Expression of ICAM-1, MHC-1 and B7 surface molecules in canine primary appendicular osteosarcoma

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ABSTRACT

Osteosarcoma is the most common primary malignant bone tumor diagnosed in dogs. The characteristic aggressive and highly metastatic behavior of this neoplasm indicates that it has developed mechanisms to elude the immune surveillance system. The aim of the study was to determine whether canine primary appendicular osteosarcomas express ICAM-1, MHC-1 and B7 surface molecules which are critical in eliciting optimum immune response. We have analyzed cell lines and tissue sections (frozen and paraffin-embedded, formalin-fixed tissues) of dogs diagnosed with osteosarcoma using fluorescence microscopy, flow cytometry and immunohistochemistry for the expression of ICAM-1, MHC-1 and B7. All of the cell lines (n=5) examined by fluorescence microscopy exhibited high ICAM-1 expression. However, 3 out of 5 cell lines showed moderate expression of MHC-1 and we observed low expression in two cell lines. We measured B7 expression using flow cytometry on 5 canine primary appendicular osteosarcome cell lines. Analysis of flow cytometry data showed that 4 out of 5 canine osteosarcoma cell lines were positive for B7 molecules, with the percentage of B7-expressing cells ranging from 73.67 to 85.13 %. Sections of frozen (n = 7) and paraffin-embedded, formalin-fixed (n = 17) canine appendicular osteosarcoma tissue sections were examined by immunohistochemistry for expression of ICAM-1 and MHC-1 surface molecules. In frozen tumor sections, expression of ICAM-1 was high in tumors from 4 out of 7 (57.1%) dogs and 3 of 7 (43%) dog tumors expressed moderate staining. MHC-1 expression in tumors was observed in 4 of 7 (57.1%) dogs with moderate staining while 3 of 7 (43%) exhibited low staining. In formalin-fixed, paraffin-embedded tumor sections, ICAM-1 showed high staining for tumors from 10 of 17 (59%) dogs and moderate staining in tumors from 7 of 17 (41.2%) dogs. MHC-1 showed low expression in tumors

from 7 of 17 (41 %) dogs and negative staining in tumors from 10 of 17 (59 %) dogs. The results of this study demonstrate a high level of ICAM-1 expression in canine primary osteosarcoma, which could possibly explain the invasive nature and rapid metastasis of this malignant tumor. While B7 expression suggests, that these tumors are able to deliver costimulatory signal but yet manage to escape from immune effector cells perhaps due to low MHC-1 expression. These results suggest that the tumor cells could use this strategy to interfere with antigen presentation by MHC-1 while possibly maintaining inhibitory signal to NK cells.

CHAPTER 1. LITERATURE REVIEW

General Introduction

"Malignant tumors are the ultimate challenge to immunologists, posing profound and basic questions concerning tolerance, escape, stimulation, death and survival." (Melief, 2000)

Osteosarcoma is the most prevalent primary malignant bone tumor in dogs (Withrow et al., 1991). It accounts for 3% to 6 % of all canine cancers and affects 8,000 to 10,000 dogs per year in North America (Brodey, 1979). Decreases in osteosarcoma incidence are not expected due to the steady popularity of large-to-giant breed dogs as pets. These breeds of dogs are the most commonly seen with this tumor. Seventy five percent of the lesions are found in the appendicular skeleton (Brodey and Riser, 1969; Misdorp and Haart, 1979). Canine osteosarcoma has a highly metastatic behavior to the lungs (Brodey 1979). Micrometastasis is present in approximately 90% of dogs at presentation (Brodey and Riser, 1969). Only 10-15% survive longer than 9 months following diagnosis and amputation (Ling et al., 1974; Straw et al., 1990; Vail and MacEwen, 2000).

Tumor cells are known to escape immune defenses and have developed numerous strategies to progress and metastasize. Canine osteosarcoma with its characteristic aggressive malignant behavior and high percentage of lung metastasis in the early stages is one such tumor that has developed mechanisms to elude the immune surveillance system. A long-standing objective in the understanding of the immunobiology and development of therapies for tumors is how to achieve an optimum anti-tumor immune response. Investigating why canine osteosarcoma continues to escape immune effector mechanisms is an important research endeavor.

To determine why canine primary appendicular osteosarcoma is able to escape immune effector mechanisms, we analyzed the expression of surface molecules involved in cell adhesion (ICAM-1), antigen presentation and recognition (MHC-1) and costimulation (B7) of immune effector cells (Tcells and Natural killer cells). These surface molecules are part of the process that leads to tumor destruction and immune memory. In this study, we used canine primary appendicular osteosarcoma cell lines and tumor tissues from dogs with histologically confirmed primary appendicular osteosarcoma to determine the expression of ICAM-1, MHC-1 and B7 molecules. Additionally, in conjunction with an ongoing related clinical study using osteosarcoma cells transfected with adenoviral vector containing a gene for human IL-2 to stimulate antitumor immune response in dogs with primary appendicular osteosarcoma, we questioned whether IL-2 altered the expression of ICAM-1, MHC-1 and B7 molecules of these tumor cells *in vitro*.

Canine osteosarcoma

Incidence

Osteosarcomas or osteogenic sarcomas are malignant tumors primarily arising in bones. They are one of the most common and rapidly growing tumors in dogs (Withrow et al., 1991). Seventy five percent of osteosarcoma arises in appendicular bones and twenty five percent develop in the skull and axial skeleton. Most affected are large breed dogs and giant breed dogs (Labrador Retriever, Boxer, Irish Setter, St. Bernard and Great Dane). The disease is most likely to occur in middle-aged to older dogs, with a median age of 7 years. Males are more frequently at risk than female with a 2:1 ratio (Brodey and Riser, 1969; Straw et al., 1990). Tumors develop mostly at the metaphyses of the major weight-bearing long bones (distal radius and ulna, proximal humerus and proximal and distal femur, and

tibia). Lesions occur almost twice in the forelegs often than the hindlegs (Brodey et al., 1959; Brodey et al.1963, Brodey, 1979; MacEwen, 1990).

Contributing factors to the disease

The exact cause of osteosarcoma in dogs is unknown. Associated factors include: spontaneously occurring, hereditary/genetic determined susceptibility (large breeds 60 times at higher risk than small breeds), ionizing radiation, preexisting bone infarcts, metallic bone implants and fracture-associated sarcoma (Brodey, 1979). In a case-control study using the Veterinary Medical Data Base (VMDB) it is reported that the risk of osteosarcoma in dogs rose with increasing age, increasing body weight, increasing standard weight and increasing standard height. Interestingly, a twofold increase in risk was observed among neutered dogs (Ru et al., 1998). Johnson and colleagues (1998) were able to demonstrate point mutations in the p53 tumor suppressor gene in 7 of 15 (47%) of dogs with spontaneously occurring appendicular osteosarcoma. Another study showed that the MET proto-oncogene, a receptor for the cytokine hepatocyte growth factor/scatter factor (HGF) which stimulates the invasive growth of neoplastic cells, is abnormally expressed at high levels in canine osteosarcoma similar to human osteosarcoma (Ferracini et al., 2000).

Clinicopathological features

Most dogs at presentation have a history of progressive lameness and usually accompanied by a firm, swollen, variably painful mass on the involved long bone (Ling et al., 1974). Pathologic fracture is not uncommon due to the destructive nature of the tumor. Osteosarcoma is locally destructive and invasive. Metastasis is primarily by invasion of veins in the tumor area with embolization of tumor cells to the lungs and to other bones (Brodey et al., 1963). Pulmonary metastasis is the major cause of death in dogs with osteosarcoma and

tends to occur early in the course of the disease. Studies suggest that micrometastasis is present in approximately 90% of dogs at presentation (Straw et al., 1990; MacEwen, 1990; Hahn et al., 1994). Grossly, a typical lesion is composed of gritty to hard tumor tissue, which involves the entire width of the medullary canal, erodes and penetrates the cortex, and extends into the neighboring tissue. The single most important histological characteristic of osteosarcoma is the presence of osteoid-forming sarcomatous cells. Sheets of spindle cells with interspersed tumor osteoid matrix are normally seen. Many osteosarcomas exhibit several variants designated as chondroblastic, fibroblastic, osteoclastic (with numerous giant cells), osteoblastic, telangiectatic (have numerous blood-filled cystic spaces), or mixed (Brodey, 1963; Ling et al., 1974).

Cell lines

Osteosarcoma cell lines provide a useful tool for studying the pathobiology of various cancers. Several cell lines have been developed and characterized from spontaneous canine osteosarcomas (Kadosawa et al, 1994; Hong et al., 1998; Barroga et al., 1999). These cell lines are morphologically a mixed cell type composed of spherical cells, fibroblast -like cells, large or small polygonal and multinucleated giant cells. Primary canine appendicular osteosarcoma cell lines that can induce transplantable tumors in nude mice were also previously described (Nieves et al., 1998). The transplantable tumor's similarity to the histologic characteristics of primary canine appendicular osteosarcomas provided a model for the future use of these cell lines in various studies about the disease In a related study, cells with increased pulmonary metastatic properties, selected from a parent canine osteosarcoma cell line, were experimentally grown in nude mice. And thus, could serve as a nude mouse model for canine lung metastatic osteosarcoma (Barroga et al., 1999).

Model for human disease

There is a tremendous similarity in the clinical presentation, radiological and histopathological features of osteosarcoma in dogs and humans (Brodey,1979). Similarities include: male sex predilection, large patient size, 75% or more affecting the appendicular site, metaphyseal location, generally unknown etiology, tumor cells producing varying amount of osteoid, and the lung as the most common site of metastasis. Dogs therefore have been suggested as a model for the human disease in studying its etiology, immunobiology and therapy in humans (MacEwen, 1990; Withrow et al., 1991; Hahn et al., 1994).

Therapy

Conventional cancer therapies involve surgery, chemotherapy, radiation therapy and immunotherapy. A hundred years have past since William Coley observed that tumor regression could be induced by activating the immune system with bacterial toxins (Gore and Riches, 1996). The 'immune surveillance theory' of cancer described by Burnet, stated that cells of the immune system continuously patrol the body, searching and eliminating cells in the process of malignant transformation. This theory further advanced the suggestion of the role of the immune system in the growth and spread of tumors (Burnet, 1970). There has been a dramatic increase over the years in papers describing the role of the immune system in cancer biology. An elegant review by Hanahan and colleagues (2000) summarized information on physiologic changes that a normal cell acquires during the process of tumor development. These alterations include: self-sufficiency in growth signals, insensitivity to growth-1nhibitory (anti-growth) signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis and tissue invasion, and metastasis.

Since transformed cells are genetically unstable and acquire multiple mutations, some of the proteins that are expressed by these mutated genes could behave as antigens. They concluded that in each of these alterations successful evasion of antitumor defense mechanism has to occur. With the tightly controlled and multiplicity of immune defenses, a malignant tumor is infrequent in an average lifespan of a host. But the enigma is, tumors do exist, they affect humans and animals alike and treatment has yet to reach the level of success as in antibiotics to treat bacterial infections of vaccines to treat viral diseases. Tumor vaccines are still in the beginning stages of development.

Few immunotherapy approaches have been used in dogs with osteosarcoma. The earliest studies recorded the use of freeze-dried bacilli Calmette-Guerin (BCG) (Glaxo strain, 50-250 x 10^6 viable organisms) given intravenously to dogs after limb amputation. Seven dogs out of 20 were alive one-year later (Owen et al., 1977). In dogs receiving macrophage activator, liposome-encapsulated muramyl tripeptide-phosphatidyl-ethanolamine(L-MTP-PE) disease-free survival time and overall survival was extended, over amputation or no treatment when used alone after amputation or after cisplastin chemotherapy (MacEwen, 1990). Interleukin-2 liposome inhalation therapy in dogs with pulmonary metastasis resulted in complete regression of metastasis in two of four dogs treated (Khanna et al., 1997). Adjuvant treatment with irradiated clonal human cytotoxic T-cell line TALL-104 (proven earlier to be effective in treating dogs with malignant histiocytosis), given to dogs with osteosarcoma after surgery and chemotherapy resulted in a prevention or significant delay of disease recurrence in 9 of 23 dogs (Visonneau et al., 1999).

Several *in vitro* experiments showed that canine osteosarcoma cells are sensitive to immune effector cells. Canine blood lymphocytes cultured with recombinant IL-2 were

shown to have tumoricidal activity towards autologous and allogeneic canine pulmonary metastatic osteosarcoma cells *in vitro* as detected by chromium release cytotoxicity assay (Mitchell et al., 1991). Activated canine pulmonary alveolar macrophages exposed *in vitro* to two recombinant canine (rc) cytokines, rcTNFα and rcIFNγ showed significant cytotoxic activity against canine osteosarcoma cells (Kurzman et al., 1999).

With the assumption that specific tumor associated antigen is present in canine osteosarcoma cells that can activate major immune effector cells such as cytotoxic T cell (CTL) and natural killer cell (NK), the remainder of this literature review focuses on the immunoregulatory surface molecules (i.e., ICAM-1, MHC-1 and B7) that need to be expressed by canine osteosarcoma and are considered critical for optimum activation of immune responses. It is theorized that, canine osteosarcoma escapes the immune response because it fails to express these immunoregulatory molecules or better yet they are expressed aberrantly. CTL provides the antigen-specific, cell-mediated immune response while NK provides a non-specific, innate immune response against tumor cells. To generate an efficient specific cellular antitumor immune response the following cascade of events must occur: (1) antigenic peptides are expressed by the tumor cells, (2) these antigenic peptides must bind major histocompatibility complex (MHC) class 1 molecules, (3) leukocyte functional antigen-1 (LFA-1) on T cells binds ICAM-1 on the target cell, (4) the first signal, the tumor peptide/MHC class 1 complex recognized by the T cell receptor (TCR) on CTL occurs, however, by itself will fail to fully activate effector functions of CTL, (5) therefore, the presence of costimulatory signals, as a second signal provided by CD28 on CTL binding to B7 on APC and/or tumor cells, then leads to optimum activation, and in this case, killing of the tumor cells. NK cells have been shown to be responsible for eliminating

growth and metastasis of tumors both *in vitro* and *in vivo* (Kiessling et al., 1976; Kim et al., 2000). The molecular mechanisms behind this lytic activity unfolded with the development of the 'missing self hypothesis', which states that NK cells survey cells for normal expression of MHC class 1 and become activated on contact with cells that have down regulated or lost MHC class 1. Target cell specifity of NK cells, is unlike that of CTL with their clonally restricted receptor, TCR. NK cells recognize tumor cells through a group of inhibitory receptors that recognize MHC class 1 and stimulatory receptors. ICAM-1 and B7 molecules are both implicated to provide costimulatory signals to enhance NK cell cytotoxic activity.

Intercellular adhesion molecule-1 (ICAM-1, CD54)

The intercellular adhesion molecule-1 (ICAM-1) is a 90-114 kd surface glycoprotein member of the immunoglobulin gene superfamily, with five homologous Ig-like domains, a single transmembrane region and a short cytoplasmic tail (Rothlein et al., 1986). It is expressed constitutively at low levels on a variety of cell types, including those of hematopoeitic and nonhematopoeitic origin such as leukocytes, vascular endothelial cells, epithelial cells, and fibroblasts. Inflammatory mediators including interleukin-1 (IL-1), tumor necrosis factor (TNF) and interferon-γ (IFN-γ) have been shown to upregulate the expression of ICAM-1 (Dustin et al., 1986). ICAM-1 serves as a ligand for the β_2-1ntegrins, LFA-1 (CD11a/CD18) found on almost all leukocytes, including T lymphocytes and Mac-1 (CD11b/CD18), which has a more restricted expression on monocytes, macrophages and granulocytes (Rothlein et al., 1986; Staunton et al., 1988; Boyd et al., 1988). Since they serve as a ligand for LFA-1 and Mac-1, their biological roles have centered on forming homotypic and heterotypic adhesions between leukocytes specifically in trafficking of inflammatory

cells (Springer, 1990), interaction between antigen-presenting cells and T cells as in the formation of immunological synapse (Grakoui et al., 1999) and in providing costimulation signals (Chong et al., 1994; Kim et al., 1999; Gaglia et al., 2000; Hubbard and Rothlein, 2000). Due to this distinctive relationship and its expression on a wide variety of cells, ICAM-1 has emerged as a highly important molecule in immune and inflammatory responses.

ICAM-1 is expressed in a wide variety of malignant tumors (Johnson et al., 1989; Maurer et al., 1998; Hakansson et al., 1999; Shimoyama et al., 1999; Sun et al., 1999). ICAM-1 expression on tumor cells increases their interaction with the immuno-surveillance system and provides costimulation to immune effector cells such as T cytotoxic cells and NK cells. Mariani and colleagues (1997), demonstrated that human osteosarcoma cell lines differ widely in their susceptibility to natural killer cell lysis *in vitro*. To explain this, they investigated the expression of some cell adhesion molecules on osteosarcomas to determine which of these can modify their susceptibility to NK lysis. They found that cytotoxicity induced by NK cells correlated with differential expression of ICAM-1 on osteosarcoma. Use of immunoflourescence confocal laser microscopy showed distribution of ICAM-1 on the different osteosarcoma cell lines and correlated the expression of ICAM-1 with the capacity of NK cells for binding and lysis of the osteosarcoma cells (Meneghetti et al., 1999).

Since ICAM-1 has been shown to strengthen interactions between immune effectors and target cells, the expectation is that its expression on tumor cells would make them a better target for cytotoxic cells and should, therefore, be associated with a good prognosis. However, contrary to this expectation, ICAM-1expression in human melanoma

suggests involvement in the progression of the tumor to metastatic disease. The role of ICAM-1 in the development of metastasis was first suggested in human melanoma by Johnson and colleagues (1989) after detecting P3.58 antigen that was identical with ICAM-1in amino acid sequences. Increased ICAM-1 expression was demonstrated as melanocytes transformed to melanomas and continued to increase as they developed to a metastatic state. It was speculated that the *de novo* increased ICAM-1 expression by melanoma cells lead to heterotypic adhesion, whereby the tumor cells attach to LFA-1 bearing leukocytes and then hitch a ride on the leukocytes as they adhere to vascular endothelium and diapedese, allowing spread of the cells from the primary tumor to secondary sites. It has since been used as a predictive parameter for the early appearance of metastasis in this tumor type (see review by Johnson, 1991). This manner of metastasis was demonstrated later in other tumors (Simmons et al., 1995; Jiang et al., 1998, Sun et al., 1999). However, the precise mechanism by which tumor cells migrate through normal vascular endothelium remains controversial. Human polymorphonuclear neutrophils (PMNs), which comprise 50-70% of circulating leukocytes, were implicated to promote tumor metastasis (Starkey et al., 1984; Welch et al., 1989). Recently this hypothesis was tested *in vitro* by Wu and his group (2001), using an *in vitro* transendothelial migration model. This model demonstrated that MDA-MB-231, human breast adenocarcinoma cells which expressed high levels of ICAM-1, attached and migrated with PMN across different endothelial monolayers when treated with tumor-conditioned media, (TCM). Interestingly, they also found that incubating the PMNs in tumor-conditioned media suppressed PMN cytocidal function as measured by respiratory burst and phagocytosis and concomitantly upregulated PMN adhesion receptor expression of CD11b/CD18. Their group speculated that the TCM suppressed PMN cytocidal function and concomitantly

upregulated PMN adhesion receptor (CD11b/CD18) expression. This may indicate that this metastatic breast cancer cell line secretes factors capable of altering the phenotype of PMN to facilitate transmigration while preventing PMN-directed tumor cell damage. The metastatic breast cancer cell line might have secreted factors capable of altering the phenotype of PMN to facilitate transmigration while preventing PMN-directed tumor cell damage. Although it is uncertain which of the soluble factors present in TCM is responsible for altered human PMN function, tumor cell-produced granulocyte-macrophage colony stimulating factor and IL-3 have been found, in previous reports, to be responsible for potentiating tumor metastases facilitated by tumor-elicited PMN (McGary et al., 1995).

Manning and collaborators (1995), analyzed the conservation of ICAM-1 amino acid sequences in man, chimpanzee, mouse, rat and dog. Their study revealed that canine ICAM-1 had 61% identity with human ICAM-1. Cys residues critical to the immunoglobulin fold structure and four sites of N-linked glycosylation are highly conserved from all species. Residues critical for human ICAM-1 binding to the β_2-1ntegrin LFA-1 are highly conserved between all species, whereas residues important in binding Mac-1, rhinovirus and malaria-1nfected red blood cells are not. This conservation supports the hypothesis that intracellular attachment is their primary function. Previous studies of canine ICAM-1 have focused on its role in inflammation, and presence on cardiac myocytes, canine endothelial cells (Smith et al., 1991), macrophages (Grigg et al., 1994), keratinocytes (Olivry et al., 1995) and histiocytoma (Moore et al., 1996).

Major Histocompatibility Complex class 1 (MHC-1)

The discovery by Zinkernagel and Shevach (1979) demonstrated that in each individual or inbred strain of mouse, T cells are limited to recognizing antigens on the

surface of that individual's or strain's cells by virtue of MHC. This phenomenon is now known as MHC-restriction. The literature has grown immensely in its understanding of the role of MHC class 1 molecules in cell-mediated immunity particularly in anti-tumor immunity (reviewed in Tanaka et al., 1988; Khanna et al., 1998; Rees and Mian, 1999). Virtually all somatic cells express MHC class 1 molecules. This class of molecules interacts primarily with CTL. They are composed of a large α (α_1, α_2 and α_3 domains) polymorphic transmembrane protein of 45 kDa, non-covalently asssociated with non-polymorphic 12 kDa light protein called β2-microglobulin. Peptide presentation to CTL is mediated through α1 and α2 domains forming the peptide-binding groove, which present peptides of a limited length (8-10 amino acids), to CD8 T^+ cells. Sub-site pockets within the grooves of α1 and α2 domains specifically interact with side-chains residues from bound peptides. In addition, critical to the character of MHC-1 molecules are that they form stable and rigid complexes with peptide antigens. The α3 domains make noncovalent interactions with the protein β2-microglobulin to add stability and rigidity to the complex and allow peptide binding to occur (Bjorkman, et al., 1987; Bjorkman and Parham, 1990; Rees and Mian, 1999). There are 3 major human major histocompatibility class 1 genes, designated as the classical HLA- A, - B, or - C and nonclassical - G and - E (Bjorkman and Parham, 1990).

MHC class 1 molecules are expressed by virtually all nucleated cells and are capable of binding antigenic peptides that are generated mostly from endogenous proteins (including, tumor-associated antigens, TAAs) by the antigen-processing machinery. The peptides are presented on the cell surface to TCRs triggering a series of signaling events (cell proliferation, cytokine production and target-cell lysis) that can result in tumor cell lysis. Several steps are involved in antigen processing and MHC class 1 cell surface expression.

Step one involves degradation of cytoplasmic proteins into short peptides that is mediated by the multicatalytic proteasome complex. Step two includes translocation of the peptides into the endoplasmic reticulum (ER) by ATP dependent transporters associated with antigen processing, TAP-1 and TAP-2. A TAP-1ndependent pathway, which directly translocates peptides into the endoplasmic reticulum lumen by hydrophobic signal sequences, is another possible mechanism of peptide transport to the ER. In step three, peptides may either bind directly to freshly synthesized MHC class 1 molecules or may be chaperoned to class 1 molecules by tapasin. Finally, once the peptide enters the ER lumen, it combines with MHC class 1 heavy and β_2- microglobulin light chains as a trimolecular complex, traversing through the trans-Golgi complex and leading to surface expression (York and Rock, 1996; Pamer and Crewell, 1998). Any of these steps can potentially be defective resulting in MHC class 1 down-regulation in tumor cells, which may render them resistant to CTL lysis. Many human tumors, particularly those of epithelial derivation, appear to express greatly reduced levels or completely lack surface class 1 molecules (Garrido et al., 1997). These abnormalities can be caused by mutations in the MHC class 1 genes themselves or through abnormalities in their regulation and/or defects in MHC class 1 dependent antigen processing (Hicklin et al., 1999). Distinct phenotypes arising from these abnormalities have been identified. They include: 1) total HLA loss or down-regulation; 2) selective loss or down-regulation of a HLA class 1 haplotype; 3) selective downregulation of the gene products of the HLA-A or HLA-B locus; 4) selective loss or downregulation of a HLA class 1 allele; and 5) complex phenotypes representing combinations of phenotypes 1-4 (Ferrone and Marincola, 1995; Garrido et al., 1997). Combinations of the phenotypes can exist within a

given tumor-cell population, generating a heterogeneous pattern of HLA class 1 expression within a tumor lesion.

NK cells provide the major innate immune defense against tumors and are a backup system in case T cells fail to recognize and eliminate tumor cells by virtue of defective MHC class 1 expression. The manner of NK killing was previously described as primitive and nonspecific in the 1980's (for review see Moretta et al., 2000). The 'missing self hypothesis' put forth by Ljunggren and Karre, (1990) along with the discovery of different activating and inhibitory receptors of NK cells in the last few years more than illustrate the complexity and functional sophistication of these cells. In a nutshell, NK cells survey cells for normal expression of MHC class 1 and are activated when there is an abnormality in MHC class 1 expression. In contrast to TCR on CTL, target cell specifity for the NK cells is through interacting signals from activating receptors and inhibitory receptors called killer cell inhibitory receptors that recognize MHC class 1, which on ligation inhibits NK cytotoxicity and cytokine secretion. NK cells in humans express distinct inhibitory receptors (Colonna et al., 2000). Some of which detect shared allelic determinants of class 1 molecules whereas others show a broader specifity for different class 1 molecules (Moretta et al., 2000). One group of inhibitory receptors belongs to the Ig superfamily of type 1 glycoprotein which include killer cell inhibitory receptors (KIRs) and the human Ig-like transcript 2 (ILT2)/leukocyte inhibitory receptor 1, that specifically recognize groups of class 1 allotypes. Another family of inhibitory receptors is a heterodimer of CD94 and NKG2 that recognizes HLA-E antigens (Colonna et al., 2000). Thus, expressions of HLA class 1-like molecules, such as HLA-E that are nonpolymorphic also provide inhibitory signals to NK cells. When transcription/translation of an MHC class 1 molecule is disrupted, or the TAP-dependent

tanslocation of their signal sequence peptides into the ER is prevented HLA-E expression is also inhibited. Since HLA-E is a ligand for CD94-NKG2, cells with reduced HLA-E expression can be eliminated by the NK cell system (Cresswell and Howard, 1999). Moreover, NK cells express at least one receptor specific for self-MHC allowing the possibility for the whole NK pool of a given individual to detect the loss of even a single class 1 allele on tumor cells (Moretta et al., 2000). If CTL cells and NK cells are working in concert to eliminate tumor cells, why then do tumor cells manage to escape cytotoxic activities? From the elegant reviews of Garrido (1997) and Ruiz-Cabello (1998), it is hypothesized that some tumor cells have devised a stealth strategy of avoiding these two cells altogether. B16/F10.9 melanoma tumor cells express very few MHC class 1 molecules on the cell surface. Nonetheless, class 1 expression is not completely lost since the tumor cells can serve as targets for class 1-restricted CTL. It could be that reduction, but not complete loss of MHC class1 expression to a level that is sufficient for tumor survival as a metastatic lesion, is occurring in this particular tumor phenotype (Gilboa et al., 1999). By selectively downgrading only certain MHC class 1 loci, tumors may be resistant to CTL and NK killing (i.e., maintaining MHC-1 that specifically interact with killer inhibitory receptors). This change would offer a unique survival advantage to tumor cells generating tumor phenotypes that can escape these anti-tumor immune effector cells (Rees and Mian et al., 1999).

Studies on the canine dog leukocyte antigen (DLA) complex demonstrate similarity to the human HLA locus. Development of principles guiding the success in human marrow grafting in the last 30 years was initially developed and described in an outbred dog model (Storb et al., 1995). There are four DLA class 1 genes namely, DLA-12, -88, -79 and - 61. In a polymorphism analysis by Grauman and colleagues (1998) DLA-88 was found to be

significantly more polymorphic than the other three genes. Active research is ongoing to determine the tissue distribution of the class 1 genes as well as their function (Wagner et al., 1999). The constitutive expression of MHC class 1 in canine mammary tumor and melanoma cell lines are reported to range from low to high and expression was increased after canine-1FN-γ treatment (Whitley et al., 1995).

B7 molecules [B7-1 (CD80) and B7-2 (CD86)]

B7 molecules are a group of costimulatory molecules belonging to the immunoglobulin (Ig) supergene family. Initially discovered on activated non-resting B-lymphocytes, they are given the name "B" (Freeman et al., 1989). There are two major family members of B7: B7-1 (CD80) and B7-2 (CD86), which contain extracellular IgV-like and IgC2-like domains. B7-1 is a 55-kDa glycoprotein made up of 288 amino acids with a transmembrane region and a short 19 amino acid cytoplasmic domain. B7-2 is a 70-kDa glycoprotein made up of 329 amino acids, a transmembrane region and a longer cytoplasmic domain than B7-1 having potential phosphorylation sites for protein kinase C (Freeman GJ, 1993). Both B7-1 and B7-2 are the natural ligands for CD28 (Linsley et al, 1990) and cytotoxic T lymphocyte antigen #4 (CTLA-4) counter-receptors present in T cells (Linsley et al, 1991a). Conserved residues found in their V- and C- like domains are critical for their binding activity to CD28 and CTLA-4 (Peach et al., 1995). Together with an antigen receptor signal (signal 1) interaction of B7 with CD28 (signal 2) results in T cell activation, clonal expansion and development of effector T cell function. In the absence of this second costimulatory signal, T cell stimulation will lead to anergy or apoptosis (Linsley et al., 1990; Greenfield et al., 1998; Slavik et al., 1999; Sansom, 2000). Numerous studies point to inhibition of activation signaling when B7 binds to CTLA-4 (Walunas et al., 1994; Krummel

and Allison, 1996). Although both B7-1 and B7-2 can provide costimulatory functions and bind similarly to CD28 (with low affinity) and CTLA-4 (high affinity), they have several distinct differences. They are only approximately 25% identical in their amino acid sequence. Another marked difference is that B7-1 has a short cytoplasmic tail while the cytoplasmic tail of B7-2 is much longer and contains three potential sites for phosphorylation by protein kinase C (PKC) (Freeman et al., 1993). Their manner of expression and distribution also differs. B7-1 is not detected on resting antigen-presenting cells (APC) such as monocytes and B cells and has only minimal expression on dendritic cells (Fleischer et al., 1996). B7-1 is induced only after activation of the cells. In contrast, B7-2 is constitutively expressed at low levels in resting APC and expressed highly in dendritic cells both *in situ* and during maturation *in vitro* (Caux et al., 1994). B7-2 appears on the surface of the cell more rapidly (i.e. within 24 hours of B cell activation) and abundantly after activation of resting APCs whereas B7-1 appears more slowly (24-48 hours later) and at lower levels (Hathcock et al., 1994). Although still controversial (Zheng et al., 1998), many expression studies, together with findings in B7-1 and B7-2 knockout (KO) mice, indicate that B7-2 is probably the major initial ligand for CD28 during T-cell activation, based mainly on its constitutive expression, and more rapid and abundant expression on APCs. Thereby, B7-2 may play a pivotal role in the decision between T cell activation and anergy. Whereas B7-1, expressed later and more slowly, may serve to amplify or regulate an immune response (Lenschow et al., 1996; Schweitzer et al., 1997; Manickasingham et al., 1998).

It still needs to be defined if both B7-1 and B7-2 have redundant, overlapping, or distinct functions. Moreover, it is somewhat confounding why two ligands share receptors that have opposing functions (Martin-Fontecha et al., 1996; Sansom, 2000). Despite these

differences in ligand binding and expression kinetics, these molecules appear to support T-cell activation equally (McAdam, et al., 1998).

The role of B7-1 and B7-2 has been studied in tumors. Unlike in APCs where B7 molecules are abundantly found, studies show that most tumor cells, particularly those of nonhematopoeitic origin (Chen et al., 1994) do not naturally express these molecules. Attempts were made to remedy the absence or marginal expression of B7 in tumors. The idea is, if tumor cells could be made to express costimulatory molecules, they would become better antigen-presenting cells and generate a T lymphocyte response capable of destroying the tumor. Expression of B7-1 and B7-2 was increased in human melanoma cells upon culturing the tumor cells with individual cytokines such as IFN-γ, GM-CSF, and IL-2, (Hersey et al., 1994). Gene transfer was another way to induce their expression in tumor cells. Studies of a number of *in vitro* tumor models such as melanoma, lymphoma, leukemia, renal carcinoma, and colon carcinoma, report that tumor cells transfected with B7-1 or B7-2 used as a vaccine are rapidly rejected and the immune response to them is able to prevent challenge with parental cells (Chen et al., 1992; Matulonis et al., 1995; Martin-Fontecha, 1996; Jung et al., 1999; Takahashi et al., 2000). This did not generalize to all cell types, as many sarcoma cell lines tested fail to elicit systemic immunity by B7 transfection (Chen et al., 1994). By introducing mouse B7-1 cDNA into a rat osteosarcoma cell line then inoculated orthotopically into the tibia of immunocompetent rats (performed to simulate the origination of osteosarcoma in the bones). Hayakawa and his group (1997) reported B7-1 transfectants generated protective as well as curative immunity against B7-1 negative parental osteosarcoma. The soluble form of murine B7-1 was first tested to bind and provide costimulatory signals for proliferation of rat T cells before transfection. The differential

function of B7-1 and B7-2 in tumors is a subject of continuing study. Interestingly, some tumors are found to express B7 molecules although the level of expression decreases as tumors progress and metastasize (Koyama et al., 1998).

Costimulation by B7-1 and B7-2 has also been shown to be necessary for natural killer cell activity against tumor cells. Human and murine tumors when transfected with B7-1 and B7-2 trigger NK-cell mediated cytoxicity *in vitro*, although the degree of triggering effect differs between individual NK cells clones (Martin-Fontecha et al., 1999; Wilson et al., 1999; Luque et al., 2000). B7-1 and B7-2 are suggested to be candidates for NK cell-activating ligands on target cells. Controversy still exists, as far as their counter-receptor in NK cells. CD28 is expressed on human fetal NK cells but is lost after NK maturation and absent on peripheral blood NK cells in adults (Nagler et al., 1989; Sanchez et al., 1993). Nevertheless, recent reports that claim detection of CD28 on NK cells could actually depend on the monoclonal antibodies used, and indicate the possibility of a variant isotype of CD28 (Lang SN 1998, Galea-Lauri 1999). These studies show another face of B7 molecules, wherein NK cells, the major nonspecific immune cells involved in eliminating tumor cells, appear to have evolved so that they also exploit costimulatory pathways.

Characterization of B7 molecules in dogs has so far been limited. Yang and colleagues (1999), in the process of characterizing dog B7 genes have discovered new, naturally occurring forms of CD80 and CD86 mRNAs that encode soluble forms of B7-1 and B7-2 molecules from a dog PBMC cDNA library. To the author's knowledge B7 expression on canine tumors particularly in canine osteosarcoma has not been reported.

Summary

In summary, this review presents a description of the clinico-pathological characteristics of canine osteosarcoma and previous preventive and multi-modal therapies undertaken for this tumor. Its significant similarity with human osteosarcoma and characteristic malignancy and rapid metastasis underlies the necessity for continued study of this tumor. The critical roles of ICAM-1, MHC-1 and B7 surface molecules in eliciting optimum antitumor response are indispensable. Tumors manipulate the expression of these molecules to evade detection and destruction by the immune system.

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CHAPTER 2. PHENOTYPIC ANALYSIS OF CANINE APPENDICULAR OSTEOSARCOMA: EXPRESSION OF ICAM-1, MHC-1 AND B7 SURFACE MOLECULES

Introduction

Osteosarcoma is the most prevalent primary malignant bone tumor in dogs (Withrow et al., 1991). It accounts for 3% to 6 % of all canine cancers and affects 8,000 to 10,000 dogs per year in North America (Brodey 1979). Decreases are not expected due to the steady popularity of large-to-giant breed dogs as pets. These breeds of dogs are the most afflicted by this tumor. Seventy five percent of the lesions are found in the appendicular skeleton (Brodey and Riser, 1969; Misdorp and Haart, 1979). Canine osteosarcoma has a highly metastatic behavior to the lungs (Brodey 1979). Micrometastasis is present in approximately 90% of dogs at presentation (Brodey and Riser, 1969). Only 10-15% survive longer than 9 months following diagnosis and amputation (Ling et al., 1974; Straw et al., 1990; Vail and MacEwen, 2000).

Canine osteosarcoma has developed mechanisms to elude the immune surveillance system with its characteristic aggressive malignant behavior and high percentage of lung metastasis in the early stages of this neoplasm. To understand why canine osteosarcoma continues to escape immune effector mechanisms, it will be necessary to analyze the expression of surface molecules such as ICAM-1, MHC-1 and B7 that are critical to eliciting optimum immune response against these tumor cells.

Several reasons may account for escape of tumor cells from immune recognition. One mechanism to avoid an immune response is that tumors may alter MHC-1 expression. Antigens must be presented in the context of MHC-1 molecules to be recognized by T lymphocytes. Abnormal expression of MHC-1 makes them susceptible to natural killer cell

killing. Some tumors can selectively downgrade certain MHC loci (as much as 70% reduction for example in human cervical carcinoma with a loss of HLA-B44) and thereby develop resistance to cytotoxic T cells (via loss of certain MHC-1 molecules) and NK killing by maintaining MHC that specifically interact with killer-cell inhibitory receptors (Garrido et al., 1997).

A second mechanism could be the absence of costimulation, which renders T cells unresponsive, or anergic. B7 molecules present on antigen-presenting cells interact with CD28 receptor on T cells to provide a costimulatory signal for optimum T cell activation. Even though some tumor cells display MHC class 1 tumor-associated antigen on their surface, they usually do not an elicit immune response and this unresponsiveness is perhaps due to the lack of B7. Unlike antigen-presenting cells on which B7 molecules are abundantly found, studies show that most tumor cells, particularly those of nonhematopoeitic origin (Chen et al., 1994) do not naturally express these molecules and this may prevent the induction of efficient antitumor immune responses.

A third potential mechanism for tumors to avoid immune surveillance involves expression of ICAM-1. The interactions between ICAM-1 and its ligand, leukocyte functional antigen-1 (LFA-1) plays a fundamental role in the course of inflammatory and immune responses. Primarily an adhesion molecule, ICAM-1 provides stable conjugates between cells through its ligand, LFA-1 (Springer, 1990). By expressing ICAM-1, tumor cells are capable of binding to CTL and NK cells with the possibility of killing the tumor or cell. ICAM-1 is not expressed by a majority of malignant tumors; however its presence has been correlated with tumor progression and metastasis (Johnson, 1991). Several studies show that in some tumors, ICAM-1 expression is not detrimental to their growth but instead is used

for metastasis (see review by Johnson, 1991; Simmons, 1995; Jiang et al., 1998; Sun et al., 1999).

In this study, we have used primary canine appendicular osteosarcoma cell lines and tumor tissues from dogs with histologically confirmed primary appendicular osteosarcoma to determine the expression of ICAM-1, MHC-1 and B7 molecules. Additionally, in conjunction with an ongoing related clinical study using canine osteosarcoma cells transduced with an adenoviral vector containing a gene for human IL-2 to direct secretion of IL-2 by the tumor cells and stimulate antitumor immune response in dogs with primary appendicular osteosarcoma, we examined if IL-2 alters the expression of ICAM-1, MHC-1 and B7 molecules on these tumor cells *in vitro*.

Materials and Methods

Preparation of Samples

Tumor specimens were aseptically obtained from surgical biopsy of dogs clinically diagnosed with primary appendicular osteosarcoma at the Iowa State University Teaching Hospital and processed for cell culture and immunohistochemistry. For immunohistochemistry, a section of the tumor tissue was formalin-fixed and paraffin-embedded, and another section was embedded in optimum cutting temperature (O.C.T.) compound (LAB-TEK products, Division Miles Lab, Inc. Naperville, IL), snap frozen in liquid nitrogen, and stored at -70 °C. All cases were immunohistopathologically diagnosed as osteosarcoma. Testing for serum alkaline phosphatase was performed at the time of surgery by the Iowa State University Clinical laboratory.

Generation of primary cell lines

The generation of primary tumor cell lines was performed as previously described (Nieves, et al., 1998). Briefly, tumor samples were aseptically obtained from surgical biopsy of dogs clinically diagnosed with primary appendicular osteosarcoma at the Iowa State University Teaching Hospital, College of Veterinary Medicine, Iowa State University. The tissues were placed in a sterile 50 ml centrifuge tube with 30 ml of digestion media containing Hank's balanced salt solution (Gibco BRL Grand Island, NY), 5μg/ml of Hyaluronidase (3,600 U/mg, Sigma Inc. St. Louis, MO), 30mg/ml of Collagenase type IV (Sigma) and 1 mg/ml of Deoxyribonuclease (Sigma) and incubated in a 37°C water bath and gently agitated every 5 minutes. After 30 minutes, the supernatant together with the cells sieved through a sterile wire mesh (size 50). The disaggregated tumor tissues were collected and centrifuged at 1,500 rpm for 10 minutes. The pellet was washed and resuspended in Dulbecco's modified essential medium, 10% fetal bovine serum and antibiotics (100 U/ml Penicillin and 100 g/ml Streptomycin). Cells were seeded in a 25 cm^2 tissue culture flask and incubated at 37°C in a humidified 5% CO_2, 95% air incubator. The following day, the culture medium was replaced to remove non-adherent cells and thereafter media was changed every other day. After reaching 80-90 % confluency, cells were detached by using trypsin/EDTA and subcultured into fresh culture media. Cells were preserved at 10^7 cells/ml cryotubes with DMEM medium containing 30% fetal bovine serum and 10% dimethylsulfoxide. After 48 hours at -70°C, frozen cultures were transferred to a liquid nitrogen freezer. Not more than 10 culture passage levels were used to minimize occurrences of intra-tumor variability.

Monoclonal antibodies and recombinant protein antibodies

The group of Smith (1991) developed two monoclonal antibodies against canine ICAM-1, CL18/D8 and CL18/6. CL18/1D8 was used for most of the studies because it demonstrated less background staining than CL18/6, although similar patterns of staining were seen in both monoclonal antibodies (Grigg et al., 1994; Olivry et al., 1995; Moore et al., 1996). The mAb H58A, which specifically binds to H-$2k^k$ equivalent MHC class 1 molecules on cells from several animals including dog was used to evaluate the expression of MHC class 1, (Whitley et al., 1995). Since monoclonal antibodies against canine B7-1 and B7-2 are not yet available at the time of this study, we used hCTLA-4Ig/Fc chimera (R & D Systems, Inc. Minneapolis, MN) to detect the expression of B7 on canine osteosarcoma cells (Pinelli et al., 1999). CTLA4-1g is a fusion protein generated by a construct in which the external domains of CTLA-4 have been fused to the Ig-C domains of human Igγ (Linsley, 1991).

Immunofluorescence and fluorescence microscopic analysis of ICAM-1 and MHC-1

Cells were grown on sterile coverslips and incubated overnight at 37°C in a humidified 5% CO_2, 95% air incubator. To detect ICAM-1 expression, cells were fixed with 4% paraformaldehyde (Polysciences, Inc., Warrrington, PA) in phosphate buffered solution for 10 minutes. After 10 minutes, the fixative was replaced with fresh fixative and incubated for another 30 minutes. To detect MHC-1, cells were fixed with ice-cold ethanol. After fixation, cells were washed with PBS three times and incubated with blocking buffer (5% normal goat serum, 4.5% bovine serum albumin (BSA) and 0.1% Triton X-100 in PBS). After the blocking solution was removed, cells were incubated with 1:100 dilution of primary specific monoclonal antibodies against ICAM-1 (CL18/ID8) and MHC-1 (H58A) overnight

in a humidified chamber at 4°C. Cells were washed three times with wash buffer (5% normal goat serum, 0.5% BSA and 0.1% Triton X-100 in PBS). For immunofluorescence detection, the cells were incubated with goat anti-mouse IgG (H+L) TRITC (Sigma Chemicals, St. Louis, MO) that was diluted 1:200 with wash buffer for 3-4 hours in the dark at room temperature. After two additional washes in PBS and three washes in distilled water, coverslips were mounted on glass slides with n-propyl gallate glycerol. Fluorescence was visualized on epifluorescence microscope. Controls included samples run in parallel that were incubated without primary antibodies, samples incubated with only primary antibodies and samples incubated with an immunoglobulin isotype control. Due to homogenous nature of the population of tumor cells, results were interpreted according to staining intensity of the cells (Table 1a).

Immunohistochemical analysis of ICAM-1 and MHC-1

Frozen Tissue Sections

Frozen tissue sections were immunostained according to standard avidin-biotin-peroxidase complex (ABC) procedure (Hsu et al., 1981). Briefly, frozen tissue sections were cut at 4μ, air-dried and fixed in acetone at 4°C, and washed for 5 minutes with PBS. Tissue sections were encircled with a water-repellent barrier PAP pen (BioGenex, San Ramon, CA) before proceeding to the rest of the immunostaining procedure. Endogenous peroxidase activity was quenched with 0.3% hydrogen peroxide, and washed in PBS. Tissues were overlaid with a protein-blocking solution containing 2% BSA and 10% normal horse serum (Vector Laboratories, Burlingame,CA) at room temperature to block non-specific staining (nonimmune binding of immunoglobulins to the tissue). After incubation, the solution was tapped off without rinsing. Tissue sections were incubated with primary mouse monoclonal

Table 1a. Scoring of immunostained cell lines.

Staining Intensity	Score
Negative	(-)
Low	(+)
Moderate	(++)
High	(+++)

antibodies against ICAM-1 and MHC-1 overnight in a humidified chamber at 4°C. The working dilution for both monoclonal antibodies was 1:100. All subsequent procedures were carried out at room temperature. After washing with PBS, sections were incubated with biotinylated horse antimouse secondary antibody (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA) diluted 1:200. Following three washes with PBS, avidin-biotin peroxidase complex, (ABC) solution (Vectastain ABC kit, Vector Laboratories, Burlingame, CA) prepared according to the manufacturer's direction was applied to the tissues. Slides were then washed with PBS and incubated with NOVAred (Vector Laboratories). Following a rinse in ultra pure water, tissues were counterstained with haematoxylin (Vector Laboratories), rinsed with water, dehydrated through increasing concentrations of ethanol, cleared in xylene and mounted with Acrytol (Surgipath Lab, Richmond, IL).

Paraffin-embedded Tissue Sections

Formalin-fixed, paraffin-embedded, tissues were sectioned at five-micron and mounted on poly L-lysine coated microscope slides. Before immunostaining, slides were heated for 30 minutes at 60 °C. Sections were deparaffinized in 2 changes of xylene and rehydrated by passage through 100%, 95% and 70% ethanol and rinsed with ultrapure water. For antigen retrieval, the slides for staining ICAM-1 were placed in a plastic staining dish with 250 ml of sodium citrate buffer (10mM,pH 6.0) while on slides for staining MHC-1, the antigen retrieval Glyca, (pH 4.0, BioGenex, San Ramon, CA) was used. The slides were then heated in a microwave at high power until the solution was boiling (2.5 minutes). Slides were heated another 10 minutes on low power. The slides were allowed to cool in the freezer and rinsed once with PBS. Tissue sections were encircled with a water-repellent barrier PAP

pen (BioGenex, San Ramon, CA) before proceeding to staining steps. Endogenous peroxide activity was quenched by incubating the tissues with 3% hydrogen peroxide at room temperature, followed by washing in PBS. The rest of the immunostaining procedure was performed as described above in frozen tissue sections using antibodies to ICAM-1 and MHC-1.

To expand the number of formalin-fixed, paraffin-embedded tissue sections examined, an additional 4 histopathological cases of dogs with osteosarcoma were included for the investigation of ICAM-1 and MHC-1 expressions. Controls included serial sections run in parallel that were incubated in the absence of the primary antibodies and sections incubated with an isotypic IgG control antibody at the same concentration as the primary mAb used in the analysis. Results were classified according to percentage of tumor cells that were stained positive (i.e., extent of staining) (Table 1b).

Immunofluorescence and flow cytometry analysis of B7

Osteosarcoma cells were detached from tissue culture flasks by incubation with 5 ml 1% Na_2 ethylenediamine tetraacetic acid (EDTA) in PBS for 10 minutes at room temperature and washed twice in ice-cold wash buffer solution (0.5% BSA, 0.025% Na azide and 25mM HEPES in PBS) at 4° C. Aliquots of 10^5 cells were stained with 6 μg of CTLA-4 Ig/Fc chimera (R & D Systems, Inc. Minneapolis, MN) diluted in wash buffer in an ice water bath. After incubation, cells were washed with ice-cold wash buffer. Biotin conjugated mouse anti-human IgG secondary antibodies, 6 μg (Sigma) were added to the cells. Cells were washed then incubated a third time with 50 ng of Strepavidin Cy-Chrome (PharMingen, San Diego, CA). After one washing, cells were fixed with 1% paraformaldehyde in PBS for flow cytometric analysis. Analysis was carried out with EPICS XL-MCL flow cytometer

Table 1b. Scoring of immunostained tissue sections.

Percent of Tumor Cells Stained	Score
< 10	(-) Negative
10 to 25	(+) Low
25 to 50	(++) Moderate
> 50	(+++) High

(Beckman-Coulter). Control staining was performed with isotype-matched human IgG (Sigma) and with the biotin-conjugated reagent only. All incubations were performed at 4°C in an ice water bath.

IL-2 stimulation of canine osteosarcoma cells

Two cell lines were grown on coverslips and were stimulated with or without rIL-2 (Sigma) at 0.5ng/ml for 24 hour at 37°C in a humidified 5% CO_2, 95% air incubator. Following staining against ICAM-1 and MHC-1, cells were examined and images stored by using X63 oil immersion objective on a Leica TCS-NT confocal laser microscope. To examine the effect of IL-2 on B7 expression, one cell line was first incubated with different concentrations of rIL-2 (0.50ng/ml, 1.25 ng/ml and 2.50 ng/ml) for 24 hours at 37°C in a humidified 5% CO_2, 95% air incubator, stained with hCTLA4-1g and analyzed by flow cytometry as described above. After staining, 0.50 ng/ml of rIL-2 was chosen as the optimum dose that affected B7 expression on canine osteosarcoma cell lines, four cell lines were additionally stimulated with or without rIL-2 at 0.5ng/ml for 24 hour at 37°C in a humidified 5% CO_2, 95% air incubator. Cells were stained with hCTLA-4Ig/Fc and analyzed with flow cytometry as described earlier.

Statistical analysis

A Student's *t*-test was used to evaluate the statistical difference ($P<0.01$) of the mean fluorescence intensity of paired samples.

Results

Patient characteristics

The dogs were diagnosed with osteosarcoma based on standard radiographs and histologic evaluation of biopsy samples following tumor excision. Table 2 summarizes the

Table 2. Summary of clinicopathological data of canine primary appendicular osteosarcoma cases.

Dog	Breed	Sex	Age Years	Location	Histological Classification	sAP IU/L[a]	DFI[b] mos	ST[b] mos	Mets[b]	Comments
1	Rottweiler	M	7.0	Humerus	osteosarcoma	217	2	8	+	w/ lung mets after 2 mos
2	Irish wolfhound	F	2.6	Distal Humerus	osteosarcoma	51	2.5	1.3	+	w/ lung/heart mets after 2.5 mos
3	Doberman Pinscher	F	6.8	Femur	osteosarcoma	336			NR[b]	leg amputation, ok as of last entry
4	Labrador Retriever	M	2.0	Proximal Humerus	chondroblastic osteosarcoma	42	1		+	w/ lung mets after 1 mo
5	Rottweiler	F	8.6	Midshaft Femur	osteoblastic osteosarcoma	101		1	+	w/ mets after 1 mo, local invasion
6	Greyhound	F	7.8	Distal Radius	osteosarcoma	26		0.1	NR	bone biopsy only
7	Mixed	F	4.5	Tibia Fibula	fibroblastic osteosarcoma	76			-	no mets, ok as of last entry
8	Labrador Retriever	F	6.0	Distal Radius	osteosarcoma	97		11	-	no mets, tumor unrelated death
9	Dalmatian	F	5.4	Proximal Humerus	osteosarcoma	53			NR	bone biopsy only
10	Mixed	F	8.0	Tibia	osteosarcoma	0			NR	bone biopsy only
11	Rottweiler	M	5.3	Distal Radius		118			NR	bone biopsy only
12	German Wirehair Pointer	F	3.6	Femur	osteosarcoma	0			NR	bone biopsy only
13	Great Pyrenes	F	7.0	Distal Tibia	pareostal osteosarcoma	99			+	w/ lymph node mets
14	Boxer	F	2.0	Proximal Humerus	osteoblastic osteosarcoma	0	1		+	w/ lung mets after 1 mo
15	Rottweiler	M	4.3	Distal Tibia	osteosarcoma	90			NR	
16	Rottweiler	F	1.0	Distal Ulna	osteosarcoma	8			NR	
17	Rottweiler	F	9.0	Distal Femur	osteosarcoma	245			NR	

[a] serum Alkaline phosphatase normal range is 0 - 100 IU/L

[b] DFI – disease-free interval time, ST – survival time, mets – metastasis, NR- not recorded

clinical features of dogs entered for the study. The dogs ranged in age from 1 year to 9 years with an average age of 5.4 years. There were 13 females and 4 males. Fifteen of 17 dogs were from purebred large to giant breeds, while 2 of 17 dogs were mixed breed. The Rottweiler was the predominant breed (6 out of 17 dogs) in this study. Tumor was found in one of four major weight-bearing bones (humerus, radius, femur and tibia) in all the cases. Foreleg lesions were found in 9 cases and hindleg lesions in 8 cases.

Table 2 also shows that 12 of 17dogs (71%) had serum alkaline phosphatase (sAP) levels within the normal range of 0 to 100 IU/L, while only 5 dogs (29%) had sAP levels exceeding the normal range. Of the Rotweiller dogs, 4 of 6 (67%) showed a sAP level exceeding the normal range.

Where data was available, 6 dogs showed metastasis and 2 dogs had no metastasis. Among the 6 dogs with metastasis, two had sAP levels above the normal range, and the other 4 dogs had sAP levels within the normal range. The 2 dogs with no metastasis had sAP levels within the normal range.

Dog 1 with lung metastasis after 2.5 months from initial diagnosis had a survival time (ST) of 8 months. While Dog 2 with lung and heart metastasis after 2.5 months from initial diagnosis had a survival time of 1.3 months. Dogs 4 and 14 after initial diagnosis showed lung and lymph nodes metastasis, respectively, and had a disease-free interval time (DFI) of one month. One dog (8) had a survival time of 11 months but died of tumor unrelated death. Data are not complete on DFI and ST because some dogs were lost during the follow up.

Detection of ICAM-1 and MHC-1 expression in canine primary appendicular osteosarcoma cell lines

All five cell lines examined expressed high level of ICAM-1. Diffuse membrane and cytoplasmic staining distribution of ICAM-1 was observed in the cells (see Figure 1a). Three of the cell lines showed moderate expression of MHC-1 while weak expression (see Figure 1b) was observed in two cell lines (Table 3). Punctate to speckled MHC-1 staining on the membrane surface of the cells is shown in Figure 1b.

Detection of ICAM-1 and MHC-1 expression in tissue sections of canine osteosarcoma

Frozen tissue sections

For frozen tumor sections (Table 4 and Figure 2) a high percentage of staining was observed for ICAM-1 in tumors from 4 of 7 (57.1%) dogs and 3 of 7 (43%) tumors expressed moderate staining. MHC-1 expression was observed in 4 of 7 (57%) tumors with moderate staining while 3 of 7 (43%) tumors expressed staining (Figure 3). Almost the same pattern of staining was seen in ICAM-1 for formalin-fixed, paraffin-embedded tumor sections (Table 5 and Figure 4). ICAM-1 showed high staining expression in tumors from 10 of 17 dogs (59%) and moderate staining expression in tumors from 7 of 17 (41.2%) dogs. MHC-1 showed low expression in tumors from 7 of 17 (29 %) dogs and negative staining in tumors from 10 of 17 (59 %) dogs (Figure 5). There were more cells positive for MHC-1 staining observed in frozen tissue sections than in paraffin tissues.

Detection of B7 expression in canine primary appendicular osteosarcoma cell lines

Flow cytometric analysis of the expression of B7 in canine osteosarcoma cell lines shows that 4 of 5 cell lines demonstrated a high proportion of positive staining ranging from 73.67 to 85.13% (Table 6 and Figure 6).

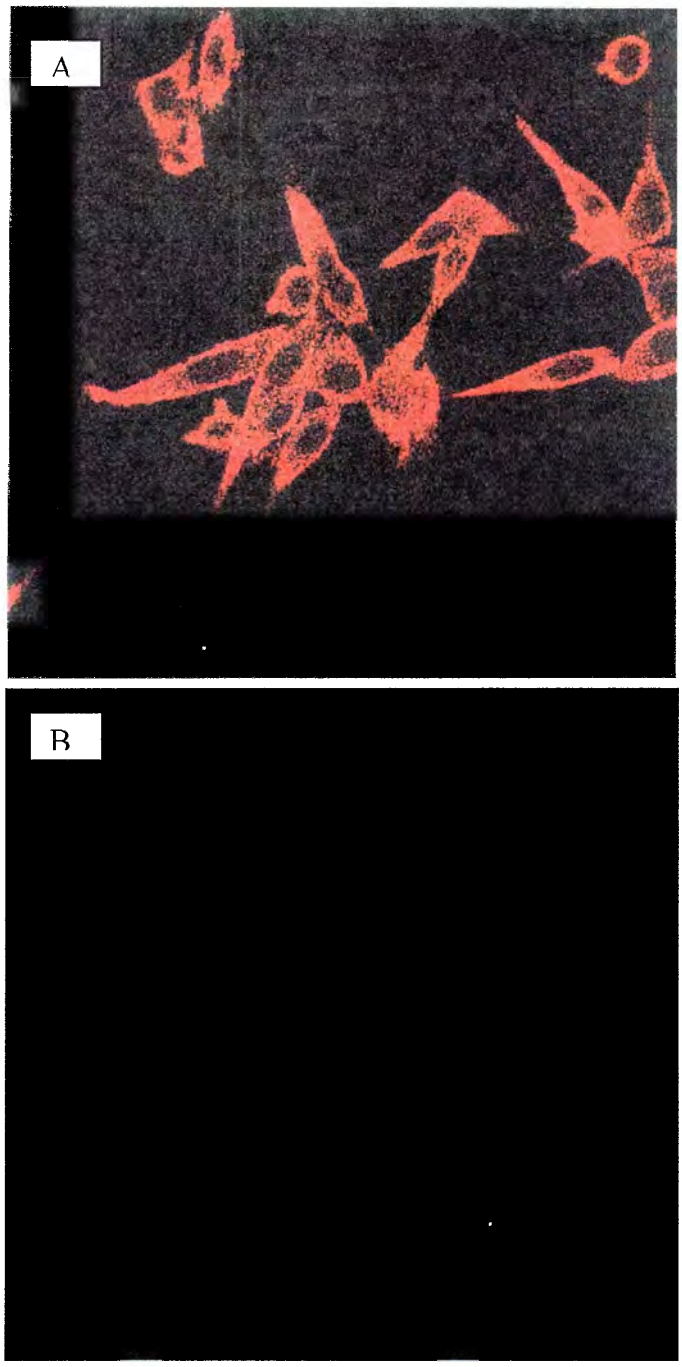


Figure 1. Indirect immunofluorescence of canine primary osteosarcoma cells. Cells from dog # 8 were fixed with paraformaldehyde were labeled with anti-ICAM-1 Mab (A) and anti-MHC-1 Mab (B) and TRITC-conjugated goat antimouse secondary Ab. Slide A demonstrates high intensity staining for ICAM-1 and slide B demonstrates low intensity staining for MHC-1. Cells were examined with X63 oil immersion objective on an epifluorescence microscope.

Table 3. Expression of ICAM-1 and MHC-1 in canine primary appendicular osteosarcoma cell lines.

Cell lines	ICAM-1	MHC-1
1	+++	++
2	+++	++
8	+++	+
9	+++	+
14	+++	++

Table 4. Immunohistochemical analysis of ICAM-1 and MHC class 1 expression in frozen canine primary appendicular osteosarcoma tissues.

Dog	ICAM-1	MHC-1
2	+++	++
3	++	++
4	+++	+
5	+++	+
6	++	+
7	+++	++
18	++	++

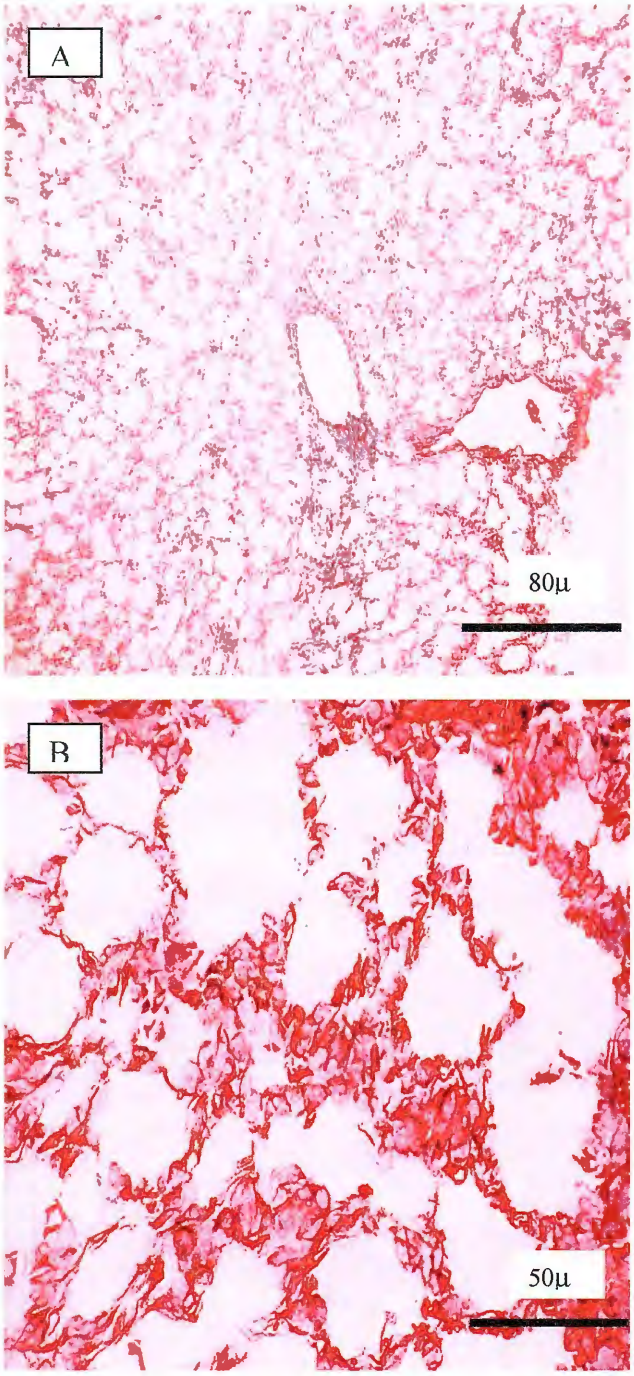


Figure 2. Immunoperoxidase staining (ABC method) for ICAM-1 in a frozen tissue section of canine primary osteosarcoma at low (A) and high (B) magnification. Slides are from dog # 2 and demonstrate high staining (+++).

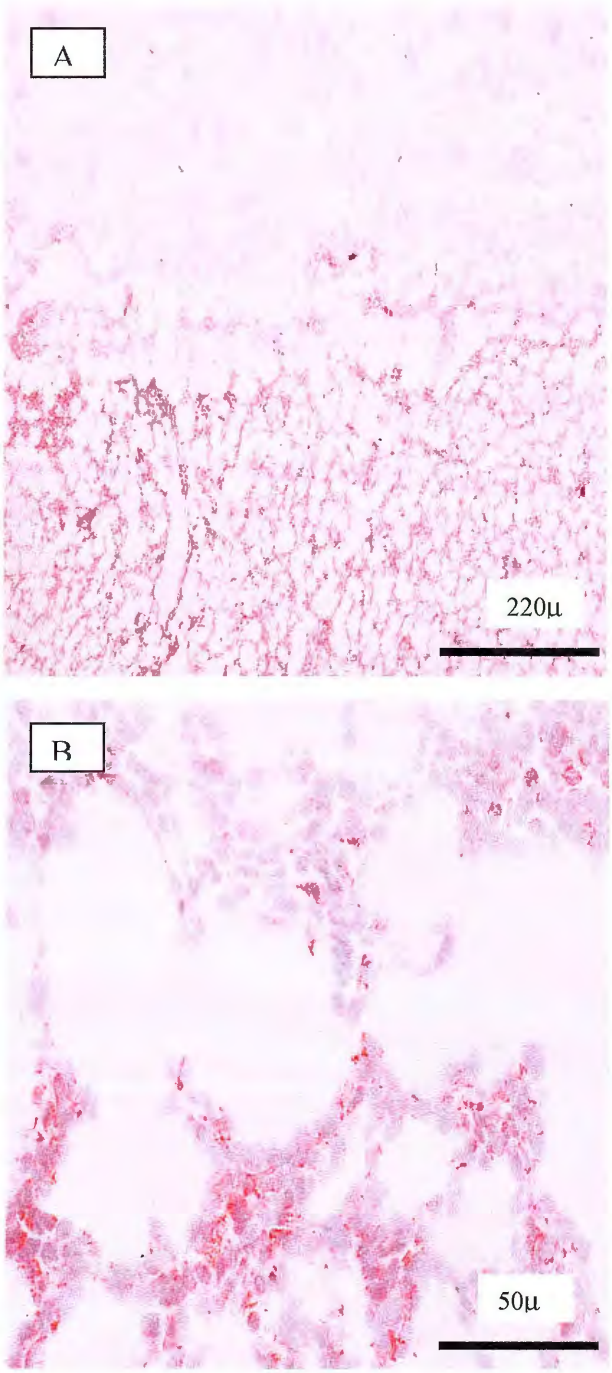


Figure 3. Immunoperoxidase staining (ABC method) for MHC-1 in a frozen tissue section of canine primary osteosarcoma at low (A) and high (B) magnification. Slides are from dog # 2 and demonstrate moderate staining (++).

Table 5. Immunohistochemical analysis of ICAM-1 and MHC class 1 expression in formalin-fixed, paraffin-embedded canine primary appendicular osteosarcoma tissues.

Dogs	ICAM-1	MHC-1
19	+++	–
2	+++	+
3	+ ++	+
5	+++	–
6	++	+
7	+++	+
9	+++	–
10	++	–
11	++	–
12	++	+
13	++	+
15	+++	–
16	+++	–
17	+++	–
20	++	–
21	+++	+
22	++	–

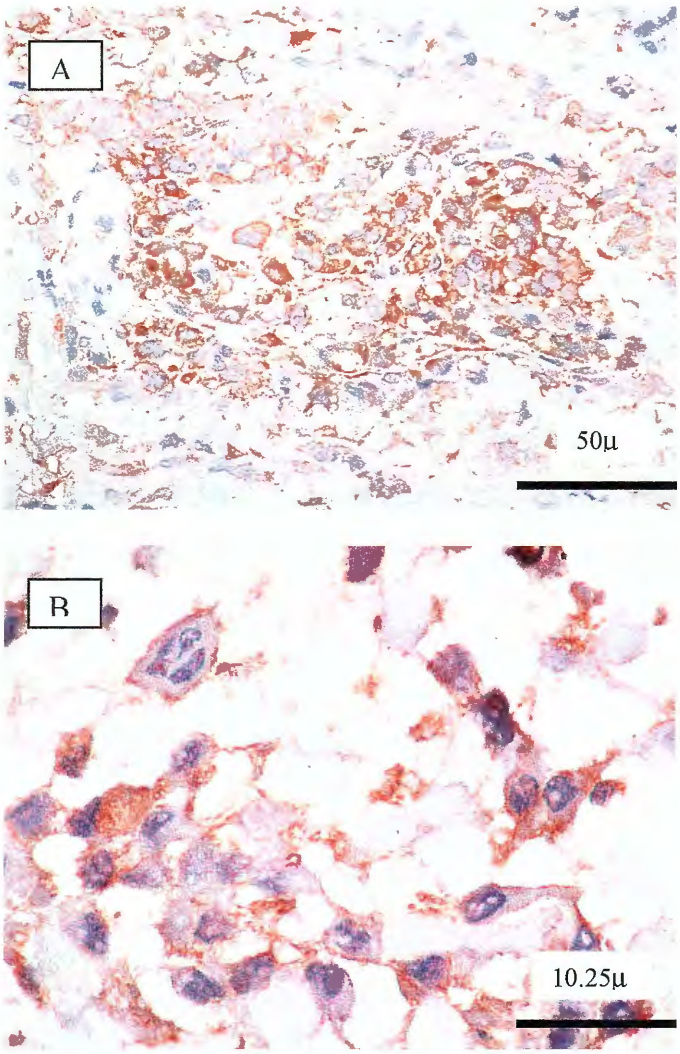


Figure 4. Immunoperoxidase staining (ABC method) for ICAM-1 in a formalin-fixed, paraffin-embedded section of canine primary osteosarcoma at low (A) and high (B) magnification showing membrane and cytoplasmic staining. Slides are from dog # 6 and demonstrate moderate staining (++).

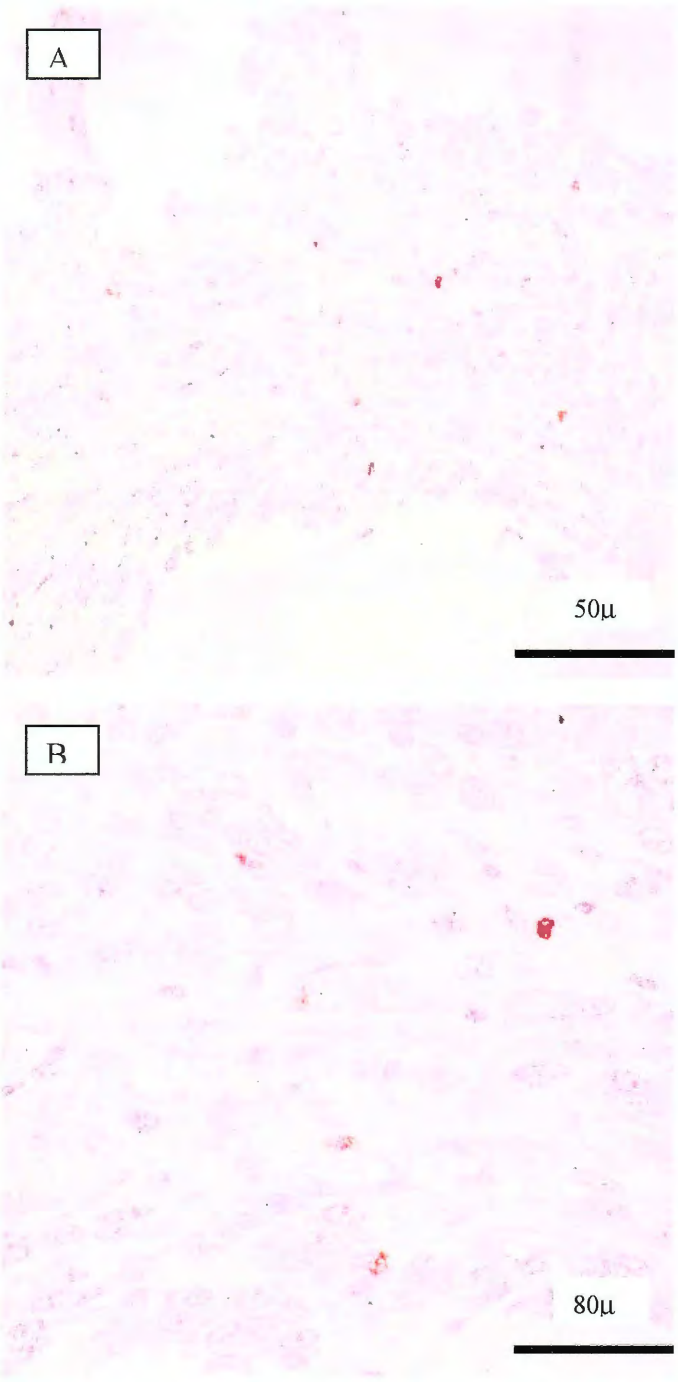


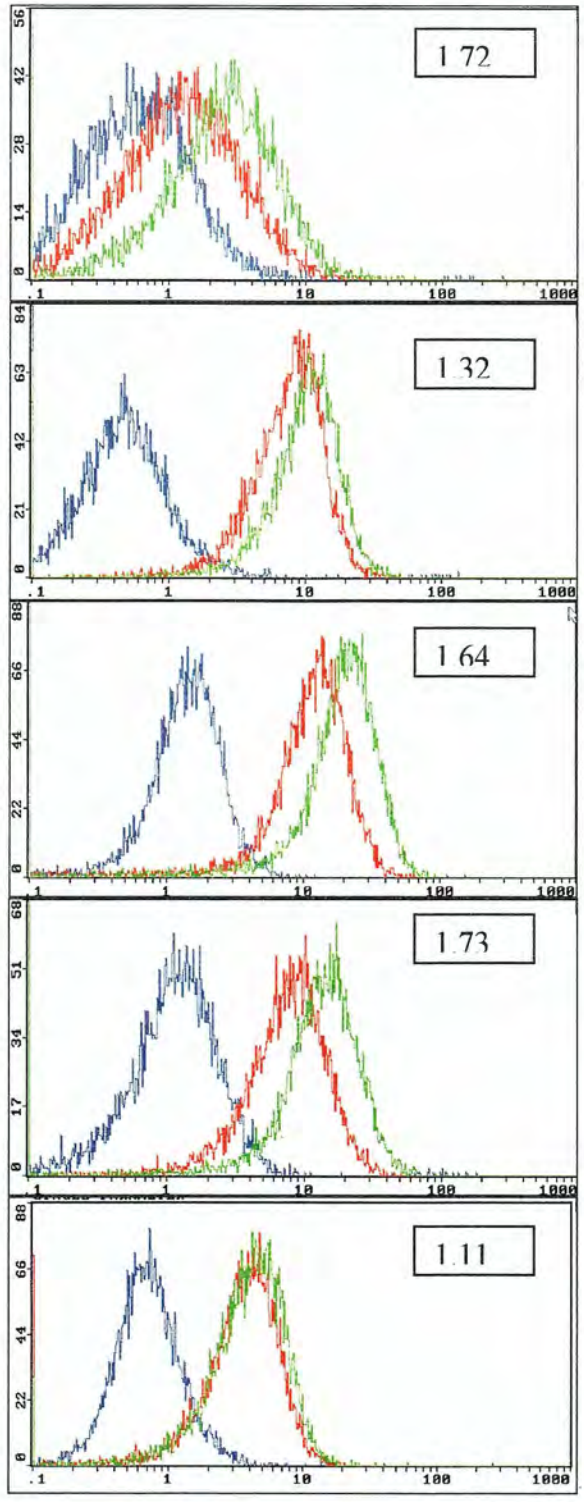
Figure 5. Immunoperoxidase staining (ABC method) for MHC-1 in a formalin-fixed, paraffin-embedded section of canine primary osteosarcoma at low (A) and high (B) magnification. Slides are from dog # 7 and demonstrate low staining (+).

Table 6. Mean fluorescent intensity (MFI) of B7 with or without IL-2 stimulation of canine primary appendicular osteosarcoma cells.

	% Positive	MFI		
Cell lines	B7[a]	Baseline	IL-2 Stimulation	Ratio Stimulation/Baseline
1	6.58	2.22	3.82	1.72
2	77.03	8.97	11.80	1.32
3	85.13	13.5	22.20	1.64
4	73.67	9.30	16.10	1.73
5	78.50	4.34	4.80	1.11

[a] Each value represents the percentage of positive hCTLA4Ig stained cells minus the percentage of cells stained by isotype control. The expression of B7 was determined by indirect immunoflourescence and FACS analysis by using hCTLA4Ig/Fc.

Figure 6. Flow cytometry analysis of B7 surface expression in five canine primary appendicular osteosarcoma cell lines. Cells were incubated with recombinant hCTLA-4Ig/Fc, biotin-conjugated mouse anti-human IgG and strepavidin cy-chrome. Human IgG was used as control antibody. All cell lines showed basal B7 expression. Cells were stimulated with (green) or without (red) 0.5 ng/ml IL-2. The blue line represents staining with control human IgG. The ratio for MFI with IL-2/MFI without IL-2 is printed in the upper right hand corner for each graph. A total number of 10,000 events were analyzed for each sample.


1 72
1 32
1 64
1 73
1 11
Fluorescence

Regulation of ICAM-1, MHC-1 and B7 expression in canine primary appendicular osteosarcoma by IL-2

IL-2 is a potent stimulator of cytotoxic T cells, NK, LAK and helper T cells. We were interested in knowing if the presence of IL-2 would alter canine osteosarcoma expression of ICAM-1, MHC-1 and B7 surface molecules. The two cell lines examined showed diffuse membrane and cytoplasmic distribution of ICAM-1 with a high level of expression. The staining intensity and distribution detected on the two cell lines after IL-2 treatment showed diffuse membrane and cytoplasmic distribution of ICAM-1 with a high level of expression. Thus, no apparent change was detected in cells that were IL-2 stimulated as compared with cells not stimulated by IL-2.

The two cell lines stained for MHC-1 showed moderate expression with punctate to speckled staining on the membrane surface of the cells. There was no change of staining intensity or staining distribution of MHC-1 detected in cells that were stimulated with IL-2 from the cells not stimulated with IL-2.

The regulatory effect of IL-2 on the surface expression of B7 was determined in five cell lines. Data is expressed as mean fluorescence intensity value (MFI), an indirect expression of antigen density. Slight shifts in the histograms were observed (Figure 5). However, with the large number of cells from each sample dog (N=10,000), it is expected that any noticeable difference in the location of the two histograms is likely to be statistically significant (Shapiro, 1988). The Student's *t*-statistics demonstrates that the paired differences in the histograms were statistically significant ($P<0.01$). However, ratios of MFI stimulated/MFI baseline less than 2 are considered small. The stimulation with IL-2 of canine osteosarcoma cell lines produced a small increase in B7 expression in 3 of the 5 cell lines (60

%) as indicated by an almost two-fold increase in the MFI of the cell lines stimulated with IL-2 compared to the without IL-2 stimulated cell lines. The remaining two cell lines (40 %) did not show any appreciable increase in B7 expression, with their MFI staying close to the baseline level, or a ratio approaching unity (Figure 5).

Relation of ICAM-1 with different clinical parameters

Where data was available, all five primary cell lines of dogs with osteosarcoma had high expression of ICAM-1 with 4 (80%) having sAP levels within normal range and one above the normal range. In frozen tissues of six dogs with osteosarcoma, 3 (50%) dogs had high expression of ICAM-1 and an sAP level within normal range, one dog had moderate ICAM-1 expression and an sAP within normal range, and two dogs with sAP level above the normal range with one moderate and one high ICAM-1 expression. In formalin-fixed, paraffin-embedded tissues of 11 dogs with osteosarcoma, 5 (45%) dogs had high expression of ICAM-1 and sAP levels within normal range, 4 (36%) dogs had moderate ICAM-1 expression and sAP levels within normal range, and 4 dogs with sAP levels above normal range with one (9%) moderate and 3 (27%) high ICAM-1 expression.

On the relation of ICAM-1 expression and metastasis, where data was present, primary cell lines of 3 of 4 (75%) dogs with metastasis had high ICAM-1 expression. On the other hand, high ICAM-1 expression was also observed in the primary cell line of one dog without metastasis. In frozen tissues of dogs with metastasis, 3 of 4 (75%) dogs exhibited high expression of ICAM-1. While one dog without metastasis showed high ICAM-1 expression. The formalin-fixed, paraffin-embedded tissues of dogs with metastasis showed moderate (1 of 4, 25%) to high (2 of 4, 50%) ICAM-1 expression. However, one dog without metastasis high expression of ICAM-1 was observed.

Discussion

Exploiting the immune system as a main modality for treatment of canine osteosarcoma is a major long-term goal of veterinary immunologists and clinicians. In the center of this approach is a need for a comprehensive understanding on how canine osteosarcoma cells relate to the major players of antitumor immunity, the CTL and NK cells, in terms of their expression of surface receptors critical to obtaining an optimum immune response.

This study provides information on the expression of ICAM-1, MHC-1 and B7 molecules in canine primary appendicular osteosarcoma cells and the possible implication of their expression in relation to tumor escape from immune effector cells. We have analyzed cell lines and tissue sections (frozen tissues and paraffin-embedded, formalin-fixed tissues) of dogs diagnosed with osteosarcoma by using fluorescence microscopy, flow cytometry and immunohistochemistry.

ICAM-1 is crucial in initiating an antitumor immune response. At the same time, tumor progression has also been linked to ICAM-1 expression. This study showed that using immunoflourescence microscopic analysis, all five canine OSA cell lines demonstrated high surface expression for ICAM-1. Immunohistochemical analysis of frozen tumor sections showed a high percentage of staining for ICAM-1 in 4 of 7 (57.1%) tumors from 7 dogs and only moderate staining in 3 of 7 (43%) tumors from 7 dogs. In addition, one frozen tumor showed a weak expression. Almost the same pattern of staining was seen in formalin-fixed, paraffin-embedded tumor sections as in frozen tissues. ICAM-1 showed high staining expression for 10 of 17 (59%) tumors and moderate staining expression for 7 of 17 (41.2%) tumors. Taken together all three techniques correlated well for high ICAM-1 receptor

expression. Some evidence is suggested by the data that there are more dogs with osteosarcoma having metastasis and high ICAM-1 expression as observed in primary cell lines, frozen tissues, and formalin-fixed, paraffin-embedded tissues. However, a few cases were observed of dogs without metastasis which had high ICAM-1 expression.

ICAM-1 present on these tumor cells should enable the adhesion and costimulation of immune effector cells optimizing their response to eliminate them. We sought to understand why canine OSA cells had escaped immune surveillance and what accounts for their rapid metastases/micrometastasis especially to the lungs and other sites. One hypothesis we tested was that they lacked adequate cell surface-adhesion molecules, rendering them unable to form stable conjugates with CTL or NK cells. Data from cell lines, frozen and paraffin-embedded tissues show that canine osteosarcoma almost uniformly express constitutively high levels of ICAM-1. For canine osteosarcoma cells to survive, expressing ICAM-1 seems to be counterproductive as increased ICAM-1 expression should lead to increased attack by cytotoxic T cells and natural killer cells. On the other hand, ICAM-1 expression on tumor cells has been shown to correlate with a higher risk of the tumor cells to metastasize (Johnson, 1989; Simmons et al., 1995; Jiang et al., 1998; Sun et al., 1999).

To metastasize, tumor cells must shed into the blood stream (intravasation) directly by invasion into the tumor-derived vasculature or indirectly by lymphatic drainage, survive in the circulation, and finally migrate through normal vascular endothelium and proliferate in the target organs (extravasation). Tumor cell extravasation plays a key role in tumor metastasis. Several hypotheses have been proposed to explain tumor cell extravasation (El Sabban and Pauli, 1994; Kebers, 1998). However the precise mechanism by which tumor cells migrate through normal vascular endothelium remains controversial. It has been

proposed that increased ICAM-1 expression by tumor cells allows leukocytes to attach to them, and thus the tumor cells can "hitch a ride" on the leukocytes as they adhere to vascular endothelium and diapedese, enhancing the potential for hematogenous metastasis (Johnson, 1991).

Although, human polymorphonuclear neutrophils (PMNs), which comprise 50-70% of circulating leukocytes, can be spontaneously cytotoxic to tumor cells, they may function to promote tumor growth and metastasis as they might assist tumor cell extravasation during metastasis by transendothelial migration. A mechanism for this process was tested by Wu et al (2001). By using an *in vitro* transendothelial migration model, they have demonstrated that tumor conditioned media-treated PMN assisted the migration of MDA-MB-231, a human breast tumor cell line, across an endothelial barrier. The transendothelial migration is due to the high levels of ICAM-1 expressed by this tumor and the upregulation of PMN CD11b and CD18 by the tumor culture media. It will be interesting to test if the canine osteosarcoma cells use a similar mechanism. This would have important clinical implications, particularly in the perioperative period when tumor cells are known to be present in the circulation and a transient leukocytosis occurs (Wu, 2001). It is also important then to consider which immunotherapy protocol (i.e., cytokine treatment) to use in the treatment of this tumor. For example, granulocyte-macrophage colony-stimulating factor (GM-CSF) which stimulates production and recruits macrophages and granulocytes to the tumor area can in fact be used by the tumor cells for a "free ride to a greener pasture".

It has been known for some time that malignant transformation of cells is frequently associated with altered MHC class 1 expression and/or function (Tanaka et al., 1988; Algarra et al., 1997; Hicklin et al., 1999). Our analysis demonstrates that three of the five canine

osteosarcoma cell lines showed moderate expression of MHC-1 expression while low expression was observed in two cell lines. In frozen tumor sections, MHC-1 staining demonstrated moderate expression in 4 of 7 (57%) dogs while low staining occurred in tumors from 3 of 7 (43%) dogs. In formalin-fixed, paraffin-embedded tumor sections, MHC-1 showed moderate expression in tumors from 2 of 17 (8.5%) dogs, low expression for tumors from 5 of 17 (29 %) dogs and negative staining in tumors from 10 of 17 (59 %) dogs. We attributed the difference in results between frozen and formalin-fixed tissues to inefficient antigen exposure. However, we could not directly test this hypothesis by comparing results from cell lines, frozen and formalin-fixed, paraffin-embedded tissues of the same animal due to the lack of clinical materials.

The expression of negative to only moderate expression for MHC-1 can provide tumor cells with avenues for escape from immune recognition due to their role in presenting immunogenic peptides to T cells. However, complete lack of MHC-1 expression can expose tumor variants to NK cell attack, since they can lyse MHC class 1-deficient targets. The present study demonstrates the presence of MHC-1, albeit at low levels, and suggests that canine osteosarcoma may be able to stimulate immune effector cells but not efficiently.

Altered expression of MHC-1, whether complete loss or partial loss, can result from the selective pressure of tumor cells, which avoid recognition both by specific CTLs, and NK cells (Marincola et al., 2000). Different altered phenotypes seem to occur at the stage when the tumor breaks through the basal membrane, invades the surrounding tissues, and starts to metastasize. It has been reported that invasive human tumors totally or partially lose HLA antigens at a very high frequency-between 40% and 90% (Algarra et al., 1997). To put it succinctly, MHC-1 loss is advantageous to tumor growth and survival because it allows

escape from specific MHC-restricted CTLs, it then follows that the minimum loss that permits CTL evasion without consequently inducing NK-cell susceptibility will be selected for tumor survival. This scenario might be occuring with canine osteosarcoma cells since they cannot be contained by immune effector cells and persist to invade and spread to distant sites particularly to the lungs. While it is possible in human tumors to differentially identify which MHC class 1 altered phenotype is expressed by an individual tumor, it is not yet feasible in canine tumors (Hinklin et al., 1999). Knowledge of MHC-1 based escape mechanisms in tumors is relevant in terms of designing and implementing clinical immunotherapeutic protocols. As in patients that display constitutive loss of MHC class 1 expression, it might be appropriate to transfer functional, active MHC class genes. In addition, decreased expression of HLA A, B, or C genes (locus-specific loss) can be partially restored by cytokines such as IFN-γ in responsive tumors. Alternatively, in tumors that have completely lost expression of MHC class 1 molecules, an appropriate therapy is to enrich for a particular NK- cell subset by IL-2 treatment that could attack the tumor cells. It is possible that some success in using IL-2 in cancer patients may be correlated with a previous loss of MHC class 1 molecules by the tumor (Rees et al., 1999; Coulie et al., 1999).

To determine whether the failure to obtain an optimum immune response could be due to lack of costimulatory molecules, B7 expression in canine osteosarcoma cell lines was analyzed. Flow cytometry analysis showed that 80% of the cell lines from 5 dogs expressed positive staining. Flow cytometry was used to measure B7 in canine osteosarcoma cells because of the existing constraints on finding specific mAb against canine B7. Since we were expecting low-density cell surface expression of B7 on canine osteosarcoma cells flow cytometry analysis was used. Flow cytometry has the advantage of detecting expression of

antigen at a lower protein concentration. To date, little is known about the expression of B7 molecules in dogs. The significance of B7 expression in canine osteosarcoma is that these cells are able to provide costimulation to both T cells and NK cells (Chen et al., 1992; Luque et al., 2000). If they are then able to render costimulatory signals, how do we explain the failure of the canine osteosarcoma cells to stimulate optimum immune effector cells response that would lead to their elimination? Unlike APC where B7 molecules are abundantly found, it has been shown that most tumor cells, particularly the nonhematopoeitic origin (Chen et al., 1994) do not naturally express these molecules. Immunogenicity of the tumors is critical to the effect of B7 costimulation on tumor immunity. When some nonimmunogenic tumors were transfected with B7, they continued to grow progressively (Chen et al., 1994). It was found that these nonimmunogenic tumors either lack molecules that can serve as tumor antigens or have deficient MHC class 1. The findings from our study show that although B7 is expressed on canine osteosarcoma cells, it is associated with a low MHC-1 expression; that appears to be inadequate to achieve the optimum antitumor immunity against these tumors. However, since a high density of B7 molecules is needed to trigger a full T cell response it could be that even with 80% of the cell lines showing high proportion of positive staining, the low MFI measured from canine osteosarcoma cells could mean a low density of B7 that is insufficient to induce a T cell response. B7 molecules have been reported to be expressed by some progressive tumors (Koyama et al., 1998; Hersey et al., 1994). Reports show that some tumors, which express B7 molecules, even at low levels, preferentially bind to CTLA-4 on T cells (Thompson and Allison 1997). Since CTLA-4 negatively regulates T cell activation this could explain their evasiveness from antitumor immunity. Since we detected B7 by using hCTLA-4Ig/Fc staining, we cannot differentiate at this time which B7 molecule

is expressed by canine appendicular osteosarcoma, B7-1, B7-2, or both, or whether the B7 has a higher affinity for CTLA-4 than CD28.

Because of the very promising use of IL-2 for therapy against canine osteosarcoma (Khanna et al., 1997; Nieves, unpublished results), we examined the effect of exogenous IL-2 in the expression of ICAM-1, MHC-1 and B7 surface molecules. We demonstrated that IL-2 did not have any effect in the expression of ICAM-1 and MHC-1 on canine osteosarcoma cells. A similar finding was reported in human melanoma cell lines incubated with IL-2 for up to 72 hours wherein IL-2 did not modulate ICAM-1 expresssion on the cells (Altomonte M, 1993). In contrast, exogenous IL-2 downregulated expression of the HLA- class 1 molecules and ICAM-1 on human carcinoma cell lines (Yasumura et al., 1994). However in a phenotypic analysis between parental and IL-2 gene transduced human squamous cell carcinoma of the head and neck cells there were no differences in expression of HLA-class 1 and ICAM-1 molecules (Nagashima et al., 1997). Human melanoma cells have been shown to express B7 molecules and increased expression is seen when these human melanoma cells are stimulated with IL-2 for 24-48 hours (Hersey et al., 1994). A marginal increase in B7 expression is detected in 3 of 5 canine cell lines tested after treatment with IL-2. Thus, our results suggest that when used as a treatment modality to canine osteosarcoma, IL-2 will basically function in recruitment of immune effector cells, expansion of these cells, and an enhancement of their cytotoxic activity but can not regulate the expression of ICAM-1, MHC-1 and B7 molecules on tumor cells.

The data that there were more dogs with osteosarcoma having metastasis and high ICAM-1 expression as observed in primary cell lines, frozen tissues, and formalin-fixed, paraffin-embedded tissues. However, a few cases were observed of dogs without metastasis

with high ICAM-1 expression. High levels of alkaline phosphatase occur in normal fully differentiated mature osteoblast cells (Rodan and Rodan, 1984). Similar high levels were observed in osteosarcoma cells (Murray et al., 1987). Several clinical studies demonstrated that high serum alkaline phosphatase is an important prognostic factor for appendicular osteosarcoma in dog and human osteosarcoma (Ehrhart et al., 1998; Bacci et al., 1993). Dogs from this study demonstrated variable serum alkaline phosphatase concentration. In addition they showed no definitive relationship between serum alkaline phosphatase level and the existence of metastasis.

In summary, the results of the study demonstrate a high level of ICAM-1 expression in canine primary osteosarcoma, which could possibly explain the invasive nature and rapid metastasis of this malignant tumor. B7 expression suggests that these tumors are able to deliver a costimulatory signal but manage to escape from immune effector cells, perhaps due to low MHC-1 expression. These results are consistent with the theory that the tumor cells could decrease MHC-1 expression to interfere with antigen presentation while possibly maintaining sufficient MHC-1 expression to deliver an inhibitory signal to NK cells.

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CHAPTER 3. GENERAL DISCUSSION AND CONCLUSION

The advantage of using clinical tumor cases (spontaneously occurring tumors) for studying the immunobiology of canine osteosarcoma was discussed by Vail and McEwen (2000) in their recent paper. They observed that most investigators have used inbred rodent models and laboratory derived canine populations to gather data for their study on canine tumors. Working with inbred rodent populations in artificial environments brings some degree of concern over the applicability of information gathered as it relates to naturally occurring tumors in dogs or people. They claim that to dispel most concerns, study of spontaneously occuring tumors in companion animals such as dogs and cats is highly recommended. These animals share a common environment with people and therefore exposure to environmental contributors to carcinogenesis should be similar. Malignancies in companion animals develop spontaneously, whereas many experimental models use induced tumors either through exposure to known carcinogens or transplantation, often in the presence of artificially induced immunologic modification.

For the purposes of this study, we analyzed canine osteosarcoma cell lines from spontaneously occurring primary appendicular osteosarcoma previously reported and some developed during the course of this work. The immunobiological behavior of canine osteosarcoma is most readily studied when the cells are growing in culture. Established tumor cell lines reflect the differentiated features of tissue origin with cellular immortality, characteristics that make them an ideal *in vitro* system for tumor studies. To minimize occurrences of intra-tumor variability, analysis was done on early cell cultures passages. Early cell cultures passages are suitable templates for immunotherapy vaccines.

On the other hand, it is possible that *in vitro* establishment of canine osteosarcoma cell lines selects a population of tumor cells with altered surface expression of ICAM-1and MHC-1. Thus, it was important to determine whether similar expression could also be demonstrated in tissues.

Since antigen is better preserved and detected in frozen state, tumor biopsies preserved as frozen tissue sections were stained for immuno- histochemistry. Important to all histological and cytological techniques is the preservation of cells and tissues in as close to life-like a manner as possible. This is accomplished by fixing them with commonly used formalin, which is superior for morphologic preservation but leads to antigen loss (Shi et al., 1991). A persistent goal in immunopathology then is to develop ways to provide maximal preservation of tissue morphology with minimal loss of antigenicity. With existing archives of formalin-fixed, paraffin-embedded tumor tissue sections of canine osteosarcoma cases available, we expanded the study in this tissue type preparation. And to solve the dilemma of possible masking of antigens due to fixation, several antigen retrieval techniques were used on the tumor tissues before proceeding to immunohistochemistry analysis.

Additionally, the present study provides new insights on how to approach immunotherapeutic strategies for canine appendicular osteosarcoma particularly on its characteristic rapid pulmonary metastasis. Considering high level of ICAM-1 expression, the beneficial effects of transducing tumor cells with GM-CSF, a pro-1nflammatory cytokine can only contribute to the availability of more leukocytes where the tumor cells can adhere and "hitch-a-ride" to metastasize to other sites, especially to the lungs. Expression of MHC class 1 is an important criterion when considering T-cell based imunotherapy. Therefore, selecting patients for clinical trials based on their ability to express normal levels of MHC class 1 is

highly recommended. Since our findings show low expression of MHC class 1 in the canine osteosarcoma cases we tested, a question of what type of loss or downregulation has occurred must be considered. This area in canine patients is not well studied yet due to the constraints imposed by unavailability of reagents and specific monoclonal antibodies to canine MHC-1. One study of canine mammary tumors and melanoma demonstrated that MHC class 1 expression in these tumors could be increased by canine IFNγ treatment (Whitley et al., 1995).

Taken together, our data provide information in the expression of ICAM-1, MHC-1 and B7 molecules on canine appendicular osteosarcoma. These surface molecules are very crucial in determining an optimum immune response of effector cells such as T and NK cells against tumor cells. The results also clarify certain aspects of canine appendicular osteosarcoma immunobiology. The presence of ICAM-1 denotes that these tumor cells can adhere and form stable interactions with immune effector cells. This can additionally contribute to costimulation of immune effector cells. Despite this functional significance it is difficult to dismiss the significance of the widespread high level of expression of ICAM-1 on these malignant cells. Based on the highly invasive and rapid metastases of canine osteosarcoma particularly to the lungs, the seeming ability to escape immune surveillance and reports from other tumor studies, we strongly suggest that these cells use ICAM-1 instead to promote their growth and metastasis. The low expression of MHC class 1 on canine appendicular osteosarcoma might render them resistant to effector T cells. In contrast, low expression of MHC class 1 on these tumors makes them susceptible to NK cells according to 'missing self hypothesis'. While this expectation might hold true the possibility still exist that canine osteosarcoma by selective pressures might generate tumor variants with

minimum MHC class 1 loss that permits CTL evasion and at the same time retaining MHC class 1 proteins that inhibit NK activity (via KIRs). Immune recognition is aided in that B7 expression is detected in canine osteosarcoma meaning that they can deliver costimulation when needed. Although to what extent is costimulation occurring is questionable because the low mean fluorescence intensity could reflect low density of B7, which might not be enough to induce sufficient T cell response. Alternatively, B7 could bind preferentially to CTLA-4, the negative regulator of T cells in which B7 molecules have higher affinity (Thompson and Allison, 1997). We believe the baseline information we gathered on these surface receptors will contribute to a better understanding of canine osteosarcoma.

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