Gy single-fraction irradiations. No statistically significant difference was observed between these groups (p = 0.135).

Morbidity

Tumor-related morbidity was noticed in all animals. All mice developed some degree of lameness as their tumor-bearing limbs approached 13 mm diameter. Swelling of the paw (i.e., distal to the xenograft) was observed in one mouse. This swelling resolved within 24 hours
without treatment. Xenograft- or treatment-associated skin changes, respiratory difficulty, bruising or death were not observed in any mouse.

**Discussion**

Tumor size, shape, location and time to reach 0.2 g were very consistent in all of the mice. 0.2 g tumors developed in approximately 7 days, which is substantially shorter than many previous reports of canine osteosarcoma xenografts in SQ locales, in which comparable tumors often developed after 6 - 12 weeks. The accelerated tumor growth seen in the mice in this study may be due to the intramuscular inoculation of our xenografts, as the rich blood supply within muscles provides stable graft reception. Another explanation could be differences in inoculation concentration or cell number. We would expect that xenografts would develop larger and faster as concentration and volume of the inoculum increases. In fact, while our xenografts developed faster, we used fewer cells per inoculation (5 x 10^5 cells) than were used in previous models (10 - 100 x 10^5 cells). The end-point of this study was a limb diameter of 13 mm (~1.0 g tumor), which is smaller than one study. Advantages of rapid growth and a relatively small tumor size end-point include shortened investigative time and thereby reduced costs.

Advantages of this gastrocnemius IM xenograft tumor model include: repeatability and uniformity (rate, location and shape of development); ease of tumor manipulation, measurement and treatment; minimal morbidity; and ease of tumor dissection for post-mortem investigation. Reports of SQ xenograft (flank, interscapular, proximal femoral locations) for canine OSA all describe irregular ellipsoid growth, necessitating three dimensional tumor measurement. While those tumor models effectively grew tumors, substantial inter-tumor variation was noted in tumor shape, growth rate and tumor location. Tumors were measured using Vernier calipers and tumor volumes were generated using mathematical formulae. Measurement with a metric circles template has been shown to accurately estimate tumor volume and weight.
using a formula and graph.\textsuperscript{62} This measurement method is much simpler and faster than previously reported techniques using three-dimensional Vernier caliper measurements.\textsuperscript{45-54} However, our model is limited by tumor size as an end-point. Therefore tumors larger than approximately 1.0 g (13 mm) made ambulation cumbersome and impaired the recipient’s quality of life, necessitating euthanasia at fairly early stages in tumor growth.

Few reports exist detailing morbidity and mortality associated with tumor xenografts. Farese \textit{et al} report 10\% of mice (including mice in both the treatment group and control group) dying or being euthanized before the end of the study due to physical deterioration.\textsuperscript{45} In this investigation, many mice developed ulceration of the skin surrounding the inoculation site, and a number of mice with tumors in the flank suffered tumor invasion into the abdominal cavity, and associated fatal intra-abdominal hemorrhage (Farese personal communication). Ulceration presumably occurred because of either osmotic necrosis ascribed to a highly concentrated inoculum that desiccated surrounding cells, avascular necrosis due to the xenograft outgrowing its blood supply, or direct invasion of tumor through the skin by tumor cell seeding of the inoculation tract. No xenograft-associated skin ulceration was noticed in the present model. The absence of ulceration may be due either to the greater vascularity of the muscular recipient bed (compared to the subcutaneous model) or the use of a lower concentration of inoculum and therefore a lower risk of osmotic necrosis and injection-site seeding.

Lameness, which developed in all mice, was the only noticeable impairment to normal function. This lameness was thought to be caused by mechanical impingement of the caudal tumor-bearing-gastrocnemius on the caudal aspect of the thigh, inhibiting flexion of the stifle joint. Tumor bearing limbs did not appear painful, and the above described lameness was only evident once tumor volumes reached approximately 0.2 g (8 mm).
Various successful non-OSA murine IM-gastrocnemius tumor models have been reported and are commonly used for radiation therapy studies. These tumor models are similar in behavior to that described here, with the most aggressive developing to useful size (0.2 g) in only 7 days, and with very little morbidity (Siemann personal communication). Recently, the University of Florida Comparative Oncology Laboratory has investigated the in-vitro radiosensitivity of canine OSA cells (Fitzpatrick personal communication). Through this work, calculated alpha/beta ratios have provided insight into the observed radioresistance with fractionated radiation therapy (Fitzpatrick personal communication). Our investigation was directed at evaluating the effect of radiation therapy on HMPOS in-vivo. External beam radiation therapy initiates time- or dose-related effects to surrounding tissues. Therefore, all efforts should be made to exclude healthy tissues from radiation or other treatment fields. The IM tumor model described here has a substantial advantage over other "axial" xenograft models in that for radiation therapy, the tumor-bearing limb may be isolated from the rest of the mouse thus minimizing radiation exposure to the remainder of the mouse and mitigating the development of systemic radiation-associated adverse effects.

Results of our study reveal that external beam radiation therapy (15 Gy) delays growth of intramuscular HMPOS xenografts and supports earlier in-vitro investigations which showed sensitivity of HMPOS cells to radiation at doses of 6 and 9 Gy (Fitzpatrick personal communication). Treatment with 15 Gy did not delay tumor growth significantly when compared to 10 Gy; however, this may be a factor of small sample size.

One of the limitations of xenograft tumor models in any location is whether these xenografts represent the true architecture of patient tumors. Immortalized and transformed cell lines employed as substrates in xenograft models can deviate significantly from the native,
complex tumor environment, thus making model and therapeutic response interpretation difficult. Similar to previous reports of subcutaneous canine OSA xenografts, the primary tumors in this study were histologically similar to the original tumors from which the cell lines were developed. Xenografts in this study displayed osteoblastic differentiation and produced both osteoid and bone, indicating histopathological similarity to naturally occurring canine osteosarcoma. Heterotopic tumor models (such as described in this report) unfortunately are inferior to orthotopic (intra-osseous) xenografts with respect to approximating spontaneously occurring appendicular OSA.

Powers et al reported a mean percent tumor necrosis of approximately 25% for spontaneously occurring untreated canine osteosarcoma. The percent necrosis increased to approximately 80% when dogs with spontaneously occurring OSA were treated with external beam radiation therapy, and the dose required to achieve 80% tumor necrosis was approximately 40 Gy when given alone. Interestingly, neither control nor irradiated tumors in this study had 25% tumor necrosis. The lower percent necrosis in this study may simply be a result of small tumor size (not outgrowing blood supply as would a larger spontaneous tumor in a dog) or dosing, as our irradiated tumors received a single fraction of either 10 Gy or 15 Gy, while dogs in Powers' study received ten equal fractions delivering a total dose of between 36 – 52 Gy. Previous reports of subcutaneous canine OSA xenografts and IM non-OSA xenografts have described similar patterns of central tumor necrosis as we report here, although no quantitative measurements were made. No significant difference was observed between groups. A post-hoc power (α = 0.05) of these comparisons (0.276) was very low and as such, our results must be interpreted with caution. The low post-hoc power was likely due to very large sample variation.
The most devastating aspect of cancer is the emergence of metastasis in organs distant from the primary tumor.\textsuperscript{54,69} Osteosarcoma is one of the most malignant tumors in both humans and animals, with a high percentage of early-stage lung metastasis.\textsuperscript{54} Tumors which grow beyond the size of 2 mm in diameter can already synthesize and secrete angiogenic factors that facilitate intravasation, shedding of tumor cells, and the formation of seeding colonies (metastases).\textsuperscript{70} At present the tumor model reported here has not been evaluated for successful pulmonary metastasis, although successful metastasis has been documented with previous heterotopic and orthotopic canine OSA murine xenograft models.\textsuperscript{45,46,50,52,54} Microscopic metastasis is reported only after 4 weeks in a SQ-model study and 10 weeks in the orthotopic model, with small macrometastases becoming evident after 6 weeks.\textsuperscript{45,46,54} Macroscopic pulmonary metastasis has been documented as soon as 17 days after inoculation in some human non-OSA IM xenografts.\textsuperscript{71}

This report details the successful use of a reliable and consistent IM gastrocnemius murine xenograft model for \textit{in vivo} assessment of canine osteosarcoma (HMPOS). This model displays substantial improvements over traditional SQ murine models with respect to host morbidity, tumor assessment and suitability for the evaluation of therapeutic modalities such as radiation, chemotherapy and therapies that target tumor vasculature.
CHAPTER 3
INVESTIGATING THE EFFECTS OF BEVACIZUMAB ON CANINE OSTEOSARCOMA XENOGRAFTS

Introduction

Canine osteosarcoma has been recognized as an accepted model for the osteosarcoma (OSA) and Ewing's sarcoma in children. The standard treatments for primary bone tumors in humans and dogs have been amputation or limb-sparing surgery with adjunctive chemotherapy. Though many appendicular tumors may be treated effectively with surgical resection, there is controversy regarding how best to treat such tumors originating from the pelvis and other less accessible anatomic locations. In addition, limb salvage procedures with hemipelvectomy may result in significant disability and loss of hip function. Furthermore, most dogs die, or are euthanized, due to metastatic or local disease progression (when limb salvage is attempted).  

In the pathogenesis of cancer, vascular endothelial growth factor (VEGF) has a number of influential pathophysiologic mechanisms. Firstly, VEGF stimulates excessive angiogenesis, allowing the tumor to embark on an exponential growth phase. Abundant vascularization also provides an avenue for dissemination of hematogenous metastases and allows tumor colonies to establish in remote locations. The critical importance of VEGF during tumorogenesis has been illustrated by a number of observations: VEGF expression is elevated in the tissues or systemic circulation in many solid tumors including some osteosarcomas; a significant correlation has been found between plasma VEGF levels and disease stage or metastasis; pre-clinical experiments showed than anti-VEGF antibodies inhibited the growth of human tumor cells injected into nude mice. Recent evidence suggests that VEGF is the single most important regulator of angiogenesis in the Ewing's sarcoma family of tumors (ESFT). Naturally occurring
OSA in human patients that are VEGF-positive are approximately 70% more likely to metastasize and have a shorter disease-free and overall survival.\textsuperscript{76}

We believe that intervention with an anti-angiogenic agent targeting VEGF, may delay tumor growth sufficiently to allow palliation of disease or cure with long-term therapy. One such anti-angiogenic agent is bevacizumab (Avastin\textsuperscript{TM}, Genentech, South San Francisco, CA). Bevacizumab is a commercially available recombinant humanized monoclonal IgG1 antibody that binds to and inhibits the biologic activity of human vascular endothelial growth factor (VEGF) in in-vitro and in-vivo assay systems. Bevacizumab contains human framework regions and the complementarity-determining regions of a murine antibody that binds to VEGF.\textsuperscript{78,79} The interaction of VEGF with its receptors leads to endothelial cell proliferation and new blood vessel formation in in-vitro models of angiogenesis. Bevacizumab binds VEGF and prevents the interaction of VEGF to its receptors (Flt-1 and KDR) on the surface of endothelial cells.\textsuperscript{79} Bevacizumab is currently approved for the initial treatment of metastatic colorectal carcinoma and non-small cell lung cancer with approval for the treatment of several other tumor types expected soon.\textsuperscript{73,80-82} Although bevacizumab has been evaluated for the treatment of various human cancers, its effect on canine tumors has not been investigated.\textsuperscript{73,81-83} In this study, we report the effect of bevacizumab on growth of intramuscular xenografted canine osteosarcomas in a murine model. We hypothesized that bevacizumab would delay tumor growth and reduce microvessel density within xenografts.

**Materials and Methods**

**Cell Line**

Canine highly metastasizing parent osteosarcoma (HMPOS) cells were obtained and cultured as described in Chapter 2 (page 29).
Induction and Measurement of Tumors

Xenografts were induced and measured as described in Chapter 2 (pages 29 – 31). When tumor weight reached 0.2 g, mice were randomly assigned to one of three groups (n=9 for each group) and treatments were begun:

1. Control group: no treatment
2. Bevacizumab-LOW: 2 mg/kg Avastin™ administered via a single intraperitoneal (IP) injection, twice weekly on Monday and Friday for two weeks for a total of four treatments
3. Bevacizumab-HIGH: 4 mg/kg Avastin™ administered via a single intraperitoneal (IP) injection, twice weekly on Monday and Friday for two weeks for a total of four treatments.

Tumor size was measured every other day, and all mice were euthanized (CO₂ + thoracotomy) when their tumors reached 1.0 g, or earlier if quality of life was compromised. All experiments were performed under institutionally approved guidelines for animal welfare (IACUC).

Drug Treatments

Neutralizing anti-VEGF antibody, bevacizumab, was diluted from the supplied concentration (25 mg/ml) to 0.76 mg/ml with the addition of sterile 0.9% NaCl. Approximately 0.01 ml/g and 0.02 ml/g bodyweight of diluted solution was then administered via intraperitoneal injection with a 27 G needle, to deliver either 2 mg/kg or 4 mg/kg bevacizumab to mice, twice weekly (Mondays and Fridays) for two weeks (total of four treatments).

Necropsy and Histopathological Examination

All mice were euthanized (CO₂ + thoracotomy) when their tumor-bearing limb reached a maximum diameter of 13 mm at the level of the gastrocnemius muscle (tumor weight ~ 1.0 g), or earlier if a mouse’s quality of life was compromised. After euthanasia, a complete necropsy was performed. The primary tumor was dissected with the limb in situ, transected at its greatest
diameter, and fixed in 10% neutral-buffered formalin. The respiratory tract (larynx to lungs), heart and mediastinal fat were dissected free and the lungs were inflated with 10% neutral-buffered formalin. After fixing in formalin for 48 hours, all tissues were then transferred into 70% alcohol. A 5-um thick section of the primary tumor was embedded in paraffin and stained with H&E for microscopic examination.

**Immunohistochemistry Protocol**

Slides were deparaffinized in xylene, rinsed in Tris buffer and incubated in 3% H$_2$O$_2$ for 10 minutes to quench endogenous peroxidase activity. Heat-induced epitope retrieval was performed for 20 minutes. After the slides were cooled and rinsed the primary antibody CD31 (rabbit polyclonal antibody) was applied (1:100 dilution) and the slides were incubated. The slides were again rinsed and the biotinylated secondary antibody (biotinylated goat anti-rabbit) was applied. Streptavidin peroxidase reagent was added and the chromogen (DAB) applied. The slides were counterstained with hematoxylin (Richard Allen Scientific). Human tonsil, Mouse Heart, Kidney. [UltraVision Detection System, Anti Rabbit, Cat. #HRP/DAB, TR-015-HD. LabVision (Cat # RB-10333L1) (Lot# 10333P608B)] were used as positive controls.

**Tumor Parameters**

The effect of bevacizumab on selected tumor parameters was studied in detail using the following techniques:

1. **Tumor Growth Delay** ($t^{0.2-1.0g}$): the number of days required for tumors to grow from 0.2 – 1.0 g was calculated for individuals and for groups.

2. **Tumor vasculature**: to evaluate the extent of angiogenesis, the areas of highest neovascularization were found by scanning the tumor sections under low power (40X and 100X) and the areas with the highest numbers of discrete microvessels staining for CD31 were identified. These areas were subjectively graded on a scale of 1 to 4+ and individual vessels
counted on a 200x field. Each count was expressed as the highest number of microvessels identified within any 200x field.

3. Tumor histomorphology: H&E staining of the same section was carried out in order to compare the distribution of tumor vasculature or necrosis with tumor architecture/structure. A single board certified veterinary pathologist (DT) performed all immunohistochemistry and histomorphology interpretation and was blinded to individual identity and treatment group.

Data Analysis

Statistical calculations were performed using a computer software program (SigmaStat for Windows Version 3.0, SPSS Inc.). All relevant assumptions, such as normality, were verified formally. Tumor growth delay (\(t^{0.2–1.0\text{g}}\)) was compared between groups using a Kruskal–Wallis one-way analysis of variance on ranks with a significance value of \(P < 0.05\). Kruskal–Wallis analysis of variance on ranks was used because the data had unequal variance. For analysis of tumor vasculature with CD-31 immunohistochemistry, mean vessel count/200x field was compared between groups using a one-way ANOVA (\(p < 0.05\)).

Results

Tumor Growth Delay

Bevacizumab at 4 mg/kg IP significantly increased the time required for tumors to grow from 0.2 – 1.0 g (median 14 ± 1.26 SEM days; range 9-21 days) when compared to control (median 8 ± 0.49 days; range: 4-9 days; \(P < 0.05\)). Bevacizumab at 2 mg/kg IP did not significantly delay tumor growth (median 10 ± 0.50 days; range: 7-11 days); no significant difference in tumor growth delay was found between high- and low-dose bevacizumab treatment groups.
Figure 3-1. Graph of tumor volume as a function of time, to generate tumor growth curves for control and bevacizumab treatment groups (2 and 4 mg/kg IP twice per week for two weeks). Data points represent daily mean tumor mass (g) ± SD.
Figure 3-2. Graph of median time for tumors to grow from 0.2 – 1.0 g (tumor growth delay). When administered at 4 mg/kg IP twice per week for two weeks, bevacizumab significantly delayed tumor growth when compared to control (p < 0.05). No significance was found between control and bevacizumab administered at 2 mg/kg IP twice per week for two weeks, or between bevacizumab treatment groups.

**Toxicity**

None of the mice were observed to have toxicities associated with administration of bevacizumab.
**Tumor Vasculature**

The number of immunohistochemically measured (CD-31) tumor microvessels per 200x field were significantly decreased in both 2 mg/kg bevacizumab (68.4 ± 9.6 SEM) and 4 mg/kg bevacizumab (53.1 ± 6.9 SEM) when compared to control (150 ± 10.4 SEM; P < 0.001). No significant difference in tumor blood microvessel count was observed between bevacizumab treatment groups (P = 0.47).

![Graph](image)

**Figure 3-3.** Graph of mean microvessel counts/200X field in control and two treatment groups. Bevacizumab, at doses of 2 and 4 mg/kg IP twice per week for two weeks, significantly reduced mean number of microvessels/200x field (p < 0.001).
Figure 3-4. Histopathologic image (CD31 with hematoxylin counterstain) of tumor sections (200X magnification) from a mouse in the control group (A) and a mouse in the bevacizumab 4 mg/kg group (B). Bevacizumab significantly decreased the number of CD31-positive staining microvessels (brown/black stain) at both doses tested (2 and 4 mg/kg IP twice per week for two weeks; p < 0.001).
**Tumor Histomorphology**

All tumors had an infiltrative growth pattern. Tumor cells were either round or polygonal, with a moderate amount of cytoplasm and round-oval, chromatin stippled nuclei. All tumors contained both osteoid and bone, and no tumor cells were seen to be invading tumor vasculature (as evidence of hematogenous metastasis).

**Discussion**

Bevacizumab administered as a single therapy (4 mg/kg IP twice weekly for two weeks) delayed tumor growth in our experimental canine OSA model without observable toxicity. When administered at both 2 and 4 mg/kg IP twice weekly for two weeks, bevacizumab also induced a reduction of angiogenesis by over 50%. The likely mechanism of this effect is that bevacizumab binds to VEGF (both soluble and bound to the extracellular matrix) and thereby prevents VEGF binding to its receptors, blocking endothelial cell growth, vascular permeability and angiogenesis.73,84,85

Human osteosarcoma has a high metastatic rate, is very locally invasive, with an abundant local blood supply.76 Some OSAs and ESFTs have been shown to produce VEGF, and its expression by greater than 30% of tumor cells has been associated with a pulmonary metastasis and poor prognosis.77,86 Therefore we hypothesized that anti-VEGF therapy may have rationale in the treatment of canine osteosarcoma. Results form the present study support this theory, shown by a dose-dependent inhibitory effect of bevacizumab on the growth of xenografted canine osteosarcomas and a reduction in tumor-associated micro-vasculature. Our results are therefore in support of previous studies where bevacizumab delayed the progression of primary soft tissue sarcomas, ESFTs and low-grade neuroendocrine tumors.74,77,87 The results of our study also support previous assessing the efficacy of bevacizumab administration to human rectal
cancer patients, which showed a decrease in tumor microvessel density as early as 12 days after a single infusion of bevacizumab.\textsuperscript{88}

D'Adamo \textit{et al} recently investigated bevacizumab in combination with doxorubicin for the treatment of metastatic soft tissue sarcoma in people.\textsuperscript{74} Following treatment with bevacizumab and doxorubicin, 65\% of the patients experienced disease stabilization for at least 3 months. Those findings suggest that the treatment combination of bevacizumab and doxorubicin provides disease stabilization for a large majority of patients with metastatic soft tissue sarcoma. Clinical results were superior to those reporting the use of doxorubicin alone.\textsuperscript{74} We did not investigate the effect of bevacizumab on tumor metastasis in this study for several reasons. First, previous studies using the HMPOS cell line in a subcutaneous murine model found that pulmonary metastatic tumor colonies were not observed histologically until approximately four weeks post-inoculation, and only when relatively high cell inoculation numbers were used (e.g. > 5 x 10\textsuperscript{6}).\textsuperscript{45,54} Second, given the anticipated time frame needed to evaluate micrometastatic tumor growth following first appearance at four weeks, the primary tumor would have likely exceeded 10\% of body weight or become ulcerated. Lung tissue was collected at necropsy, but only to fully characterize the model (tumor cells were not expected to be present). We speculate that similar effects would have been observed in pulmonary metastatic tumor colonies. Bevacizumab may, in fact, be beneficial in the control of micro-metastatic tumor colonies, as radiographic responses are rare when bevacizumab is used in a salvage setting, suggesting that bevacizumab may have greater effect when used earlier in the disease course.\textsuperscript{89} Clearly, further investigation into the anti-tumor effects of bevacizumab on metastatic canine OSA and both human ESFT and OSA is warranted.
An emerging consideration from the studies by D'Adamo et al, is the implication of bevacizumab in observed treatment toxicities. Preclinical and phase I studies have suggested that anti-angiogenic therapy should not have the typical acute toxicities of cytotoxic chemotherapy. Most important of the reported toxicities is the exacerbation of doxorubicin-associated cardiomyopathy associated with the use of combination doxorubicin and bevacizumab therapy. That is, cardiomyopathy is magnified with concurrent bevacizumab, but repaired when bevacizumab is discontinued, suggesting that low-grade cardiomyopathy caused by doxorubicin is reparable in a VEGF-dependent fashion. While previously reported toxicities such as cardiomyopathy, hypertension, epistaxis and proteinuria were not observed in our study, we did not specifically evaluate our mice for these toxicities through diagnostic testing. Nevertheless, new cardiovascular toxicities are being observed with increasing use of bevacizumab and, despite promising results in colon cancer, safety and efficacy studies should be undertaken before routine incorporation into clinical regimens.

Bevacizumab is a humanized monoclonal antibody and therefore we would not expect bevacizumab to block host derived murine VEGF completely, thereby limiting the observed growth delay in this study. Using a humanized monoclonal antibody is a limitation of our study and, although proof of principle could be demonstrated for dogs, treating dogs with a humanized monoclonal antibody would only be effective in the short-term before the antibody is recognized as foreign and rejected (in immunocompetent dogs). Even if a canine conspecific anti-VEGF antibody was available, the need for consistent long-term dosing may be cost-prohibitive. These results provide translational support for investigating the use of anti-VEGF therapies for human ESFT and OSA.
The small body-weight of our mice (mean 16.8 g; range 13.8 - 19.3 g) made accurate dosing at 2 mg/kg and 4 mg/kg bevacizumab difficult. This difficulty was somewhat addressed with dilution to deliver a consistent volume of drug to individuals. Although it is possible that some minor variation in bevacizumab dosing may have resulted from the small injection volumes, the low amount of standard error seen in the vessel counts and tumor growth delay suggest that absorbed dose was fairly consistent. While intraperitoneal administration of drugs has become the standard for murine models, it is possible that blind IP administration could result in delivery of the drug into the urinary bladder, hollow viscus organ or intravenously.

Grier et al showed that as little as 40% of intended IP injections, are actually delivered into the peritoneal space.92 This limitation could be addressed with indwelling intraperitoneal or intravenous catheter placement and drug administration; however, the morbidity associated with indwelling devices may preclude their use. Furthermore, for bevacizumab, route of administration is apparently not important in reaching therapeutic, circulating concentrations.93 More important than inadvertent intravisceral administration of IP injections, is the risk of visceral (especially cecal) perforation and peritonitis with blind IP injection.94 Despite this risk, no evidence of local or diffuse peritonitis was noted in this study.

Another limitation of this study was that control mice did not receive a placebo injection. While we were unable to definitively assess that the effect in tumor growth delay and inhibition of angiogenesis in this study is due to bevacizumab rather than the act of intraperitoneal injection, previous evidence suggests that no placebo effect is seen with volume-matched IP administration of diluent or vehicle.95

Finally, the present study uses intramuscular (heterotopic) xenografts rather than intraosseous (orthotopic) xenografts. The heterotopic model used here is easy to establish, very
reliable and repeatable, and yielded encouraging preliminary results. These results may not represent those seen with orthotopic xenografts, and therefore may not predict clinical performance or outcome of bevacizumab used to treat clinical cases of OSA in dogs or human patients. Compared to intramuscular or subcutaneous tumors, high interstitial pressures within osseous tumors may limit drug delivery.67

In the present study our findings show a dose-dependent inhibitory effect of bevacizumab on the growth of xenografted canine osteosarcomas. This effect is likely a result of the anti-angiogenic properties of bevacizumab. Further investigations are warranted to investigate the effect of combination therapies and the effect of bevacizumab on micrometastatic disease for dogs and humans with osteosarcomas and other cancers.
CHAPTER 4
ANTI-TUMOR EFFECTS OF RADIATION THERAPY, CARBOPLATIN AND COMBRETASTATIN-A4 PHOSPHATE COMBINATION THERAPIES IN A MOUSE MODEL OF XENOGRAFTED CANINE OSTEOSARCOMA

Introduction

In the management of spontaneous canine osteosarcoma, radiation therapy (RT) has been a beneficial therapeutic modality in both palliative and curative intent strategies.\textsuperscript{1-5,14,15,18,20-29,32,33} When fractionated, RT is not uniformly effective, presumably due to the relative radioresistance of the canine osteosarcoma cell population. This limitation has been somewhat addressed in dogs through the application of stereotactic radiosurgery, but the need for a widely available clinical alternative remains.\textsuperscript{3,15}

Tumor vessels are highly disorganized, have an incomplete underlying basement membrane, and exhibit increased permeability. While endothelial cells of normal tissues are largely quiescent, those of tumor vessels are activated and are more responsive to angiogenic cell signaling.\textsuperscript{96} Vascular associated anti-cancer therapy can be broadly classified into two groups: the anti-angiogenic agents, directed at inhibition of tumor neovascularization; and the vascular disrupting agents (VDAs), directed at destruction of tumor associated vasculature. Vascular disrupting agents exploit physiologic differences between tumor and normal vasculature to selectively shut down blood flow in solid tumors.\textsuperscript{97} One class of VDAs of particular interest is the tubulin binding agents, of which vinblastine, colchicines and recently combretastatin are members. Tubulin binding agents like Combretastatin A4-Phosphate (CA4P), disorganize the microtubules within the endothelial cells; specifically binding to the beta-subunits and preventing the formation of microtubules.\textsuperscript{57,97-99}

Combretastatin A4-Phosphate is a water-soluble, simple phosphate monoester (prodrug) of the phenolic natural product combretastatin A-4, isolated from African Bush Willow,
Combretum caffrum.\textsuperscript{100-102} It is rapidly dephosphorylated in vivo to yield the active, hydrophobic parent drug (CA4) by membrane-bound phosphatases, which are widely expressed on endothelial cells.\textsuperscript{100,101} CA4 has a very short plasma half-life and induces immediate and selective shutdown of the tumor vasculature through induction of endothelial cell apoptosis.\textsuperscript{100,103} After treatment with CA4P, newly formed daughter endothelial cells undergo shape changes as a result of cytoskeletal alterations. These shape changes lead to an increased vascular permeability. Endothelial cells detach, the vascular wall collapses, and tumor cell death occurs as a consequence of tumor blood flow obstructions.\textsuperscript{57,97-99,104,105} It produces a characteristic early necrosis in the center of a tumor, while leaving a viable rim of cells that may be supplied with oxygen and nutrients by surrounding host vasculature. A heterogenous pattern of tumor blood flow reduction has also been reported, and the viable rim may also be a result of incomplete shutdown of vasculature in the outer regions of the tumor. While this viable rim may ultimately limit the activity of VDAs administered as single agents, the susceptibility of this cell population to ionizing radiation and chemotherapy could result in a powerful effect of combining vascular targeting with conventional therapy.\textsuperscript{57,97} Furthermore, a great advantage of CA4P is that is shows antivascular activity even at doses far below the maximal tolerated dose (MTD) and, in addition to its effects on tumor vasculature, CA4P has direct antineoplastic activity against different tumor cell lines.\textsuperscript{103,106,107}
Figure 4-1. An artist’s sketch of the Combretum family of trees, depicting the characteristic four-winged pods and simple leaves. Combretastatin A4-Phosphate is derived from Combretum caffrum, the African Bush Willow. Source: www.bushwillow.com/html/bushwillow_tree.htm

Figure 4-2. Molecular structure of Combretastatin A-4 and its phosphorylated pro-drug CA4P.107

Carboplatin is a platinum-derivative chemotherapeutic agent that inhibits DNA synthesis.109,110 It is used extensively in human and veterinary oncology, in particular, it is used as both a radiosensitizing agent and as an adjunctive chemotherapeutic agent in the treatment of
canine appendicular OSA.\textsuperscript{3,15,23} The rationale for coordinating the administration of carboplatin with radiation to achieve enhancement of cancer therapy is developed. Two major effects include radiosensitization (RS) of hypoxic cells with platinum present during irradiation, and potentiation of cell kill with platinum complexes administered after irradiation. Both these effects are expected to result in an improved therapeutic ratio. The latter effect may include inhibition of recovery from radiation-induced potentially lethal damage (PLD) and sublethal damage (SLD).\textsuperscript{33,111} Radiation therapy has been shown to increase cellular uptake and DNA-binding of carboplatin in vitro and subsequently increase chemotoxicity.\textsuperscript{112-114} As such, most clinical regimens involving canine OSA and radiosensitization with carboplatin involve administration of carboplatin immediately after local tumor irradiation, though it is not known whether greatest radiosensitization is achieved when carboplatin is administered before, during or after radiation therapy.\textsuperscript{3,15,23} Despite the widespread use of carboplatin as a radiosensitizer for treatment of canine OSA, no objective data exists to support its use.

The objectives of this study were 1) to investigate the effect of CA4P alone on xenografted canine OSA, and 2) to investigate combination therapies including CA4P, external beam radiation therapy and carboplatin.

Our hypotheses were as follows:

1. CA4P would induce significant central tumor necrosis without affecting tumor growth.

2. Combination therapies (CA4P/XRT, Carboplatin/XRT and CA4P/Carboplatin/XRT) would provide superior tumor growth delay than single agent therapies.
Materials and Methods

Cell Line

Canine highly metastasizing parent osteosarcoma (HMPOS) canine osteosarcoma cells were obtained and cultured as described in Chapter 2 (page 29).

Induction and Measurement of Tumors

Xenografts were induced and measured as described in Chapter 2 (pages 29 – 31).

When tumor weight reached 0.2g, mice were randomly assigned to one of seven groups (n=9 for each group) and treatments were begun:

1. Control group: no treatment
2. CA4P alone
3. Carboplatin alone
4. Radiation therapy alone
5. Radiation therapy + carboplatin
6. Radiation therapy + CA4P
7. Radiation therapy + CA4P + carboplatin

Tumor size was measured every other day, and all mice were euthanized (CO₂ + thoracotomy) when their tumors reached approximately 1.0g (i.e. 13 mm in diameter), or earlier if quality of life was compromised. All experiments were performed under institutionally or nationally approved guidelines for animal welfare (IACUC).

Radiation Therapy Treatment

Conscious mice were restrained in customized plastic jigs and tumor irradiations were performed using a 6-MV Clinac 600c linear accelerator (Varian Oncology Systems, Palo Alto, CA). The tumor-bearing limb was extended through an opening in the side of the jig, allowing for local tumor irradiation (10 Gy) at a rate of 4 Gy/min. 8mm thick gelatin radiation boluses
were suspended above the tumor-bearing limb to ensure uniform tumor irradiation. (See image in tumor model). Sham radiation treatments were performed, whereby all mice (regardless of group) were transported to the radiation facility to coincide with radiation treatments in groups receiving radiation therapy.

**Drug Treatment**

CA4P (OXiGENE Inc, Lund, Sweden) was dissolved in 0.9% sterile saline spiked with sodium carbonate immediately before each experiment, to achieve a final concentration of 18mg/ml. The CA4P was administered at 100mg/kg animal weight. In all mice (except control groups), IP injections were administered by use of a ½ cc insulin syringe (Terumo U-100 insulin, 1/2cc, 27G x 1/2-inch needle). Combretastatin A4-P administration was initiated on a Monday-Wednesday-Friday (or similar) regimen, for a total of 6 treatments. In combination therapy groups, the first CA4P dose was administered 60 minutes after irradiation.

Carboplatin (Paraplatin, Bristol Myers Squibb, Princeton, USA) was administered at 60mg/kg, via a single IP injection (Terumo U-100 insulin, 1/2cc, 27G x 1/2-inch needle) either as a single agent therapy or 60 minutes after radiation therapy.

**Necropsy and Histopathological Examination**

After euthanasia, a complete necropsy was performed. The primary tumor was dissected with the limb in situ, transected at its greatest diameter, and fixed in 10% neutral-buffered formalin. The respiratory tract (larynx to lungs), heart and mediastinal fat were dissected free and the lungs were inflated with 10% neutral-buffered formalin. After fixing in formalin for 48 hours, all tissues were then transferred into 70% alcohol. A 5-um thick section of the primary tumor was embedded in paraffin and stained with H&E for microscopic examination.
Tumor Parameters

The anti-tumor effects of radiation therapy, carboplatin and CA4P combination therapies were studied in detail using the following techniques:

1. Tumor Growth Delay \((t^{0.2-1.0g})\): The number of days required for tumors to grow from 0.2 – 1.0g was calculated for individuals and for groups.

2. Tumor Necrosis: during slide examination for tumor histomorphology, digital images of tumor sections (maximum tumor diameter) were captured with an Olympus BX41 microscope with attached Olympus DP70 10 megapixel camera and processed using DPController version 1.2.1.108 and DPManger version 1.2.2.107, Olympus Optical Co., Ltd. (2001-2003). Analysis was completed with Image Tool for Windows version 3.00, The University of Texas Health Science Center in San Antonio (1995-2002) and percent tumor necrosis was calculated with the following formula:

\[
\%TN = \frac{NTA}{TTA} \times 100 \quad \text{or} \quad \frac{1}{1}
\]

where NTA = Necrotic Tumor Area and TTA = Total Tumor Area.

3. Morphology: Hematoxylin and eosin (H&E) staining of the same section was carried out in order to compare the distribution of tumor vasculature or necrosis with tumor architecture/structure. A single Board Certified Veterinary Pathologist (DT) performed all immunohistochemistry and histomorphology interpretation and was blinded to individual and group identity.

Data Analysis

Statistical calculations were performed using a computer software program (SigmaStat for Windows Version 3.0, SPSS Inc.). All relevant assumptions, such as normality, were verified formally. Significance was set at \(p < 0.05\). Tumor growth delay \((t^{0.2-1.0g})\) was compared between
groups using a one-way ANOVA (p < 0.05). Analysis of percent tumor necrosis was made using a Kruskal–Wallis one-way analysis of variance on ranks. Kruskal–Wallis analysis of variance on ranks was used because the data were not normally distributed. A pairwise multiple comparison procedure was performed using Tukey’s method. Because treatment groups were uneven for evaluation of percent tumor necrosis (one missing data-point), Dunn’s method of multiple pairwise comparisons was used for this evaluation.

**Results**

**Tumor Growth Delay**

Combination therapy including XRT/CA4P/Carboplatin (mean 18.3 ± 1.3 SEM days) induced significantly greater tumor growth delay than all single-agent treatments (XRT mean 12.1 ± 1.2 SEM days, CA4P mean 7.3 ± 0.5 SEM days, Carboplatin mean 7.4 ± 0.6 SEM days) and control (mean 6.2 ±0.7 SEM days; p<0.001). No significant difference was evident between XRT/CA4P/Carboplatin and either XRT/Carboplatin (mean 14.2 ± 0.8 SEM days; p = 0.061) or XRT/CA4P (mean 16.2 ± 1.3 SEM days; p = 0.726). While XRT, XRT/Carboplatin and XRT/CA4P groups all induced significantly greater tumor growth delay compared to Control, CA4P and Carboplatin groups (p < 0.001), no significant difference was observed between XRT, XRT/Carboplatin and XRT/CA4P groups (p > 0.73). Similarly, no significant difference in tumor growth delay was observed between Control, CA4P and Carboplatin groups (p > 0.97; Figure 4-3)
Figure 4-3. Graph of Tumor Growth Delay. Significant differences are indicated by letters above error bars. 

- a = no significant difference between control, CA4P and carboplatin single agent treatment groups.
- b = no significant difference between XRT alone, XRT/carboplatin and XRT/CA4P combination therapy groups.
- c = no significant difference between XRT/carboplatin, XRT/CA4P and XRT/CA4P/carboplatin combination therapy groups.
- d = significant difference between XRT/CA4P/carboplatin combination therapy and control, CA4P, carboplatin and XRT single agent treatment groups.

**Tumor Necrosis**

Treatment with CA4P alone (median 32.8 ± 4.3 SEM %) induced significantly greater tumor necrosis than all other treatment groups (XRT median 7.9 ± 3.3 SEM %, Carboplatin median 9.7 ± 4.9 SEM %, XRT/Carboplatin median 12.0 ± 4.4 SEM %, XRT/CA4P/Carboplatin median 18.0 ± 2.7 SEM %, XRT/CA4P median 21.2 ± 9.0 SEM %) and control (median 8.4 ± 3.0 SEM %); p>0.05 (Figures 4-4 – 4-11).
Figure 4-4. Graph of Median Percent Tumor Necrosis. Significant differences are indicated by letters above error bars. a = no significant difference between control, carboplatin and XRT single agent treatment groups as well as XRT/CA4P and XRT/CA4P/carboplatin combination therapy groups. b = significant difference between CA4P single agent treatment and all other treatment groups.
Figure 4-5. Comparative images of low power (40x) slides from the 9 control tumors stained with H&E. Median tumor necrosis was 8.4 ± 3.0 SEM %; range 1.92 – 24.93 % and significantly lower than tumors treated with CA4P alone.
Figure 4-6. Comparative images of low power (40x, stained with H&E) slides from the 9 tumors treated with 60 mg/kg carboplatin IP once. Median tumor necrosis was $9.7 \pm 4.9$ SEM %; range $0.31 – 45.67$ % and significantly lower than tumors treated with CA4P alone.
Figure 4-7. Comparative images of low power (40x, stained with H&E) slides from the 9 tumors treated with XRT alone (single fraction 10 Gy). Median tumor necrosis was $7.9 \pm 3.3$ SEM %; range 0.61 – 33.05 % and significantly lower than tumors treated with CA4P alone.
Figure 4-8. Comparative images of low power (40x, stained with H&E) slides from the 9 tumors treated with CA4P alone (100 mg/kg IP on a Monday-Wednesday-Friday regimen or similar, for 6 treatments. Median tumor necrosis was 32.8 ± 4.3 SEM %; range 9.05 – 53.94 % and significantly greater than all other treatment groups.
Figure 4-9. Comparative images of low power (40x, stained with H&E) slides from the 9 tumors treated with XRT 10 Gy and a single dose of 60 mg/kg carboplatin IP, 60 minutes after irradiation. Median tumor necrosis was 12.0 ± 4.4 SEM %; range 1.6 – 44.38 % and significantly lower than tumors treated with CA4P alone.
Figure 4-10. Low power (40x, stained with H&E) images from the 9 tumors treated with XRT 10 Gy, followed 60 minutes later by CA4P (100 mg/kg IP on a Monday-Wednesday-Friday regimen or similar, for 6 treatments). Median tumor necrosis was 21.2 ± 9.0 SEM %; range 9.62 – 84.82 % and significantly lower than tumors treated with CA4P alone.
Figure 4-11. Low power (40x, stained with H&E) images from tumors treated with XRT 10 Gy, followed 60 minutes later by a single dose of 60 mg/kg carboplatin IP and CA4P (100 mg/kg IP on Monday-Wednesday-Friday or similar, for 6 treatments). Median tumor necrosis was $18.0 \pm 2.7$ SEM %; range $8.37 - 30.87$ % and significantly lower than tumors treated with CA4P alone. Histomorphology and percent necrosis data is not available for one tumor.
Table 4-1. Percent tumor necrosis data for control, 10 Gy irradiation, carboplatin 60 mg/kg, CA4P 100 mg/kg, 10 Gy irradiation + carboplatin 60 mg/kg, 10 Gy irradiation + CA4P 100 mg/kg and 10 Gy irradiation + carboplatin 60 mg/kg + CA4P 100 mg/kg treatment groups. Values are represented as % of total tumor cross-sectional area at the level of bisection. * - Data not available.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mouse Number</th>
</tr>
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<tbody>
<tr>
<td></td>
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<td>Control</td>
<td>24.44</td>
</tr>
<tr>
<td>10 Gy</td>
<td>11.46</td>
</tr>
<tr>
<td>Carboplatin 60mg/kg</td>
<td>11.47</td>
</tr>
<tr>
<td>CA4P 100mg/kg</td>
<td>35.32</td>
</tr>
<tr>
<td>10Gy + Carboplatin 60mg/kg</td>
<td>2.92</td>
</tr>
<tr>
<td>10Gy + CA4P 100mg/kg</td>
<td>21.22</td>
</tr>
<tr>
<td>10Gy + Carboplatin 60mg/kg + CA4P 100mg/kg</td>
<td>9.41</td>
</tr>
</tbody>
</table>
Tumor Morphology

All tumors were described histopathologically as having infiltrative growth patterns. All tumor cells were either round or polygonal, with a moderate amount of cytoplasm and round-oval, chromatin stippled nuclei. All tumors contained both osteoid AND bone, and no tumors/cells were seen to be invading tumor vasculature (as evidence of hematogenous metastasis).

Many of the tumors (in all groups) had very large cystic areas filled with blood between the aggregates of tumor cells. Such areas were included in the total tumor area for calculations of percent tumor necrosis. Where there was evidence of tumor cell necrosis within the cystic areas, these were considered necrotic and reflected in the percent necrosis. However, if the cystic areas had no evidence of tumor cell necrosis, they were not considered as necrotic. Among all groups, areas of active tumor necrosis were frequently closely aligned with cystic areas, without necrosis in the cystic area.

Discussion

Results from this study support our hypothesis that Combretastatin-A4-Phosphate (CA4P) would induce significant central tumor necrosis without delaying tumor growth. In-fact, CA4P (single agent) treatment was the only treatment to provide significantly greater tumor necrosis than control tumors. Also, we were able to partially accept our hypothesis that combination therapies would provide superior tumor growth delay than single agent therapies. This hypothesis was true for XRT/CA4P/Carboplatin compared to all single agent therapies, but only true for XRT/CA4P and XRT/Carboplatin compared to groups not treated with radiation.

All tumors, regardless of treatment, were histomorphologically similar and the characteristics of these tumors demonstrated the successful xenografting of osteosarcomas in the intramuscular model. Many of these tumors in this study had very large cystic areas filled with
blood, between the aggregates of tumor cells, and often closely aligned with areas of active
tumor necrosis. Such areas were included in the total tumor area and where there was evidence
of tumor cell necrosis within the cystic area this was considered tumor necrosis and reflected in
the percent tumor necrosis data. However, if the cystic areas had no evidence of tumor cell
necrosis, then they were not considered part of the necrotic tumor fraction. These cystic areas
with only blood may represent areas of tumor degeneration that occurred during early tumor
growth and that have persisted until this point in time, and they are probably not associated with
the recent treatment. The theory that cystic regions without obvious tumor necrosis reflects
"normal" HMPOS xenograft morphology is supported by a report of HMPOS xenografts in
which untreated tumors frequently had a cystic center. At higher magnification, some tumor
cells in the CA4P treatment group showed clear evidence of a transitional zone of nuclear
fragmentation, bordering areas of coagulative necrosis, as has been described in previous studies
(Figure 4.15). These findings are consistent with an ischemic injury, and while they were
present in both control and CA4P treated tumors, the percentage area occupied by necrotic cells
was significantly greater in CA4P treated tumors.

Despite substantial reductions in tumor perfusion, as suggested by our percent tumor
necrosis data, our results support previous reports indicating that CA4P has minimal effect on
tumor growth. CA4P-treated tumors frequently have a central necrotic cavity with a peripheral
viable rim of tumor cells, which allows continued eccentric tumor growth. The rationale for
combination of radiation therapy and CA4P is that radiation should be more effective in the well-
oxygenated tumor periphery (where CA4P is ineffective due to the presence of mature normal
vasculature), while CA4P targets the tumor center where radioresistant areas of tissue hypoxia
may exist. Several studies have investigated the importance of timing and sequence between
VDA and radiation treatment.\textsuperscript{117-119} Improved tumor control has been demonstrated with both tumor growth delay and \textit{in-vivo/in-vitro} colonogenic cell survival.\textsuperscript{117-119} By far, the greatest anti-tumor activity was observed when the VDA was administered within a few hours after irradiating.\textsuperscript{119} Administration of CA4P before irradiation has little or no benefit.\textsuperscript{118} With fractionated radiation schedules it would therefore be important to give CA4P after the last radiation treatment each week, to achieve maximal effect.\textsuperscript{119} In our study, both CA4P and carboplatin were administered 60 minutes after tumor irradiation. 10 Gy external beam radiation administered in a single fraction, alone or in combination with either CA4P or Carboplatin or both, significantly delayed tumor growth compared to other single agent treatments and controls. The use of either carboplatin or CA4P in combination with radiation therapy did improve tumor growth delay when compared to radiation therapy alone in this study, but this trend was not significant. Lack of significance is either because the tumor growth delay effect in these combination therapy groups was due primarily to radiation therapy, or sample sizes were too small to observe statistical significance.\textsuperscript{117} Interestingly, the use of both CA4P and carboplatin, in addition to radiation therapy, significantly delayed tumor growth compared to radiation therapy alone. This suggests that either some radiosensitization is provided by combining carboplatin and CA4P, or that the tumor growth delay effect observed in this group is an effect of interaction between CA4P and carboplatin. While not investigated in the present study, combretastatins (CA4P and CA1P) have shown enhancement of the therapeutic effect in a combination treatment with chemotherapy, including platinum-derivatives.\textsuperscript{101,116,121,122} As with CA4P-radiation therapy combinations, greatest enhancements are seen when CA4P is administered within a few hours after chemotherapy.\textsuperscript{116,120} Larger sample sizes may have
improved the statistical significance of radiation-combination therapies, compared to radiation therapy alone in this study.

At the end-point of the tumor growth delay model (when tumor volumes reached 1.0g), the extent of tumor necrosis in untreated mice was approximately 8%. In comparison, CA4P treated mice displayed significantly greater tumor necrosis than control mice, with mean tumor necrosis being approximately 33%. Clinically it has been demonstrated that approximately 25% necrosis is present at the time of treatment with naturally occurring canine OSA, so it is important to note that control tumors displayed less tumor necrosis than detailed in previous reports of naturally occurring canine OSA.68 This difference is likely a result of altered local tumor biology and tumor size in our model. Percent necrosis data is not available for comparison from reports of orthotopic canine osteosarcoma xenografts.46

Combretastatin A-4 induces immediate and selective shutdown of the tumor vasculature through induction of endothelial cell apoptosis. Peak effect on tumor blood flow is demonstratable after 6 hours and is sustained until 24 hours, inducing approximately 90% vascular shutdown when administered at 100mg/kg in some models.103,108 While the significant difference in percent tumor necrosis in the present study is encouraging, maximum percent tumor necrosis in any tumor was ~85%. This tumor was harvested 24 hours after CA4P treatment, and 10 days after radiation therapy. The large percent necrosis in this (and other mice receiving CA4P many days after other combination therapies) tumor is possibly a reflection of CA4P-effect rather than true combination therapy. All mice in our study that received CA4P, were sacrificed between 24 and 72 hours after single agent or combination CA4P treatment. This variation was, presumably, the result of using a single end-point (tumor growth to 1.0g) for assessment of all variables. Therefore, in some mice, peak-effect of CA4P treatment had likely
passed and percent tumor necrosis values are potentially lower than might be expected with universal assessment either 6 or 24 hours after treatment. Due to large variation in percent tumor necrosis, one-way ANOVA was performed on ranks, which may have limited the statistical significance of group differences. Despite this variation, CA4P single-agent treatment induced significantly greater tumor necrosis compared to all other treatment groups.

In 1998, Li et al reported CA4P and radiation combination therapy against KHT sarcoma murine xenografts. This study identified a significant increase in tumor cell kill when CA4P (100mg/kg) was administered 0.5-1 hr after radiation (15Gy), compared to radiation alone.\textsuperscript{115} We did not perform colonogenic cell survival assays in our study, and therefore cannot make an objective assessment on cell survival. Using percent tumor necrosis as an estimate of tumor cell kill, we noticed a similar trend towards greater tumor cell kill with combined CA4P and radiation, compared to radiation therapy alone. The significance of this trend is likely limited by large intra-group variation and variable intervals between treatments and histologic assessment.

In this murine intramuscular model, CA4P as a single-agent therapy induced a significant degree of tumor necrosis, but did not delay growth of HMPOS xenografts. This is most likely due to the remnance of a viable peripheral tumor rim that continues to grow with a healthy vascular supply. The extent of tumor necrosis in this model was substantially lower than previous reports of between 60-90% tumor necrosis in various human tumor xenograft models. Therefore the extent of tumor necrosis may be dependent on tumor type and inherent blood vessel density. Radiation therapy was effective at delaying tumor growth; however, this effect was only enhanced with the addition of both CA4P and carboplatin to the treatment regimen.
Figure 4-12. High-powered (200x) microscopic image of a CA4P treated tumor section illustrating a zone of transition from relatively normal and viable tumor cell mass, through an area characterized by karyolysis, to an area of substantial coagulative necrosis.
CHAPTER 5
CONCLUSION

Canine osteosarcoma (OSA) is the most common primary bone tumor in dogs, and has been recognized as an acceptable model for the relatively rare osteosarcoma in children. In both species, radiation therapy plays an important role in case management, especially with appendicular disease. External beam radiation therapy is effective in palliating specific symptoms associated with osteosarcoma, including pain and lameness. Until recently, with the advent of stereotactic Radiosurgery (SRS), curative-intent radiation therapy has been ineffective without combining tumor irradiation and surgery.

Chapter 1 described, in detail, the objectives and reported protocols for both palliative and curative-intent radiation therapy for canine osteosarcoma. Importantly, this literature review highlights one reason why conventional fractionated radiation therapy may be ineffective with curative intent. When Fitzpatrick et al reported inherent radiosensitivity of some canine OSA cell lines in-vitro (through relatively low alpha:beta ratios and high survival fraction at 2 Gy), these results indicated that high fraction size is required to kill canine OSA cells. For clinicians without access to SRS, radiation-induced adverse effects prevent the clinical use of large-dose single fraction tumor irradiation. Likewise, intra-operative tumor irradiation is technically demanding and associated with frequent complications. As such, a technique must be developed that allows delivery of effectively high-dose radiation while sparing surrounding tissues from radiation-associated adverse effects.

Using techniques described for human intramuscular (IM) xenografts, we developed an IM murine xenograft model using canine highly metastasizing pulmonary osteosarcoma (HMPOS) cells. The xenograft model is described in Chapter 2 of this thesis, and proved to be very reliable and applicable to investigation of therapies that may complement radiation. By
creating distal appendicular IM xenografts, we were able to use specially designed jigs to isolate the tumor-bearing limb and spare the remainder of the mouse from radiation exposure.

Vascular endothelial growth factor (VEGF) allows tumors to grow rapidly, achieve vascular invasion and establish metastatic tumor colonies. With respect to OSA, VEGF has been associated with more frequent metastasis and poor outcome in humans. We hypothesized and confirmed in Chapter 3 that, by blocking VEGF with the anti-VEGF humanized antibody bevacizumab, we could delay tumor growth and reduce tumor neovascularization. At both doses tested, bevacizumab significantly reduced tumor micro-vessel density by at least 50%. When bevacizumab was administered at 4 mg/kg IP twice weekly for two weeks, tumor growth delay was significantly longer (almost twice as long) than control. These results are very encouraging not only for local disease control, but potentially for metastatic disease as well. Frequent dosing, treatment cost and problems associated with the administration of a humanized monoclonal antibody will likely prohibit the clinical use of anti-VEGF antibodies in canine OSA. However, our findings do demonstrate proof-of-principle and justify close investigation of bevacizumab with human OSA.

Chapter 4 details the investigation of the vascular targeting agent Combretastatin A4-Phosphate (CA4P). By damaging tumor associated vasculature through tubulin-binding, CA4P produces a characteristic central necrosis in human xenografts. Adjacent host vasculature that supplies the tumor periphery is less affected by CA4P, and an outer viable rim of tumor cells often remains. While this viable rim may ultimately limit the activity of CA4P administered as a single agent, the susceptibility of this cell population to ionizing radiation (i.e. well oxygenated tumor periphery) could result in a powerful effect of combining vascular targeting with conventional therapy. We investigated radiation therapy, carboplatin and CA4P alone, and as
combination therapies and found superior tumor necrosis when CA4P was administered alone compared to all other treatment groups. A predictable necrotic center was not seen in the present study, and percent tumor necrosis did not correlate directly to tumor growth delay for CA4P single-agent therapy, presumably because tumors continue to grow from the remaining peripheral rim. Radiation therapy, and its use in combination therapies, was the only treatment that significantly delayed tumor growth compared to control. A trend towards tumor growth delay was observed with the addition of carboplatin and CA4P to tumor irradiations, but only became significant when all three treatments were combined and compared to radiation alone. While carboplatin is used frequently in combination with tumor irradiation in a clinical setting, we were unable to document any local therapeutic advantage of this combination. Used in the combinations (timing and dose) reported here, neither carboplatin nor CA4P provided enough radiopotentiation to justify dose-reduction for curative-intent (non-SRS, non-IORT) irradiation in a clinical setting. However, while carboplatin has well established potential as a radiopotentiating agent, in investigative research, no ideal dose has been established and we did not test a wide enough range of doses to make these conclusions. Therefore, further in-vivo and clinical investigation with these combination therapies is justified.

Using this IM HMPOS murine xenograft model, tumor irradiations were delivered safely and evaluated easily. This model is suitable for further investigations of HMPOS, and could be used as a template for other canine tumor models and human OSA. Studies evaluating percent tumor necrosis with CA4P and naturally occurring canine OSA are justified.
LIST OF REFERENCES


Notes: -MAb TES-23


BIOGRAPHICAL SKETCH

Alastair R. Coomer began his veterinary education by being accepted to the College of Veterinary Science, Massey University, Palmerston North, New Zealand, after 6 months of a pre-veterinary program. He completed a 5 year program in veterinary medicine and graduated in 2003 with a Bachelor of Veterinary Science. After graduation, he practiced veterinary medicine at Riverside Veterinary Services, Ashburton, New Zealand, for 18 months. He then completed a clinical internship in small animal medicine and surgery at the Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada, between 2004 and 2005. After completing the internship, Alastair moved to Gainesville, Florida, in 2005, where he entered the Master of Science degree program at the University of Florida’s College of Veterinary Medicine. In combination with the master’s degree, Alastair is also completing a residency in small animal surgery, and is scheduled to be board eligible in the summer of 2009.