

A NOVEL APPROACH TO THE CLINICAL DIAGNOSIS AND TREATMENT OF
CANINE HISTIOCYTIC SARCOMA

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CANINE HISTIOCYTIC SARCOMA

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Chapter 1: Introduction

Histiocytic diseases are well documented in veterinary medicine with at least four defined disease courses being present in dogs. The term histiocyte has been used to describe cells which arise from stem cell precursors and differentiate into dendritic cells (DC) or macrophages (Moore, 2014). DC and macrophages both have important roles in the immune response as antigen presenting cells. DCs are present throughout the body and arise from stem cells in the bone marrow, with the exception of Langerhans cells which are located in the skin and develop from embryonic precursors (Abbas, 2014). They are concentrated in areas of the body such as the skin, gastrointestinal tract, respiratory tract, and lymphatics where they can efficiently sample their environment for foreign microbes or antigen. Once a DC comes in contact with antigen in the body, it phagocytizes the antigen and upregulates surface receptors such as major histocompatibility complex II (MHC II) and co-stimulators (CD40, CD80, CD86) that turn it into a mature or active DC (Abbas, 2014). Once activated, it leaves its resident site due to decreased adhesiveness to tissues and travels to the lymph node where antigen can efficiently be presented to the lymphocytes. This typically results in the differentiation of naïve T cells into effector T cells as part of the cell-mediated immune response. DCs may also phagocytize entire cells which contain antigen to present to naïve T lymphocytes in a process called cross-priming. Macrophages are also a type of antigen

presenting cell; however macrophages have lower levels of MHC II expression and are less effective than DCs (Abbas, 2014). Macrophages can be recruited to areas of inflammation through cytokines and may ingest and kill microbes they come in contact with. Both DCs and macrophages serve important roles within the immune system, and canine histiocytic diseases involve an abnormal proliferation of one of these cell types.

Histiocytomas and Cutaneous Langerhans Cell Histiocytosis

Langerhans DCs are resident cells found primarily within the epidermis of the skin. A proliferation of Langerhans DCs typically forms a solitary lesion on the skin called a cutaneous histiocytoma (Moore, 2014; Mastroilli, 2012).

Histiocytomas are small, benign lesions most commonly found in young dogs on the head and neck, specifically the pinna. These lesions have been shown to spontaneously regress which is thought to be mediated by the activity of CD8+ cytotoxic T lymphocytes (Moore, 1996). In late stages, the diagnosis of cutaneous histiocytoma can be confused with epitheliotropic lymphoma due to the increased numbers of lymphocytes present (Moore, 2014).

Immunohistochemistry for lymphocyte markers and Langerhans DCs are useful in differentiating these lesions.

Less commonly, dogs may present with multiple cutaneous lesions similar to that of histiocytomas. This presentation has been classified as cutaneous Langerhans cell histiocytosis (LCH) (Moore, 2014). The cutaneous lesions on

these dogs may be erythematous or ulcerated and can occur anywhere on the body. As opposed to cutaneous histiocytomas, LCH may involve draining lymph nodes and rarely other areas such as the lungs, and involvement beyond the skin carries a worse prognosis (Moore, 2014). Dogs with a diagnosis of LCH have been treated with various immunomodulatory agents with little to no success, and many of these dogs are euthanized due to a decreased quality of life.

Reactive Histiocytoses

The reactive histiocytoses are histiocytic inflammatory diseases with immune system dysregulation (Moore, 2014). Cutaneous histiocytosis (CH) is characterized by multiple cutaneous lesions which typically involve the deep dermis and possibly panniculus. Unlike cutaneous LCH, reactive histiocytosis arises from activated DCs which gives them a unique characteristic on immunohistochemistry (Moore, 2014). Due to the immune dysregulation involved, dogs with CH tend to respond to immune suppression; however the lesions may also undergo spontaneous resolution with time.

A more widespread form of cutaneous histiocytosis that may involve the eyes, nasal mucosa, and lymph nodes in addition to skin is called systemic histiocytosis (SH). Systemic histiocytosis is more commonly seen in young to middle aged Bernese Mountain dogs, Irish wolfhounds, Rottweilers and Labradors; however any breed may develop the disease. Dogs affected with SH often have periocular and nasal planum involvement as well as enlarged

peripheral lymph nodes. As with cutaneous histiocytosis, these lesions have a unique immunohistochemistry profile consistent with activated interstitial dendritic cells. Activated DC will express high levels of Thy-1 and costimulators such as CD80/86 along with CD1a, CD18/CD11c and MHCII compared to Langerhan's cells which do not express Thy-1 and the other costimulators (Moore, 2014). This can be particularly useful in differentiating diseases that arise from Langerhans cells from those that arise from activated DC.

Histiocytic Sarcoma

Interstitial DCs have been found in almost all tissues in the body with the only exception being the brain (Moore, 2014). A neoplastic proliferation of interstitial dendritic cells is termed a histiocytic sarcoma (HS) or, if the neoplastic population is a proliferation of macrophages, a hemophagocytic HS (Moore, 2014; Mastroilli, 2012; Moore, 2006).

Histiocytic sarcoma (HS) is a disease that is commonly seen in middle aged to older dogs with Bernese Mountain Dogs, Flat Coated Retrievers, and Rottweilers overrepresented (Moore, 2014; Abadie, 2009; Skorupski, 2009; Affolter, 2002). Bernese Mountain Dogs have been shown to have polygenic mode of inheritance of HS and point mutations have been documented in several tumor suppressor genes including deletions in PTEN, RB1, and CDKN2A/B (Hedan, 2011). HS is seen in localized and disseminated forms, each with their own clinical presentation. Dogs presenting with the localized form typically have

lesions which arise from a single site including skin, subcutaneous tissue, lung, bone, or joints (Klahn, 2011; Skorupski 2009 and 2007; Affolter, 2002).

Disseminated HS, which is the most common clinical presentation and was formerly known as Malignant Histiocytosis, may represent progression of localized disease or may arise as its own entity. Multiple organs are involved at the time of diagnosis including lungs, skin, bone, joints and extension into lymph nodes and liver (Klahn, 2011; Affolter, 2002). While a smaller number of dogs present with the localized form, 70-91% of these will eventually develop distant metastasis (Skorupski, 2009).

Hemophagocytic HS is a type of histiocytic sarcoma which is derived from macrophages of the splenic red pulp or bone marrow rather than from interstitial dendritic cells (Moore, 2014; Moore, 2006). Dogs with the hemophagocytic form of HS have a unique presentation as well as diagnostic findings. Moderate to severe regenerative anemia, thrombocytopenia, hypoalbuminemia, and hypocholesterolemia have been frequently documented in dogs with hemophagocytic HS (Moore, 2006). Due to the regenerative anemia and thrombocytopenia seen, these dogs may often be confused with those that have immune mediated hemolytic anemia or Evan's syndrome; however dogs with hemophagocytic HS should be negative on Coomb's test (Moore, 2006). On imaging, these dogs also tend to have more diffuse changes seen in the spleen and/or liver when compared to nodules noted in dogs with HS arising from interstitial DC. The disease also often involves the bone marrow, pulmonary

vasculature, and lymph nodes. Another common finding in this form of HS is that erythrophagocytosis is seen more readily compared to other HS. The diagnosis of hemophagocytic HS can be made by using immunohistochemistry to determine macrophage origin (Moore, 2014; Moore, 2006).

The prognosis for dogs with HS is considered very poor with the course of disease being rapid despite aggressive treatment. Depending on the location, treatments for HS vary and include surgery, radiation therapy and/or chemotherapy. Due to the high presence of disseminated disease, chemotherapy is necessary in combination with local therapy such as surgery or radiation therapy. Varying results have been published with HS localized to the joint. A 2002 study of 18 dogs with periarticular HS had a MST of 5.3 months; however, this population of dogs ranged from receiving no treatment to local control and various chemotherapy agents (Craig, 2002). A 2011 study comparing periarticular to non-periarticular HS found that dogs with periarticular HS had longer overall MST (391 days) with 13/19 dogs having metastasis (Klahn, 2011). Eleven of 19 dogs in this study received both local treatment including surgery and/or radiation therapy along with chemotherapy, and 8/19 dogs received chemotherapy as the sole treatment (Klahn, 2011). Another study similarly evaluating dogs with localized HS that were treated with adequate local control along with lomustine chemotherapy had prolonged survival times of 568 days (Skorupski, 2009). This is in contrast to dogs with disseminated HS or gross disease treated with chemotherapy. A 2007 study of 59 dogs with gross disease

who were treated with lomustine showed a 46% response rate to lomustine chemotherapy and the responders had a median survival time of 172 days (Skorupski, 2007). Dogs with hemophagocytic HS also have an extremely poor prognosis with a mean survival of 7 weeks from diagnosis (Moore, 2006).

The diagnosis of HS can be a challenge and is typically made on cytology or histopathology of the affected tissue. Cytologically, histiocytic sarcoma is characterized by round to mesenchymal cells with marked cellular atypia, anisocytosis, anisokaryosis, and binucleation. The cells may be very large and bizarre with several mitotic figures and can be seen phagocytizing other cells (hemophagocytic form) (Moore, 2014). On histopathology, large pleomorphic cells with pronounced cellular atypia and frequent multinucleation and bizarre mitotic figures are typically seen (Moore, 2014). Unfortunately, due to the mesenchymal appearance of the cells, there are many cases in which histiocytic sarcoma cannot be differentiated from soft tissue sarcomas based on histopathology alone and additional tests are needed. Differentiating HS from other sarcomas or round cell tumors is very important clinically due to the significant difference in treatment and prognosis of the tumor types.

The most common diagnostic challenge is differentiating HS from high grade or undifferentiated soft tissue sarcomas. Soft tissue sarcomas are locally aggressive neoplasms arising from various connective tissues in the body. They are commonly seen in middle aged to older large and medium breed dogs (Dennis, 2011). Based on histopathology, soft tissue sarcomas are classified

into 3 grades which are defined by a scoring system using mitotic index, amount of necrosis, and degree of differentiation of the cells (Dennis, 2011). In contrast to HS, soft tissue sarcomas develop metastasis in approximately 40% of cases for high grade tumors and rarely (<20%) for low grade tumors (Kuntz, 1997). Treatment for soft tissue sarcomas involves aggressive surgery and, in some cases, radiation therapy for tumor control. Those dogs with low grade sarcomas that can be removed with complete surgical margins have a better prognosis than high grade sarcomas as these high grade sarcomas have a higher metastatic rate and increased risk of local recurrence. Chemotherapy is not indicated in low grade soft tissue sarcomas due to the low metastatic rates and long survival times. In one study evaluating surgery for soft tissue sarcomas in dogs, the median survival time was greater than 1,000 days with the mitotic index and percentage of tumor necrosis being the most prognostic factor (Kuntz, 1997). In high grade sarcomas, the use of chemotherapy has been evaluated due to the higher metastatic and local recurrence rates. Doxorubicin was evaluated in a phase II study in a variety of neoplasms with response rates of 22% seen in dogs with sarcomas (Ogilvie, 1989). A retrospective study (Selting, 2005) evaluated the use of doxorubicin following surgery for dogs with high grade soft tissue sarcomas and did not find a statistically significant difference in survival time between dogs receiving adjuvant doxorubicin and those that did not.

Due to the significant differences in both treatment and prognosis for dogs with histiocytic sarcoma compared to high grade soft tissue sarcomas, a proper diagnosis is critical. While undifferentiated soft tissue sarcomas can be similar in appearance to histiocytic sarcomas on histopathology, additional information with immunohistochemistry to determine the cell origin is needed for a diagnosis. As it is standard for biopsy samples to be placed in formalin, the use of a unique and specific immunohistochemistry marker that can be used on fixed tissue samples is ideal.

Immunohistochemistry

Historically, immunohistochemistry has been used to evaluate cell surface markers to differentiate HS from other tumor types. Because histiocytic sarcoma arises from a proliferation of dendritic cells, the immunohistochemistry profile should be characteristic of interstitial DC. Several different cell surface markers are known to be expressed in histiocytic diseases; however, these markers are frequently expressed on other leukocytes and are not specific to dendritic cells. CD18 is part of the beta subunit in the beta-2 integrin family which is involved in leukocyte adhesion. CD18 is expressed as a heterodimer with CD11a, b, c, or d and all leukocytes express at least one of these heterodimers. Cells derived from macrophages in the splenic red pulp or bone marrow express CD11d; macrophages from other tissues and a subset of interstitial DC express CD11b

(Moore, 2014; Mastrorilli, 2012). Interstitial DC typically express CD11c along with CD18 (Moore, 2014; Mastrorilli, 2012). Other cell markers involved in antigen presentation such as CD1a and major histocompatibility complex (MHC) are known to be expressed by histiocytic sarcoma cells. CD1a and MHC I and II are responsible for presentation of peptides, lipids, and proteins to T lymphocytes (Moore, 2014). CD1a is consistently expressed by dendritic cells whereas MHC I and II are more broadly expressed (Moore, 2014). While CD1a and CD11c are expressed in HS and would be useful in diagnosis, these are only detectable in fresh smear or snap-frozen tissue. When performing surgery to remove a mass in veterinary medicine, it is standard that the biopsy sample is placed in formalin for preservation. This makes the use of immunohistochemical markers that are only available in fresh frozen tissue of limited use when trying to obtain a diagnosis. CD11d has been used on formalin fixed samples to specifically differentiate histiocytic tumors of macrophage origin, but CD11d may be negative in HS arising from interstitial dendritic cells (Mastrorilli, 2012).

Due to the role of dendritic cells as antigen presenting cells, some cell surface markers are known to be expressed upon activation or upregulation of the DC by its interaction with T lymphocytes. These are called co-stimulators (CD80, CD86, and Thy-1) and the binding of these to T cells is required for full activation of the T lymphocyte (Heinrich, 2014; Mastrorilli, 2012). Because these co-stimulators are expressed in activated DC, unstimulated DC may only express low levels (Heinrich, 2014). The use of immunohistochemistry for costimulators

is particularly helpful in differentiating cutaneous/systemic histiocytosis from cutaneous Langerhans cell histiocytosis as the former arises from activated interstitial DC. Once activated, DC will express Thy-1, CD4 and CD80/86 along with CD1a, CD11c/CD18 and MHCII. In contrast, the diseases of Langerhans DC will not express Thy-1 and the other costimulators, but they do express E-cadherin particularly along the epidermis (Moore, 2014). Histiocytic sarcoma, which arises from interstitial DC will also express the co-stimulatory molecules, but in lower levels as they are typically from inactive DC. HS may also express other molecules involved in presenting antigen to T cells, as dendritic cells and macrophages are both antigen presenting cells in the body (Moore, 2014).

Because there has not been a single marker specific to histiocytic cell origin evaluated, a panel of stains is used to assist in the diagnosis of HS. Currently, a diagnosis of histiocytic sarcoma is made in those tumors with appropriate morphology which are positive for CD18 and negative for lymphocyte markers (CD3, CD20 and CD79a) (Moore, 2014; Mastroilli, 2012). HS arising from interstitial DC should have positive expression of CD18/CD11c and CD1a with negative expression of CD11d and lymphocyte markers (CD3, CD20 and CD79a). Because histiocytic sarcoma can often take on the appearance of an undifferentiated sarcoma or pleomorphic round cell tumor, it is important that CD18 be performed in conjunction with other markers to rule these out. The presence of negative lymphocyte markers is important as CD18 is a pan-leukocyte marker and CD18+ lymphomas are reported (Moore, 1990) as well as

CD3-/CD79a- (null phenotype) lymphomas (Ponce, 2010). In contrast, hemophagocytic HS is expected to express CD18/CD11d and lack expression of CD11c with a weak expression of CD1a (Moore, 2014). Recently, research has been done to evaluate other cell markers which may be more specific for histiocytes and be beneficial in diagnosis of HS in formalin fixed tissue.

Treatment

Various chemotherapy treatments have been evaluated for use in dogs with histiocytic sarcoma; however no systemic therapy has shown significant improvements in survival times and there are a lack of prospective clinical trials performed with control groups or comparison to current protocols used. The prognosis remains poor even with systemic therapy especially for dogs with the disseminated form of disease. Due to the prognosis with current therapies, several studies have been performed to investigate the response of new potential chemotherapy drugs for canine histiocytic sarcoma in vitro.

Historically, lomustine has been considered the chemotherapy drug of choice for dogs with disseminated histiocytic sarcoma or as adjuvant therapy to those with local disease due to the high metastatic rate. Lomustine is an alkylating agent that has been evaluated for use in dogs with histiocytic sarcoma both in retrospective and prospective studies. In 2007, Skorupski et al. retrospectively evaluated dogs with histiocytic sarcoma and their response to lomustine therapy. In this group of 56 dogs, there was a 46% overall response

rate and median survival time of 172 days (Skorupski, 2007). In 2010, Rassnick et al. evaluated the response rates of dogs with both disseminated and non-resectable localized histiocytic sarcoma in a prospective study where the dose and follow-up could be standardized. Out of 21 dogs, 3 dogs had a complete response and 3 dogs had a partial response to lomustine (90 mg/m² by mouth every 4 weeks). The overall response rate for this group of dogs was 29% and the median survival time was 96 days (Rassnick, 2010).

Prior studies evaluating the response of various malignancies to doxorubicin have been performed and responses were noted in a small number of dogs with histiocytic neoplasia. A preclinical trial of liposomal-pegylated doxorubicin performed in 1997 included 2 dogs with a diagnosis of malignant histiocytosis involving the lymph nodes. A complete response was noted in 1 of the 2 dogs with a remission time of 160 days (Vail, 1997). The liposomal form of doxorubicin evaluated in this study is thought to be an improvement over early forms of the drug as it is encapsulated with polyethylene-glycol (PEG) derivitized phospholipid which helps to decrease the rapid uptake and clearance of the liposomes by phagocytic cells in the body previously noted with liposomal drugs. Given that histiocytic sarcoma is a disease which arises from antigen presenting cells in the body, it could be theorized that liposomal forms of chemotherapy may be effective as they are readily taken up by the cancerous population.

With minimal to no improvements noted in survival time for dogs with histiocytic sarcoma, there is a significant amount of ongoing research evaluating the use of different chemotherapy drugs both in vitro and in vivo. In 2004, the use of paclitaxel was studied in dogs with various measurable tumor types. This study included 2 dogs with malignant histiocytosis and showed a partial response in 1 of the dogs. The dose of paclitaxel used in this study resulted in significant adverse side effects (hypersensitivity and neutropenia) and would not be feasible for most clinical patients (Poirier, 2004).

Clodronate is a type of bisphosphonate that has been shown to kill osteoclasts and other macrophages via apoptosis in mouse studies. In 2010, this concept was tested both in vitro with histiocytic cell lines and in vivo in a pilot study of 5 dogs with histiocytic sarcoma. In vitro, the clodronate was added to the cells in a liposomal form and showed that histiocytic cells were much more susceptible to cell killing from the liposomal clodronate compared to other cell types. However, when free clodronate was added to the cell lines, the histiocytic cells still showed sensitivity but to a lesser extent than with the liposomal form. The other cells lines showed significantly improved responses to the free form of clodronate (Hafeman, 2010). In the same study, clodronate was given intravenously to 5 dogs with HS and an objective response was reported in 2/5 dogs (Hafeman, 2010).

While many studies have been performed to find a more promising treatment protocol for canine HS, very few have investigated multi-drug protocols. In 2012, an in vitro study evaluated masitinib as a chemosensitizer to multiple cell lines including histiocytic sarcoma and showed that masitinib strongly sensitized cells to vinblastine (Thamm, 2012). Hafeman et al, also looked at the effectiveness of bisphosphonates to sensitize histiocytic cells to various chemotherapeutic agents (Hafeman, 2012). The only study to date that has evaluated a multi-drug protocol for treating canine HS in vivo combined doxorubicin with lomustine for a median survival time of 185 days (Cannon, 2015). Unfortunately, despite the numerous in vitro and in vivo studies investigating different treatments for dogs with HS, we have yet to make improvements in the overall survival times for these patients.

Biomarkers and monitoring

A growing area of research in veterinary oncology involves the evaluation of dogs for biomarkers which may be detected prior to gross disease or relapse of disease. Due to the poor prognosis associated with histiocytic sarcoma, any biomarker that may be used to detect disease and start treatment earlier would be beneficial. Biomarkers also have a potential role in serial monitoring to help determine response to treatment and remission status. There is extensive precedent for this in human cancer treatment but reports are less common in veterinary medicine and often compare biomarkers in tumor-bearing to healthy

animals rather than to animals with other disease states. In addition, reports of serial biomarker monitoring to assess response to therapy are sparse in veterinary medicine. Because the immune system is closely tied to cancer with many different cytokines being produced, research is being done to investigate increases in these cytokines as it may relate to the tumor. Other areas of interest of measuring various proliferation markers which may be increased in certain types of neoplasia.

C-reactive protein (CRP) is an acute phase protein in the body which is known to increase in blood earlier than other markers of inflammation such as leukocytosis. Unfortunately, C-reactive protein is not a specific marker for neoplastic processes and may be increased with any cause of inflammation in the body. The acute phase proteins such as CRP have been evaluated in many different tumor types in both dogs and humans. A 2007 article investigated CRP in dogs with lymphoma and found that an increase in CRP correlated with dogs who were not in remission (Neilsen, 2007). A 2009 study by Planellas, evaluated both CRP and haptoglobin in dogs with mammary tumors as well as controls and found that CRP was significantly higher in dogs with carcinoma than controls; however this could be attributed to more inflamed tumors (Planellas, 2009). In 2015, a study was performed which evaluated CRP along with thymidine kinase for use in screening dogs for occult neoplasia and found that the use of these biomarkers together was more effective than a single biomarker alone (Selting 2015).

Thymidine kinase (TK1) is another biomarker which has been studied in both human and veterinary medicine. Thymidine kinase plays a role in the synthesis of thymidine, a DNA precursor, and is specific to the S phase of the cell cycle. Due to its activity in the S phase, TK1 acts as a proliferation biomarker and may be useful in evaluating tumors with high turnover rates (Selting, 2015; Thamm, 2012; von Euler, 2011). While other markers of proliferation such as Ki67 and proliferating cell nuclear antigen (PCNA) have been evaluated in different tumor types and associated with prognosis, these are typically noted on initial biopsy tissue samples and not easily monitored serologically throughout the disease process (von Euler, 2011). When evaluated in different types of neoplasms, TK1 has been increased more in proliferative diseases such as lymphoproliferative disorders when compared to solid tumors (Selting, 2015; Thamm, 2012). TK1 was evaluated in a group of dogs with a variety of neoplasms including 9 dogs with HS and found to be greater than 2 U/L in all 9 dogs and greater than 6 U/L in 5/9 dogs (Selting, 2016). TK1 was also increased in dogs with lymphoma and hemangiosarcoma in the same study. In a 2013 study, TK1 was evaluated in dogs with pyometra and was shown to be increased in these dogs when compared to controls; however, a conclusion was not drawn as to the role or prognostic value of increased TK1 in bacterial infections (Sharif, 2013). In humans, increases in TK have been associated with a worse prognosis for certain cancers such as breast, colon, lung, and other hematologic cancers (von Euler, 2011).

Because biochemical abnormalities have frequently been associated with signs of inflammation in dogs with HS, more research has been done to evaluate whether this inflammation is associated with tumor associated cytokines inciting an inflammatory response. In a 2013 paper by Neilson, Bernese Mountain Dogs were evaluated along with healthy controls for any changes in serum C-reactive protein (CRP), fibrinogen, monocyte-chemotactic protein (MCP-1), and other interleukins. Significant increases were noted in fibrinogen, C-reactive protein, and MCP-1 in the dogs with HS (Neilson, 2013). While the fibrinogen and CRP increases are not specific findings for HS, the MCP-1, which was significantly increased, may be a promising finding as MCP-1 is a cytokine released from dendritic cells and macrophages during inflammatory responses to help recruit monocyte derived cells from the bone marrow. In humans, MCP-1 (CCL2) as well as tumor necrosis factor-alpha have been evaluated as potential poor prognostic indicators in patients with nasopharyngeal carcinoma (Lu, 2011).

Another biomarker, big endothelin-1, has been evaluated in dogs with neoplastic diseases as well as cardiopulmonary disease. Big endothelin-1 is the inactive precursor to endothelin-1 and is easier to measure as it is more stable in blood. In a 2014 study, dogs with both neoplasia and cardiac disease were evaluated and an increase in big endothelin-1 was found in dogs with pulmonary hypertension when compared with controls as well as dogs with hemangiosarcoma, adenocarcinoma, HS, hepatocellular carcinoma, chondrosarcoma, and osteosarcoma (Fukumoto, 2014). With the largest

increase found in the dogs with hemangiosarcoma, vascular endothelium may be a source for increases in big endothelin-1 and warrant further investigation.

Chapter 2: Immunohistochemistry

Histiocytic sarcoma (HS) is a rapidly progressive neoplasm of dendritic cell origin common in middle aged to older dogs with Bernese Mountain Dogs, Flat Coated Retrievers, and Rottweilers overrepresented (Moore, 2014). Clinically, HS presents in a localized or disseminated form, and of the dogs presenting with the localized form, 70-90% will eventually develop distant metastasis (Skorupski, 2009). Localized histiocytic sarcoma is commonly found in the subcutaneous tissue of the limbs and is difficult to differentiate from soft tissue sarcomas (STSAs), which have a similar presentation. The prognosis for HS is poor with rapid disease progression and metastasis despite aggressive therapy, while STSAs have a comparatively lower metastatic rate and are often successfully treated with surgery and radiation therapy if indicated (Kuntz, 1997), making an accurate and early diagnosis crucial to guide staging and treatment.

The current gold standard for the diagnosis of HS utilizes morphologic appearance with immunohistochemistry (IHC) for CD1 and CD11c, both of which are specific to histiocytes. However, these stains can only be used on fresh frozen tissues. Following surgical removal, it is standard practice for tissues to be formalin fixed, as HS is not often suspected at the time of surgery and the equipment necessary for preparing and storing fresh frozen tissues is not available in general veterinary practice. Therefore, CD1 and CD11c are not accessible for confirmation of HS in most cases. Currently, the diagnosis of HS is based on morphologic appearance of cells combined with positive staining of

CD18, which is strongly expressed by histiocytic cells as well as other leukocytes, and negative staining for lymphocyte markers such as CD3, CD20, and CD79a (Affolter, 2002). However, it is important to note that CD18 is not specific for dendritic cells, and null cell (CD3, CD20 and CD79a negative) lymphomas have been reported (Ponce, 2010). Furthermore, pathologist review of immunohistochemistry must take into account not just overall staining of cells, but specifically staining of the cancerous cells versus staining of tumor infiltrating immune cells, including macrophages, which are CD18 positive. This can further complicate obtaining an accurate diagnosis. If cellular morphology combined with currently available IHC stains does not provide a clear diagnosis, a diagnostic and therapeutic dilemma results.

Part 1: CD206

CD206, or the mannose receptor, is a C-type receptor which is an important cell marker on immature dendritic cells but is not typically expressed in mature dendritic cells such as Langerhans cells (Wollenberg, 2002). A previous study evaluated canine dendritic cells in healthy, atopic, and non-allergic inflamed skin, and found that CD206 positive cells were noted in the dermis of inflamed skin and represented a population of monocyte derived dendritic cells (Ricklin, 2010). Another study evaluating CD206 found that it was expressed highly in granulocytic-monocytic-bone marrow derived dendritic cells (Ricklin Gutzwiller, 2010). Recently, canine and feline adipose tissue macrophages were

showed to express CD206 on flow cytometry similar to the M2 phenotype expression shown in mice (Ampem, 2016). The specificity of CD206 expression on immature dendritic cells and macrophages makes it a promising cellular marker for diagnosis of canine histiocytic diseases. To date, no studies have been performed evaluating the use of CD206 for diagnosing canine HS. The objective of this study was to evaluate the use of CD206 in diagnosing dogs with HS on formalin fixed tissue. The hypothesis was that CD206 would be a more specific marker of tumors arising from DC and thus be useful in differentiating HS from other types of sarcomas.

Materials and methods

To validate the CD206 antibody in canine formalin fixed tissue, canine lymph nodes were fixed in 10% neutral buffered formalin, processed, and embedded in paraffin wax. Paraffin-embedded tissues were sectioned into 4 μm slices for immunohistochemistry. The primary antibody used was anti-human rabbit polyclonal antibody (CD206; GeneTex; Irvine, CA). A heat-induced antigen retrieval method was performed initially using Diva decloaker (Biocare; Concord, CA) and EnVision™ + system (Dako; Carpinteria, CA). The primary antibody was tested at dilutions of 1:100, 1:300, and 1:500 in Tris buffered saline (TBS). The primary antibody was incubated for 30 and 60 minutes. The heat induced antigen retrieval method was repeated using Diva decloaker (Biocare; Concord, CA) and EnVision™ + system (Dako, Carpinteria, CA).

Immunohistochemistry was performed on fresh frozen canine lymph node. A fresh canine lymph node was collected upon necropsy of an apparently healthy dog euthanized for reasons unrelated to the current study. The lymph node was sectioned into two pieces with one half frozen using liquid nitrogen and stored in -80 degrees Celsius until assay, and the other fixed in buffered formaldehyde, processed, and embedded in paraffin wax. Cryosections of the frozen lymph node were brought to room temperature and fixed with acetone for 8 minutes prior to being rinsed with phosphate-buffered saline (PBS) three times. The anti-human rabbit polyclonal antibody (CD206; GeneTex; Irvine, CA) was used as the primary antibody and a negative control was included by omitting the primary antibody. Romulin AEC Chromogen (Biocare Medical, Concord CA) was used to visualize the immunoreactivity and hematoxylin was used as a counterstain.

Results

In formalin fixed tissues, the primary antibody was incubated for 60 minutes at a dilution of 1:100 in TBS. No staining was detected. Next, proteinase K (Dako, Carpinteria, CA) was used to enzymatically expose the antigen. The primary antibody was added in a 1:100 dilution and incubated for 60 minutes. A MACH™ polymer detection kit (Biocare; Concord, CA) was used and no staining was detected in the positive control.

To determine whether the lack of CD206 staining on the paraffin embedded tissue was due to the antibody or the tissue processing, the study was

repeated utilizing lymph node positive control, both fresh frozen and formalin fixed tissue. Similar to the formalin tissue, no CD206 staining was noted in the fresh canine lymph node sample. When immunohistochemistry was performed on the portion of the paraffin embedded lymph node as stated previously, again no staining was noted.

Conclusion

CD206 could not be validated in canine tissues. Based on the lack of success using CD206 in positive control fresh frozen and formalin fixed samples, we conclude that the CD206 anti-human rabbit polyclonal antibody (GeneTex; Irvine, CA) is not useful for evaluation of canine tissues.

Part 2: CD204 and Iba-1

Recent studies found CD204 to be promising as a more specific cell marker for histiocytic sarcoma. CD204, also known as the class A macrophage scavenger receptor, plays a role in host defense including macrophage adhesion, phagocytosis, production of reactive oxygen species, and host defense (Kelley, 2014; Tomokiyo, 2002). CD204 is expressed in the tissue macrophages of several organs including Kupffer cells in the liver, sinus macrophages in the lymph node, alveolar macrophages in the lung, and other organs (Tomokiyo, 2002). While CD204 is expressed in the dermal macrophages in the skin, it is invariably negative in the Langerhans cells, epithelial cells, lymphocytes, and

granulocytes (Tomokiyo, 2002). CD204 has recently been evaluated in canine histiocytic sarcoma both in formalin fixed tissue and on cytologic samples via immunocytochemistry. CD204 was evaluated on immunocytochemistry in 10 HS and 45 other tumors (13 round cell tumors, 18 epithelial tumors, and 14 non-epithelial tumors). All of the HS samples showed intense positive staining for CD204 in at least 50% of the cells compared to no staining seen in the other tumor tissues (Kato, 2014). In a second study utilizing IHC, all undifferentiated sarcomas which included melanoma, poorly differentiated hemangiosarcoma, and rhabdomyosarcoma were negative for expression of CD204 with variable expression for CD18 and MHCII, however, these tumors have specific IHC markers currently validated for use on formalin fixed tissues and can be therefore be readily diagnosed (Kato, 2013). Although CD204 has not been evaluated in canine soft tissue sarcomas and compared to canine histiocytic sarcomas, it is commonly used in practice as a single IHC stain to rule in or rule out canine HS, thus dramatically changing the treatment protocol.

Ionized calcium binding adaptor protein (Iba-1) is a calcium binding protein that plays a role in rearrangement of the actin skeleton and aids in the early phases of phagocytosis. Iba-1 is specifically expressed in cells of the monocyte/macrophage lineage including microglial cells (Schulze, 2008; Kohler, 2007). Iba-1 and CD204 were positive in 15 dogs with subdural histiocytic sarcoma as well as in 23 dogs with intracranial histiocytic sarcoma (Thongtharb, 2016; Ide, 2010). Iba-1 also showed strong staining in all cases of canine

cutaneous histiocytoma, histiocytic sarcoma, and reactive histiocytosis as well as feline progressive dendritic cell histiocytosis and macrophages in cutaneous mycobacteriosis. Other round cell tumors (melanoma, lymphoma, mast cell tumor and plasmacytoma) were all negative for Iba-1 (Pierezan, 2014). Despite these studies, Iba-1 has not been evaluated in canine soft tissue sarcomas and compared to canine histiocytic sarcomas.

While previous research confirms the use of CD204 and Iba-1 in canine formalin fixed tissues, and while clinicians utilize these antibodies to differentiate histiocytic sarcoma from other round cell tumors, an important clinical dilemma that has not been addressed is differentiating histiocytic sarcoma from high grade soft tissue sarcoma in formalin fixed tissues. Thus, the primary objectives of this study are to confirm expression of CD204 in a canine histiocytic cell line and to determine the diagnostic utility of CD204 and Iba-1 alone or in combination to differentiate canine histiocytic sarcoma from other canine soft tissue sarcomas (STSA). The hypothesis is that CD204 expression will be seen in the DH82 canine histiocytic cell line and that CD204 and Iba-1 will be specific and useful markers in differentiating HS from STSA in formalin fixed tissue.

Materials and Methods

CD204 expression in a canine histiocytic cell line:

Cell culture conditions: The canine histiocytic cell line DH82 (ATCC, Manassas, VA) was cultured in DMEM supplemented with 10% fetal bovine serum and incubated at 5% CO₂ and 37°C.

Western blot: Western blot was performed to demonstrate the protein expression of CD204 in DH82 cells. Fresh canine liver and spleen were used to extract protein for positive controls as the CD204 receptor has been shown to be present in these cells in previous studies (Tomokiyo, 2002). The fresh samples of liver and spleen were collected upon necropsy of an apparently healthy dog euthanized for reasons unrelated to the current study.

The western blot was performed on canine DH82 cells and tissue lysate from fresh canine liver and spleen that were treated with tissue protein extraction reagent (Thermo-Fisher, Rockford, IL) based on the manufacturer's directions. The DH82 cell line was treated with mammalian protein extraction reagent along with a protease inhibitor to extract out proteins. The Bradford method (Thermo-Fisher, Rockford, IL) was used to determine the protein concentration needed from the liver, spleen, and DH82 cell line in order to have equal amounts of protein. The proteins were separated on a 10% SDS polyacrylamide gel along with a molecular weight marker and then transferred to a nitrocellulose membrane. Following transfer to the membrane, the blot was blocked for 1 hour

at room temperature using tris-saline buffer containing 0.1% Tween 20 and 10% nonfat milk. Following this, the membrane was incubated with 40 microliters of the primary antibody for CD204 (SRA-E5; Transgenic Inc.) at -4 degrees Celsius overnight. The membrane was then washed with tris-saline buffer and incubated with a horseradish peroxidase-labeled secondary anti-mouse antibody. After incubation at room temperature for 3 hours, a chemiluminescence detection kit was used for visualization, and the blot imaged using a Kodak imaging station (Carestream Health, Rochester, NY).

Clinical cases: The database of the University of Missouri Veterinary Medical Diagnostic Laboratory was searched for cases from 2004-2014 with a previous diagnosis of sarcoma. Of the archived cases, 10 cases were then selected that had previously been diagnosed on histopathology with histiocytic sarcoma and 10 cases were selected that had previously been diagnosed with sarcoma. Prior diagnoses of histiocytic sarcoma were made based on morphology as well as positive staining for CD18 and negative staining for lymphocyte markers (Affolter, 2002). The described morphology for HS utilized in this study for diagnosis of histiocytic sarcoma includes large pleomorphic cells with pronounced cellular atypia and frequent multinucleation and bizarre mitotic figures as previously reported (Moore, 2014). Prior diagnoses of STSA were made based on morphology alone or morphology combined with a lack of CD18 staining. Cases were excluded if not enough tissue remained to repeat IHC

staining or the diagnosis was uncertain. Clinical data including age, weight, sex, breed, treatment pursued, and survival times were recorded if available.

All tissues were previously fixed in 10% neutral buffered formalin, processed, and embedded in paraffin wax. Paraffin-embedded tissues were sectioned into 4 μm slices for immunohistochemistry. A heat-induced antigen retrieval method was performed using Diva decloaker (Biocare; Concord, CA) and EnVision™ + system (Dako; Carpinteria, CA) for CD18, Iba-1, and CD3. For CD204 and CD79a, a heat-induced antigen retrieval method was performed using Borg decloaker (Biocare, Concord, CA). Information on the specific antibodies used can be seen in Table 1. A canine lymph node obtained during a necropsy of an apparently healthy dog euthanized for reasons outside of this study was used as a positive control; the same lymph node was also used as a negative control by omitting the primary antibody.

All of the slides were reviewed by a single pathologist (DYK) who was blinded to the prior diagnosis. Both cellular morphology and CD18 staining were used to confirm the prior diagnosis. The slides were evaluated for overall staining and semi-quantitatively scored by assessing the number of neoplastic cells staining positively. The following scoring system was used: 0 for 0-10% of cells staining positively, 1 for 10-25% staining positively, 2 for 25-50% staining positively, 3 for 50-75% staining positively, and 4 for > 75% of cells staining positively.

Table 1: Primary antibodies used in this study

	Antibody	Dilution	Source
CD18	Mouse monoclonal	1:80	UCD
CD204	Mouse monoclonal; SRA-E5	1:100	TransGenic
Iba-1	Rabbit polyclonal	1:1000	Wako
CD3	Rabbit polyclonal	1:400	DAKO
CD79	Mouse monoclonal	1:400	AbD Serotec

Statistics

Statistical analysis was performed using commercially available software (SigmaStat, Systat Software Inc.). To compare individual markers with diagnosis, the Shapiro-Wilk test was used to test normality assumptions. The Mann-Whitney rank sum test was utilized to compare IHC scores among the two groups (HS and STSA). A pairwise multiple comparison procedure (Holm-Sidak method) was used to compare the combined IHC score between HS and STSA groups. A p value of < 0.05 was considered significant.

Results

CD204 expression in the DH82 cell line: A western blot was performed to show expression of CD204 in the DH82 canine histiocytic cell line with fresh canine liver and spleen used as positive controls. The results showed weak expression of CD204 in the DH82 cell line lysate as seen in figure 1 with a light band at approximately 68 kDa.

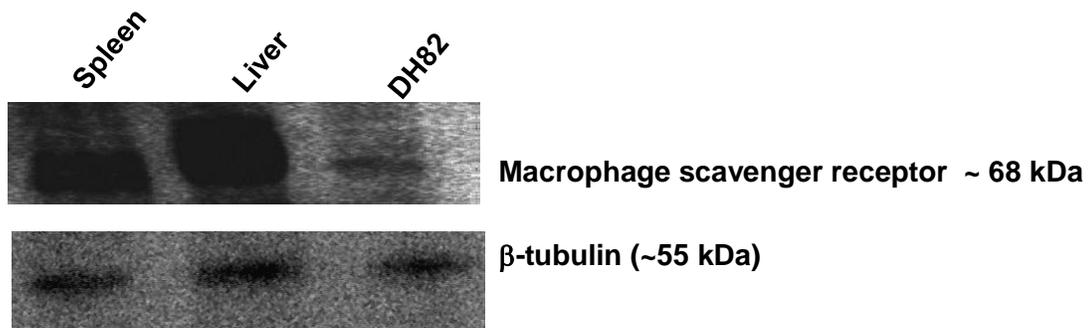


Figure 1: Western blot analysis using DH82 canine histiocytic cell line and fresh canine liver and spleen as positive controls. CD204 expression seen as a single band at approximately 68 kDa. Beta tubulin used a loading control.

CD204 and Iba-1 in the diagnosis of HS: Tissue samples from eight dogs with HS were evaluated. The median age was 10 years (range 2-14 years) and median weight 33.2 kg (range 6.7-47.4 kg). Breeds included Golden retriever (n=2), and one each of the following: Labrador retriever, Weimaraner, English springer spaniel, miniature schnauzer, bulldog, and Great Pyrenees. Tissue samples from nine dogs with soft tissue sarcoma were evaluated. The median age was 12 years (range 2-13 years) and median weight 20.2 kg (range 1.7 – 47.8 kg). Breeds included Siberian Husky (n=2) and one each of the following breeds: Cairn terrier, Beagle, Boston terrier, American Staffordshire terrier, Labrador retriever, Golden retriever, miniature poodle, and mixed breed dog. All of the dogs had surgery. 5/8 dogs with histiocytic sarcoma had adequate local

control defined as surgery with either complete or incomplete margins following by radiation therapy, and 6/8 had chemotherapy following surgery. 5/9 dogs with STSA had adequate local control and 4/9 were treated with chemotherapy. The median survival time available for dogs with HS was 259 days (5-600 days), and for dogs with STSA the MST was 446 days (3-1580 days).

Evaluation of CD204 staining revealed a median score for HS samples of 2, and similarly, the median score for STSA samples reviewed was 2 ($p=0.920$). Evaluation of Iba-1 showed a median staining score of 2.5 in HS samples and 2 in STSA samples ($p=0.015$). The median score for CD18 was 2.5 in HS samples and 1 for STSA samples ($p=0.088$). When combining IHC scores of the three stains, with the goal of using a panel of markers to better differentiated HS from STSA, the median score for HS was 8 and for STSA was 6 ($p=0.044$). The sensitivity and specificity of using Iba-1 alone with a cut-off of 2.5 for staining score of HS was 50% and 100% respectively. However, when using the combined IHC scores with a cutoff of 7.5 where a staining score greater than 7.5 was consistent with HS, the sensitivity and specificity were 75% and 100% respectively.

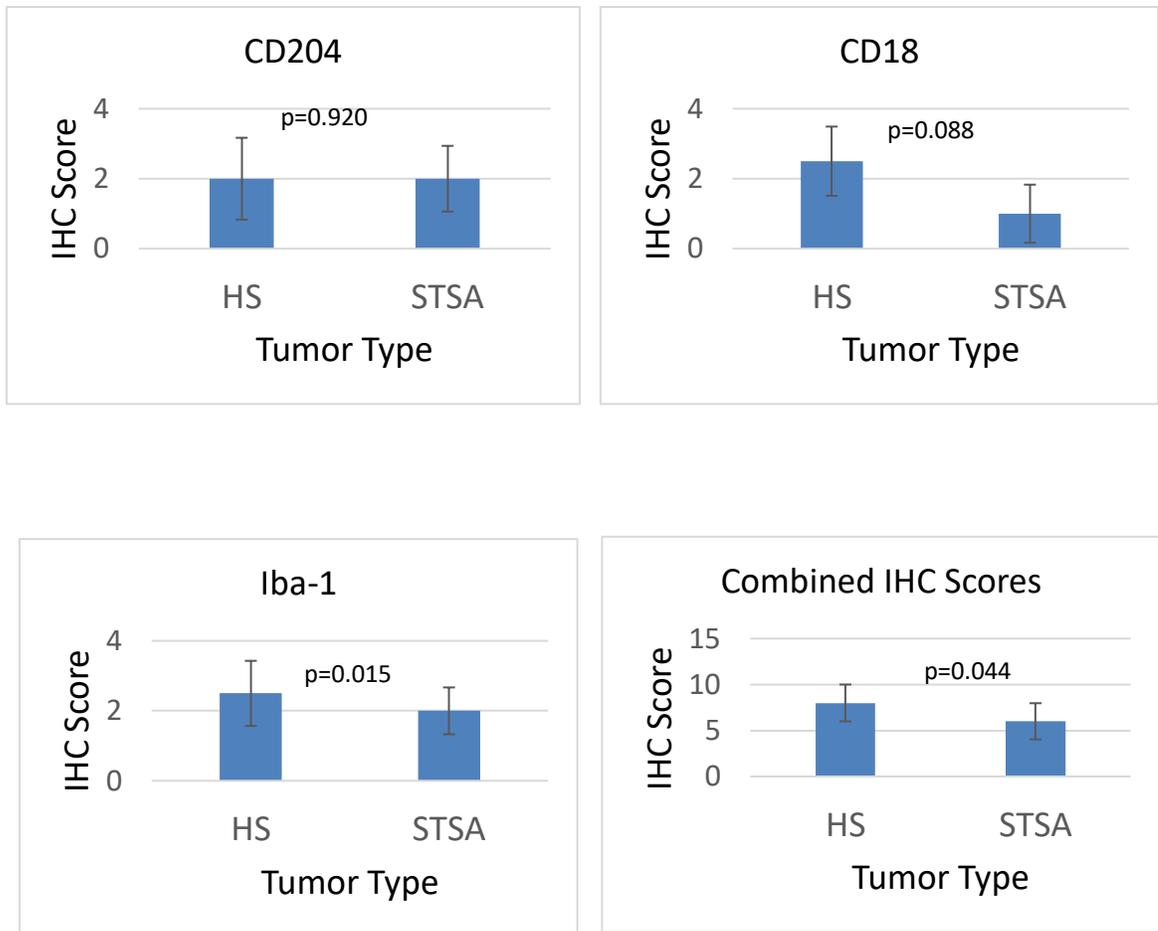


Figure 2: Immunohistochemistry score by stain and tumor type. There is no difference in CD204 or CD18 staining between HS and STSA. Iba-1 staining score is higher in HS compared to STSA, and the overall IHC score is higher in HS compared to STSA. Error bars denote standard deviation.

Discussion

Differentiating histiocytic sarcoma from other types of sarcomas in dogs, although vital to therapy and prognosis, remains a clinical dilemma. Ideally, fresh

frozen tissue biopsies would be submitted for immunohistochemical analysis and confirmation of tumor type; however, this diagnostic problem of HS versus STSA is often not discovered until after the microscopic review of formalin fixed samples. In addition, fresh frozen tissue is not regularly obtained during surgery at the majority of institutions, nor is this possible in most veterinary clinics where excision is performed, and therefore these samples are not available for analysis and definitive diagnosis of HS. Since HS is often not suspected until this initial histopathology is performed, it is important to discover a way to reliably differentiate between the two tumor types on formalin fixed tissue. Currently, due to cost, a single IHC stain is frequently requested to rule in or rule out histiocytic sarcoma. For example, CD18 negative tumors can be considered STSA, whereas CD18 positive tumors with the typical morphologic characteristics are considered HS. The recent development and availability of CD204 and Iba-1 antibodies has resulted in the increased clinical utilization of these stains as single markers to diagnose HS. However, no study has directly compared the two antibodies or evaluated their effectiveness in differentiating HS from STSA.

Given the difference in metastatic potential, chemotherapy recommendations, and prognosis, it is important to develop a clinically useful tool for the diagnosis of HS. The objective of this study was to confirm histiocytic cell expression of CD204 and to determine if a single stain or panel of stains could be utilized to reliably differentiate between HS and STSA in formalin fixed tissues. DH82 cell line expression of CD204 was confirmed and provided the basis for

investigating the IHC markers of CD204 and Iba-1 in canine formalin fixed HS and STSA. One single marker was not specific or sensitive for a diagnosis of HS. However, when multiple stains were used and subjective scores of staining combined, dogs with HS had a higher overall combined score compared to dogs with STSA. Given this data, we cannot recommend utilizing a single antibody for the diagnosis of HS in formalin fixed tissue in clinical practice. A combination of the studied stains including Iba-1, CD18, and perhaps CD204 may be more useful for differentiation of canine histiocytic sarcoma and soft tissue sarcomas.

Previous studies have compared CD204 and Iba-1 to tumor types that can be ruled out by other means (such as other round cell tumors or melanoma), but these are not helpful to the veterinarian in clinical practice faced with differentiating HS from STSA (Pierezan, 2014; Kato, 2013). Kato et al in 2013 found that CD204 was useful in differentiating HS from round cell tumors and from sarcomas such as melanoma, hemangiosarcoma, and alveolar rhabdomyosarcoma. While these tumors showed no staining for CD204 compared to positive CD204 staining in all HS samples, these sarcomas have specific IHC stains that can be utilized for their diagnosis and represent a different population than that studied here. Importantly, we showed that there was no difference in staining of CD204 between the HS and STSA, a population of tumors which is clinically imperative to differentiate and which often represent a diagnostic dilemma. Furthermore, due to expense, CD204 is commonly being utilized as a single IHC stain to differentiate HS from STSA. Since the STSAs

utilized in this study are representative of the tumor population from which we are clinically differentiating HS, the data shown here suggest CD204 alone is not a helpful diagnostic tool.

One limitation of this study is that the definitive diagnosis was obtained utilizing cell morphology, and, in the case of HS, cell morphology and CD18 positivity with negative lymphocyte markers, diagnostics based on previously published studies. While cases chosen were those that were confidently diagnosed by the pathologist, it is possible that cases represented as HS were truly STSA, and vice versa. Ideally, the evaluation of Iba-1 and CD204 in HS should be performed prospectively using tissues known to be histiocytic sarcoma based on fresh frozen evaluation and IHC for CD1a and CD11c. To overcome these limitations, one pathologist (DYK), who was blinded to the previous results, reviewed H&E slides for confirmation of the diagnosis in addition to review of the IHC slides. Another limitation is the small sample size evaluated, as a higher number of tissue samples may have resulted in a difference detected between the different stains.

In conclusion, despite current clinical use, a single IHC stain is not effective for differentiating HS from STSA in canine formalin fixed tissues. Given the differences in therapy and prognosis, this is an important dilemma to solve. A combination of IHC stains may be more appropriate than a single stain for the diagnosis of HS. This study provides the necessary rationale for a prospective study utilizing fresh frozen and formalin fixed tissues from the same tumor to

definitively evaluate the sensitivity and specificity of CD18, CD204, and Iba-1 in the diagnosis of canine HS.

Chapter 3: Cell Culture

Canine histiocytic sarcoma (HS) is a cancer of dendritic cells with a poor prognosis due to the frequency of disseminated disease and lack of promising treatment protocols. Currently, the treatment of choice for dogs with HS is single agent CCNU (Rassnick, 2010; Skorupski, 2007); however, even with this treatment the survival time is short. Several other treatments have been evaluated both in vitro and in vivo for dogs with HS, but none has significantly altered the clinical prognosis. Paclitaxel, liposomal doxorubicin, liposomal clodronate, vinorelbine, and lomustine have all been used clinically in dogs with HS (Wouda, 2015; Rassnick, 2010; Skorupski, 2009; Skorupski, 2007; Poirier, 2004; Vail, 1997). In vitro, a variety of compounds have been evaluated for efficacy against canine histiocytic cell lines including bisphosphonates such as liposomal clodronate, taxanes, microtubule inhibitors, and small molecule inhibitors (masitinib and dasatinib) (Asada, 2015; Ito, 2013; Thamm, 2012; Hafeman, 2012; Hafeman, 2010). While many studies have been performed to find a more promising treatment protocol for canine HS, very few have investigated multi-drug protocols. Thamm et al. in 2012 evaluated masitinib as a chemosensitizer to multiple cell lines including histiocytic sarcoma and showed that masitinib strongly sensitized cells to vinblastine. Hafeman et al. in 2012, also looked at the effectiveness of bisphosphonates to sensitize histiocytic cells to various chemotherapeutic agents. The only study to date looking at use of a multi-drug protocol for treating canine HS in vivo included alternating doses of

doxorubicin and lomustine for a median survival time of 185 days (Cannon, 2015).

Two drugs reportedly used as single agent treatments in canine HS, lomustine and vinca alkaloids, can be given in combination to dogs with minimal side effects (Fahey, 2011; LeBlanc, 2006) and are used frequently for treatment of refractory lymphoma. The primary aim of this study was to evaluate the effectiveness of vinblastine, vincristine, vinorelbine, and lomustine in vitro against canine histiocytic sarcoma and determine if a combination of these drugs would lead to additive cytotoxicity. The hypothesis was that an additive effect would be seen with combinations of vinca alkaloids and lomustine leading to increased apoptosis and decreased cell viability in the DH82 cell line.

Materials and Methods

The canine DH82 malignant histiocytosis cell line was purchased from ATCC. Cells were maintained with RPMI media containing 1 mM sodium pyruvate, 2 mM L-glutamate, 10 mM HEPES, 0.4 mg/mL gentamicin, and supplemented with 10% fetal bovine serum at 37 degrees C in an incubator containing 5% carbon dioxide. An Invitrogen™ Countess™ II cell counter (Thermo Fischer Scientific, Inc.) was used to determine the number of viable cells per milliliter of media, and approximately 5,000 cells per well were plated in a 96-well plate adding RPMI culture media for a total volume of 100 ul per well. The cells were incubated for 24 hours until confluence was achieved. Stock

solutions of lomustine and vinca alkaloids (Sigma Aldrich, St. Louis, MO) were prepared and stored in -20 degrees C. To make the stock solutions, lomustine was diluted with 25% DMSO solution and sterile distilled water, and the vinca alkaloids were diluted with sterile distilled water. The stock solutions were further diluted to make working solutions prior to adding various concentrations of each drug to the DH82 cells.

The cells were incubated at 37 degrees C and 5% CO₂ for 24 hours until they reached confluence, and each of the above mentioned chemotherapeutic agents was then added to the cells at varying concentrations and in combinations to evaluate cell viability, cytotoxicity, and caspase activity as described below. The lomustine was diluted to final concentrations of 2000 ng, 4000 ng and 6000 ng and each of the vinca alkaloids were diluted to final concentrations of 25 ng, 50 ng, and 100 ng. Lomustine was added at concentrations of 4000 ng and 6000 ng in combination with 100 ng of each individual vinca alkaloid to evaluate any additive effects of the drugs. The DH82 cells were incubated with the chemotherapeutic agents for 36 hours at 37 degrees C with 5% CO₂. Initially, data was collected on the 3 different concentrations of the vinca alkaloids for cell proliferation using a BrdU colorimetric assay (Sigma Aldrich, St. Louis, MO) according to manufacturers' instructions. The experiment was then performed on each of the vinca alkaloids, lomustine, and drug combinations using an ApoTox-Glo™ Triplex Assay (Promega; Madison, WI) to measure cell viability,

cytotoxicity, and caspase 3/7 activation through fluorescence and luminescence, respectively.

Results

The initial cell proliferation assay performed on the vinca alkaloids in DH82 showed a decrease in cell proliferation correlating with increasing concentrations of vincristine and vinorelbine. With vinblastine, a decrease in cell proliferation was seen in the high concentration compared to the low concentration of the drug; however, the intermediate concentration was not consistent (Figure 3).

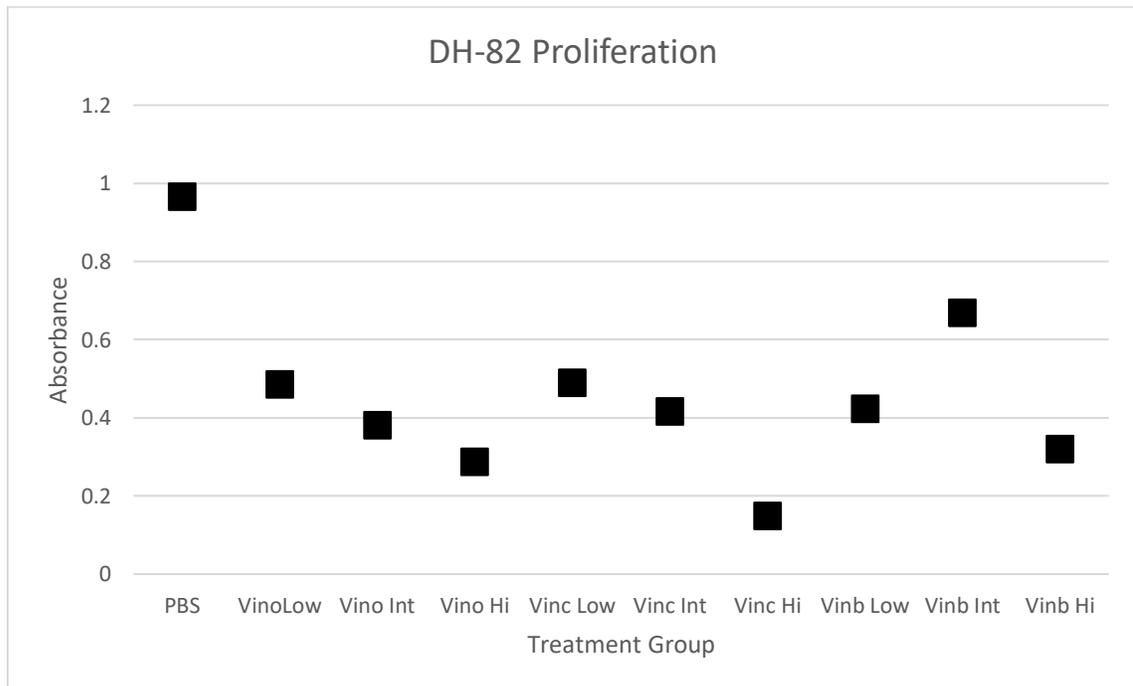
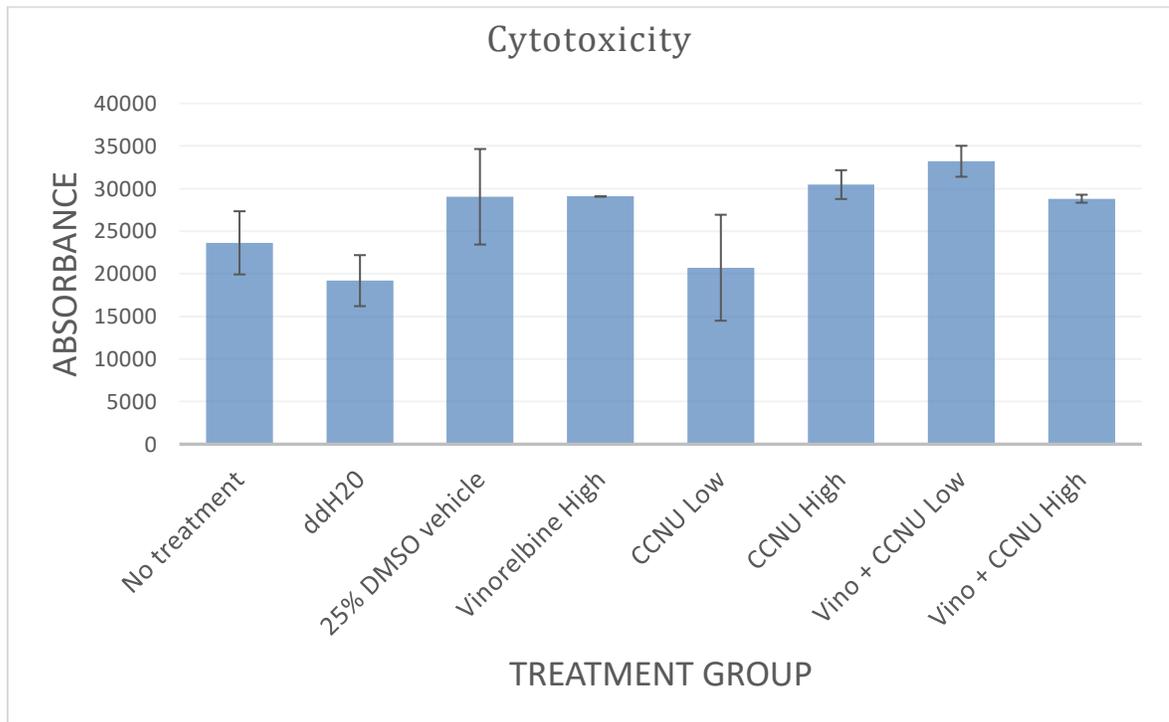


Figure 3: Cell proliferation assay in DH82 cell line

When the experiment was repeated using the Triplex assay, vincristine, vinblastine, and vinorelbine appeared to result in a concentration dependent increase in apoptosis and cytotoxicity as expected. When each vinca alkaloid was added at a concentration of 100 ng/ml to the low and high concentrations of lomustine, no consistent increase in apoptosis, cytotoxicity, and decreased cellular viability was seen with the drugs in combination compared to alone. When vincristine was combined at a high concentration (100 mg/ml) with the high concentration of lomustine (6000 ng/ml), a slight increase in apoptosis and cytotoxicity was seen compared to these concentrations alone; however, no apparent change was seen in cell viability (Figures 4-6).



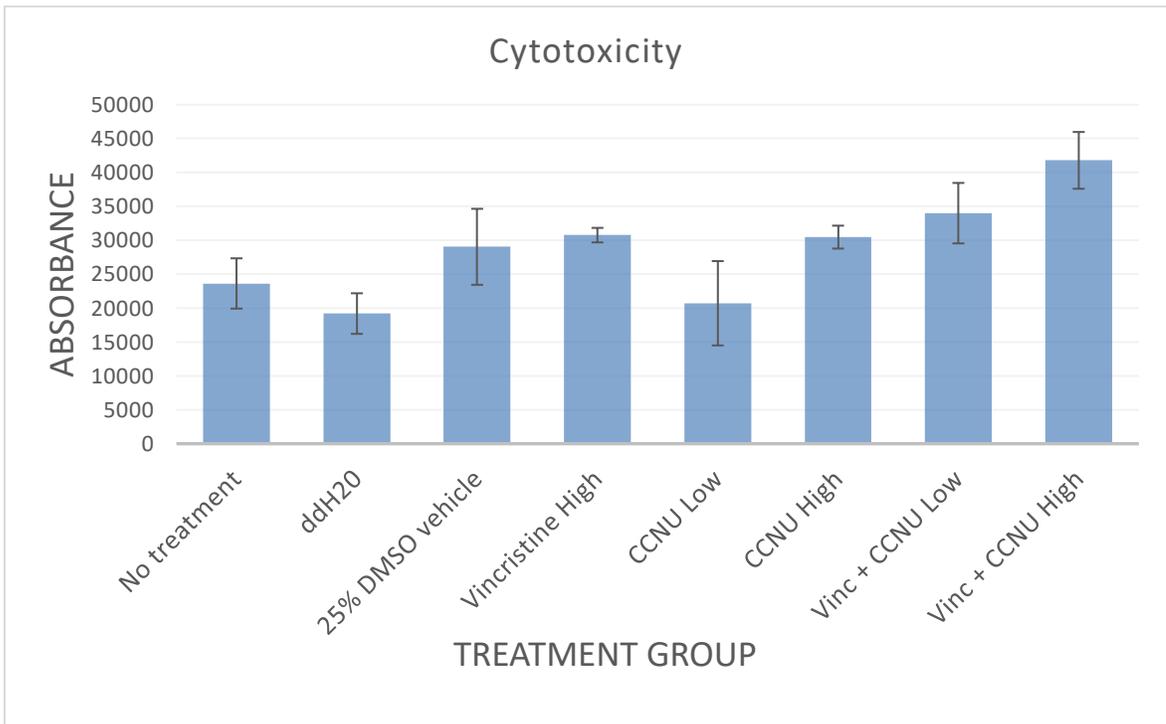
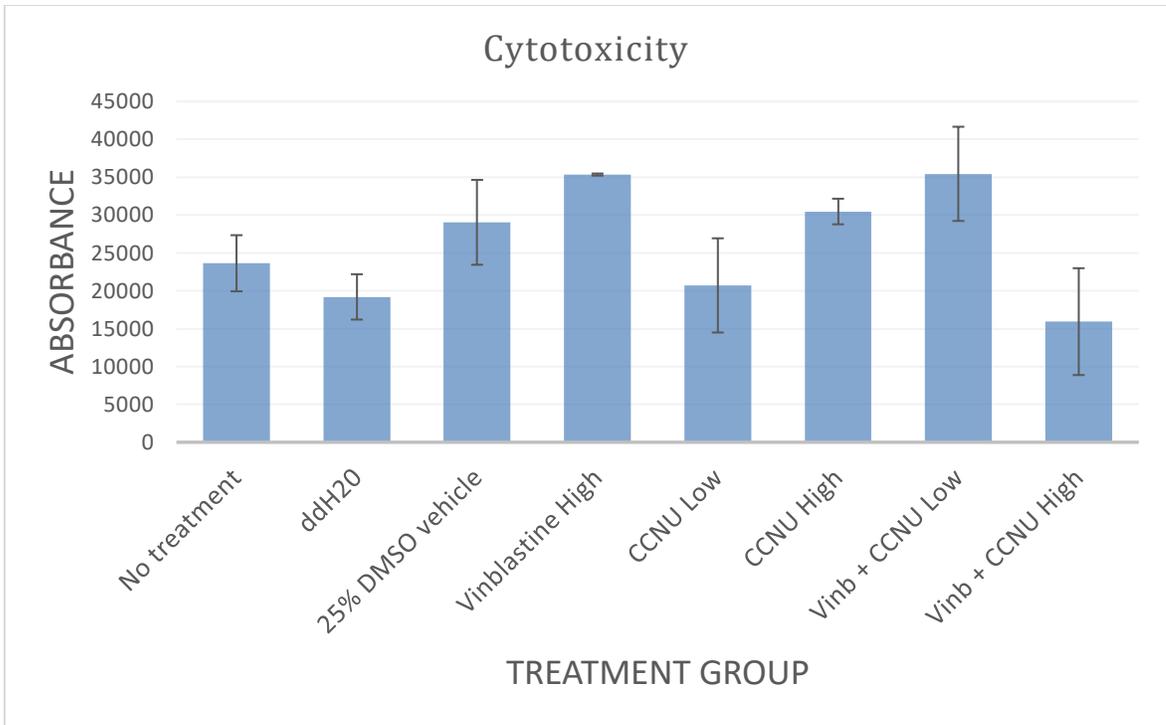
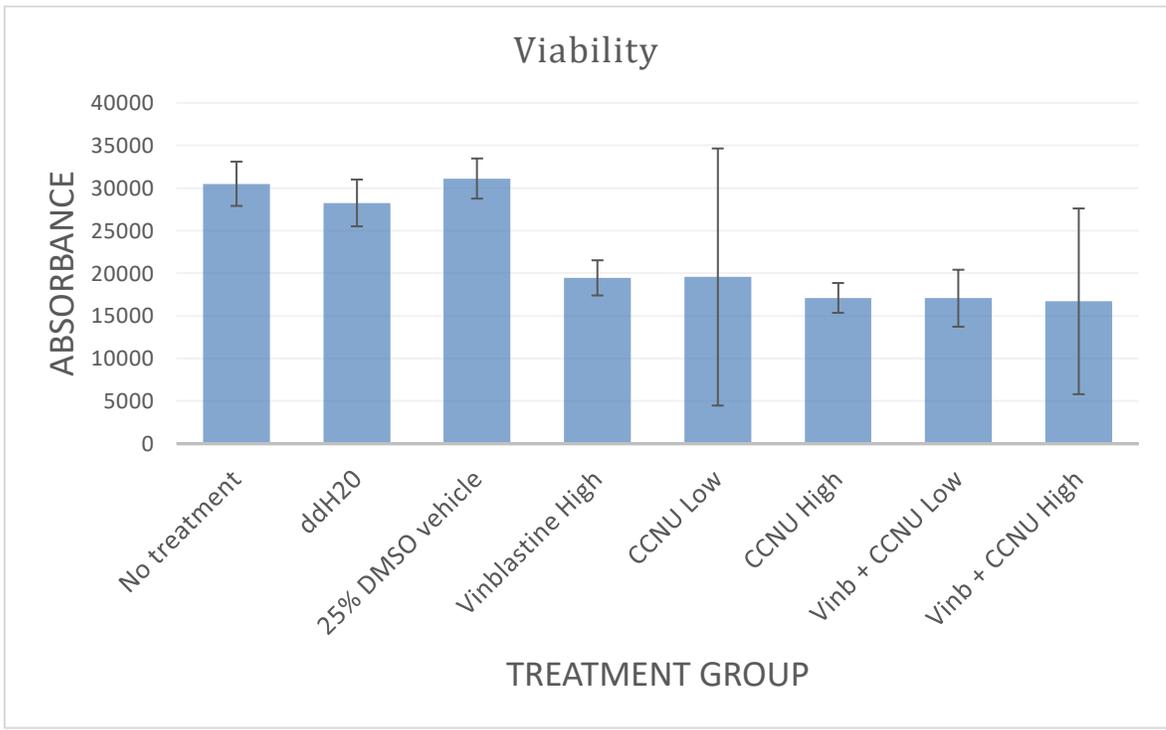
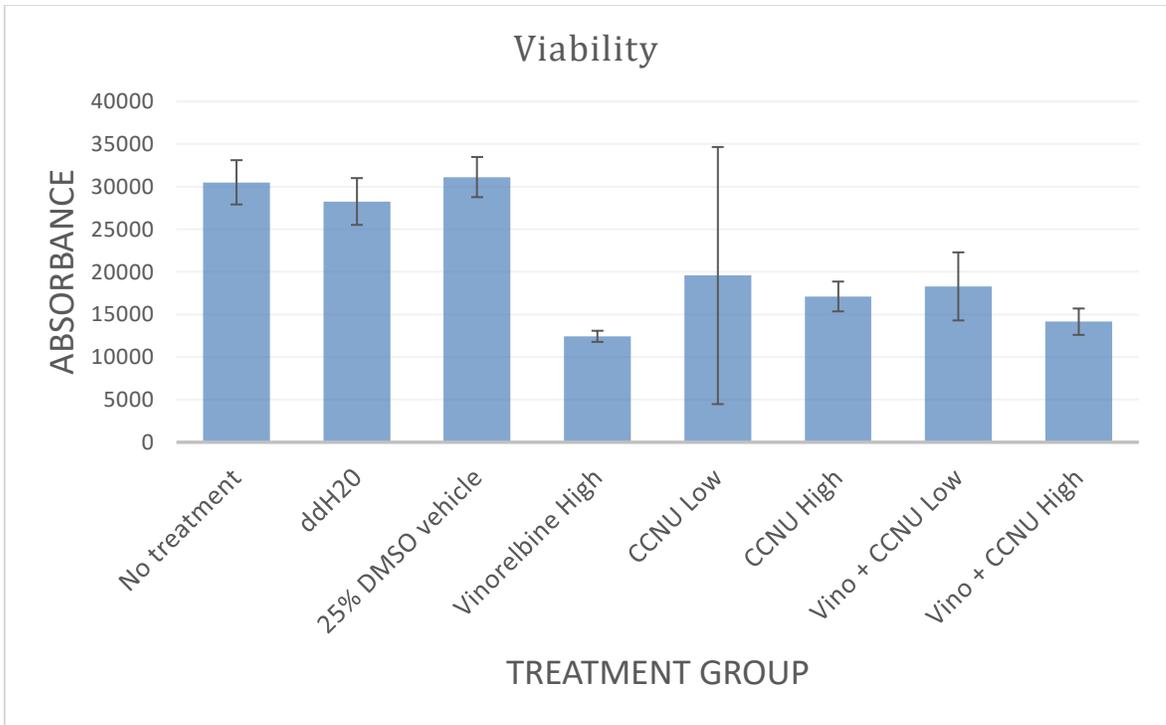


Figure 4: Cytotoxicity in the DH82 cell line



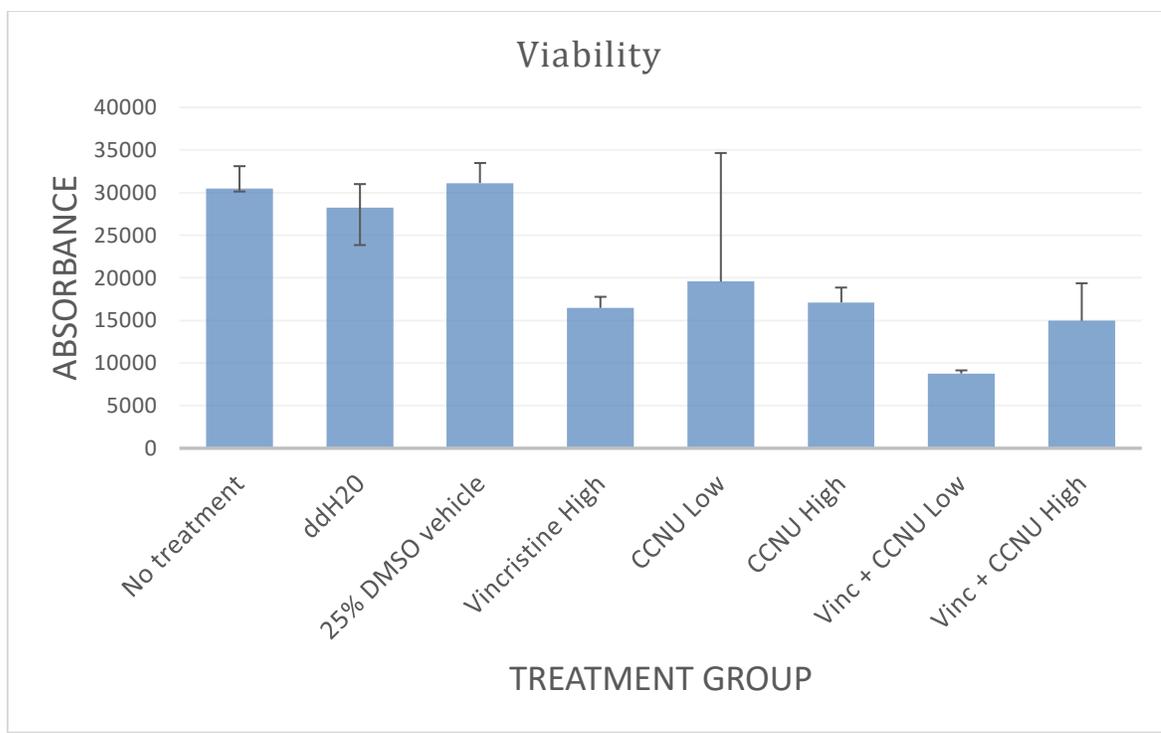
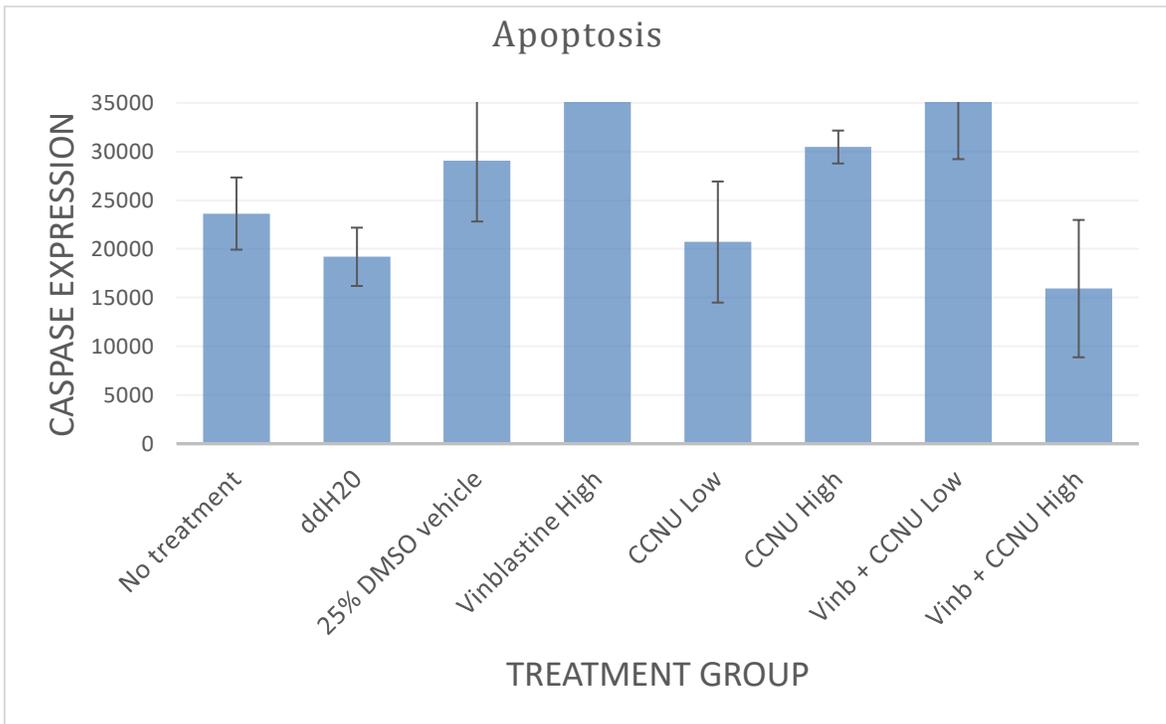
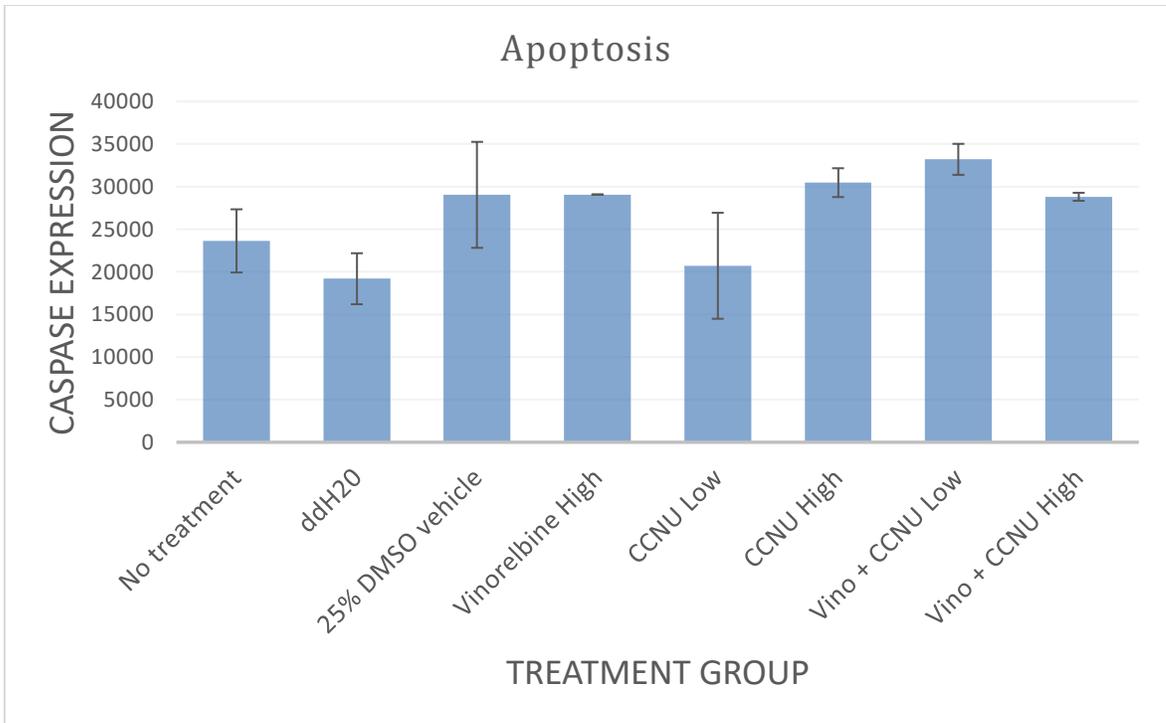


Figure 5: Cell Viability in the DH82 cell line



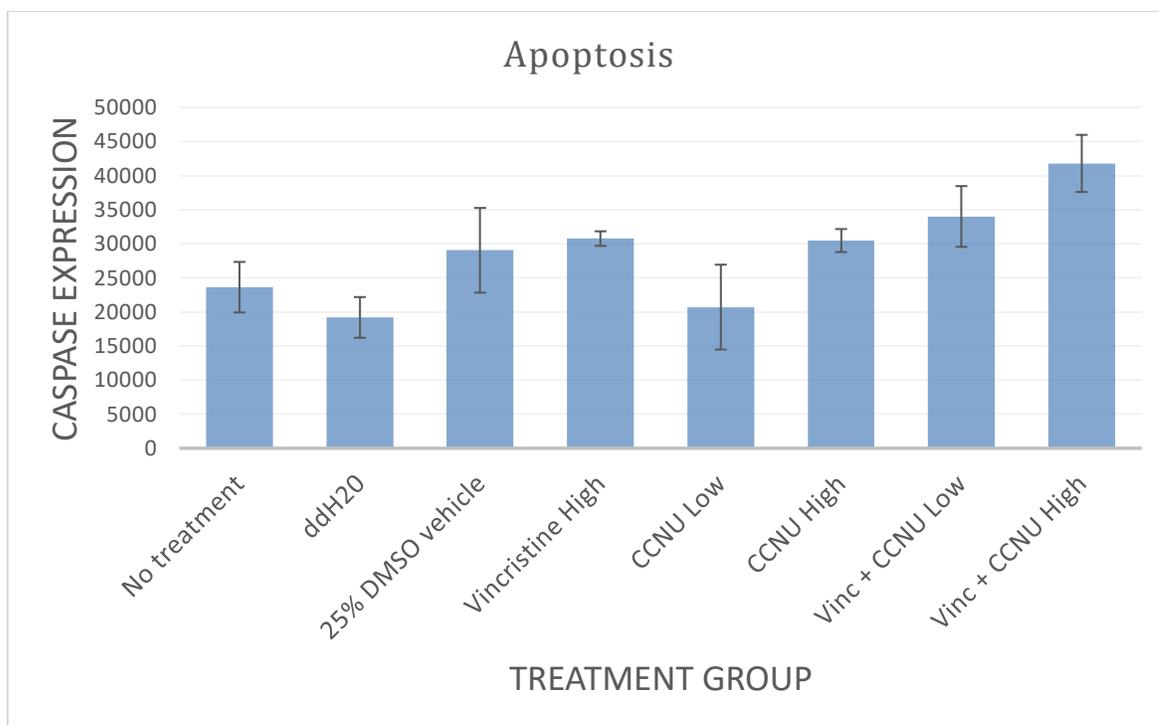


Figure 6: Apoptosis in the DH82 cell line

Discussion

The initial results of the cell proliferation assay showed that a decrease in proliferation was seen with increasing concentrations of the vinca alkaloids in the DH82 cell line. Similarly, a concentration dependent increase in apoptosis and cytotoxicity was seen with vincristine, vinblastine, and vinorelbine individually; however, when the vinca alkaloids were combined with lomustine, there was not an overall additive effect seen. Only the high dose of vincristine combined with the high dose of lomustine showed a slight increase in apoptosis and cytotoxicity compared to these drugs alone; however, no apparent change in cell viability was seen.

Overall, the results obtained in this experiment were not consistent. This is potentially due to error in pipetting both during the initial plating process leading to unequal numbers of cells in each well or potentially during the assay itself leading to variability in the results. Additionally, the drugs were plated in duplicates so that interpreting the variability of the results was difficult. Another possible cause for the lack of additive effects seen in vitro could be the use of lomustine as the parent compound rather than the active metabolites of the drug. In dogs, lomustine undergoes hepatic metabolism to trans-4-hydroxylomustine and cis-4-hydroxylomustine which are the active metabolites and reportedly have enhanced alkylating activity (Chakkath, 2014). A study in 2014 evaluated lomustine in vitro compared to its metabolites in canine lymphoma cell lines and found no difference in the cytotoxicity of the three compounds (Chakkath, 2014); however, the lack of use of active metabolites may have played a role in the decreased effectiveness seen in our experiment when combining lomustine and vinca alkaloids in vitro.

These data suggest that a combination treatment protocol of lomustine and vinca alkaloids may not be more effective than vinca alkaloids alone for HS. The concentrations of each vinca alkaloid and lomustine used in this experiment were selected based on previously published pharmacokinetic data available in dogs (Chakkath, 2014; Hantrakul, 2014; Niwa, 2011). This should allow for any results obtained in vitro to be translated to safely give to dogs in vivo without unacceptable toxicity. While no significant additive effects were seen in

combination of the drugs initially and in light of the notable variations in wells, additional studies performed in triplicate should be completed to confirm these results prior to clinical application.

Chapter 4: Biomarkers and monitoring

Thymidine kinase 1 (TK1) is a biomarker with growing potential in veterinary oncology both in diagnosing neoplastic processes as well as in monitoring remission status. Thymidine kinase 1 is a marker of proliferation as it plays a role in the synthesis of thymidine, a DNA precursor, and is specific to the S phase of the cell cycle (von Euler, 2011). With the dysregulated growth seen in cancer cells, an increase in the TK1 enzyme in the bloodstream is expected to correlate with diagnosis (Selting, 2016; von Euler, 2011). Similarly, a subsequent decrease in the value is expected to correlate with remission status and should be useful in monitoring for certain cancerous processes.

TK1 has been shown to correlate better with hematologic malignancies when compared to solid malignancies in dogs. TK1 has been evaluated in dogs with lymphoma as well as several occult types of cancer including hemangiosarcoma (Selting, 2016; Thamm, 2012). In lymphoma, it has been shown to increase with the severity of disease as well as decrease in states of remission (Elliot, 2013; Sharif, 2012). In 2015, Selting et al. evaluated TK1 and CRP as useful biomarkers when screening dogs for occult disease and determined that these could be combined in a proprietary dual algorithm created to moderate the impact of one biomarker as part of a panel called neoplasia index. Further studies utilizing these biomarkers showed that TK1 and the neoplasia index were increased in the dogs with cancer and were the highest in dogs with lymphoma, hemangiosarcoma, and histiocytic sarcoma (Selting, 2016).

The primary goal of our study was to evaluate TK1, C-reactive protein, and neoplasia index in dogs with a diagnosis of histiocytic sarcoma and to determine if a correlation was seen when monitoring biomarkers and response to treatment. The hypothesis was that TK1 and neoplasia index would be increased at baseline in dogs with histiocytic sarcoma and a decrease would be seen in serial monitoring in those patients who responded to therapy.

Materials and Methods

Cases were recruited with a diagnosis of histiocytic sarcoma made either on cytology or histopathology from multiple institutions. The cases selected had a baseline serum sample collected as described below and serial samples were collected at 3-4 weeks following treatment. Treatment allowed included surgery, radiation therapy, and/or chemotherapy at the treating clinician's discretion. Cases were excluded if a diagnosis other than histiocytic sarcoma was made. Serum was collected by venipuncture (jugular or peripheral vein) at time points described above using plain red top or serum separator tubes. Serum was allowed to clot for 30 minutes, then spun at a minimum of 1500g for 10 minutes. Within 30 minutes of collection, serum was withdrawn from the tube, transferred to cryotubes provided, and frozen at minus 20°C until shipment. Serum was then shipped on ice to VDI Lab using the shipping package provided. Assays for TK1 and CRP were performed at VDI Laboratory as previously published (Selting,

2016; Selting, 2015). A Neoplasia Index was also determined on each sample based on previously determined algorithms (Selting, 2016; Selting, 2015).

Results

Samples from 19 dogs with a diagnosis of histiocytic sarcoma were obtained. The median age was 8 years (range 4 to 12 years). There were 12 castrated males, 6 spayed females, and 1 intact female. Breeds represented included 4 Golden retrievers, 3 Bernese Mountain dogs, 3 Australian Shepherds, 2 Rottweilers, and 1 each of the following breeds: Labrador retriever, Flat Coated retriever, Bull Mastiff, Bulldog, Great Pyrenees, German Shepherd Dog, and Scottish terrier.

Of the 19 dogs, 11 (57.9%) were diagnosed on cytology alone and 8 (42.1%) were diagnosed on histopathology and IHC. CD18, Iba-1, and CD204 were all used for diagnosis on histopathology depending on the pathologist. Nine (47.4%) dogs were considered to have disseminated histiocytic sarcoma, 4 (21.1%) had HS localized to the lungs, 2 (10.5%) had hemophagocytic HS based on laboratory abnormalities and cytologic abnormalities, and 4 (21.1%) had localized HS.

Fifteen (78.9%) dogs were treated with either surgery, radiation therapy, chemotherapy, or a combination of these. Four dogs (21.1%) did not elect to undergo any therapy. Of those receiving treatment, 5 dogs were treated with surgery, 2 were treated with radiation therapy, and 12 received various chemotherapy agents. For the dogs treated with radiation therapy, one dog

underwent a fractionated course of treatment following incomplete surgical resection and the other received radiation as treatment prior to chemotherapy with lomustine. Lomustine was the most commonly used chemotherapy drug with dogs also receiving doxorubicin, vinorelbine, Palladia, zoledronate, cyclophosphamide, and dasitinib. Additional medications that patients were receiving included prednisone, Denamarin, levothyroxine, meloxicam, carprofen, and tramadol.

The median TK1 for dogs with HS at baseline was 8.2 U/L (range 2.1 U/L to 61.9 U/L), the median CRP at baseline was 13.5 mg/L (range <0.5 mg/L to > 50 mg/L), and the median neoplasia index was 8.2 (range 1.8 to 9.9). While the median TK1 results for the dogs with histiocytic sarcoma were at the high end of the equivocal range (2.0 U/L - 8.9 U/L), 11 samples came back in the high range (> 9.0 U/L). The median neoplasia index at baseline was in the high range for dogs with HS. 8 of the patients received follow up samples as monitoring of their biomarkers during treatment; however no correlation could be made between these results and response to treatment due to small sample size and most only having 1 follow-up sample.

Table 2: Baseline TK1, CRP, and Neoplasia index (NI) results in dogs with HS

Baseline results	Normal values
TK1: Median 8.2 U/L (2.1 to 61.9 U/L)	Normal: <1.9 Equivocal: 2.0-8.9 High: >9.0
CRP: Median 13.5 mg/L (< 0.5 to > 50 mg/L)	Normal: < 3.9 Low Inflammation: 4-9.9 Moderate Inflammation: 10-39.9 High Inflammation: > 40
NI: Median 8.2 (1.8 to 9.9)	Negative: < 5.3 Equivocal: 5.3 Positive: 5.4-8.9 High Positive: > 9.0

Discussion

In this study, we evaluated thymidine kinase, C-reactive protein, and the combined neoplasia index as biomarkers in canine histiocytic sarcoma and monitored them during treatment of patients receiving therapy to determine if any correlation was seen with treatment response. During the study period, only 19 patients with HS were enrolled with 8 of these patients receiving multiple biomarker samples. The results of our study showed that the median TK1 and NI at baseline diagnosis were 8.2 U/L and 8.2 respectively. This was similar to the findings in previous studies where patients with lymphoma, hemangiosarcoma, and histiocytic sarcoma had the highest biomarker values (Selting, 2016).

Only 8 of the patients had follow-up serum samples monitored during treatment and of these patients, many only had 1 additional sample obtained after baseline. In one of these patients, the TK1 and NI increased from baseline

to 3-week recheck and this correlated to a decline in the patient clinically prior to euthanasia elected by the owner. In another patient, the biomarker values slowly increased over time which correlated with progressive disease despite treatment with a variety of chemotherapy agents. Three dogs had biomarkers which were in the equivocal range at baseline and remained stable throughout monitoring. Two dogs had clinical remission of their disease and both the TK1 and NI decreased on follow-up biomarkers. However, due to the small sample size and minimal follow-up samples obtained during treatment, we determined that no conclusion could be made as to the utility of TK1, CRP, and neoplasia index in monitoring dogs with HS during treatment.

There are obvious limitations to this study due to the small sample size and limited number of patients who underwent treatment. Of those patients who underwent treatment, the follow-up monitoring was not standardized and some patients were lost to follow-up making evaluation of remission status difficult. Another limitation was that not all patients were diagnosed the same way with 11 (57.9%) diagnosed on cytology alone and the remaining 8 diagnosed on histopathology but with different pathologists and therefore differing immunohistochemistry that had been performed.

Despite these limitations, TK1 and NI were increased at baseline in patients with histiocytic sarcoma similar to previous studies. This gives us evidence that these biomarkers could be used as potential screening tests in patients and potentially breeds that are predisposed to histiocytic sarcoma.

Given the small number of patients with serial biomarker samples submitted and the variety in results, no conclusion can be made at this time as to the utility of these biomarkers in monitoring remissions status in dogs with HS.

Chapter 5: Conclusions and Future Directions

Despite extensive research, canine HS remains a difficult disease to diagnose, treat, and monitor. When facing the challenge of diagnosing HS on biopsy, immunohistochemistry is often needed to differentiate HS from other types of sarcomas. Currently, there is no single specific marker unique to dendritic cells in dogs that can be used on formalin fixed tissue. With CD206 also unable to be validated for use in canine tissue and showing lack of staining on formalin fixed tissue, we further evaluated CD204 and Iba-1 for differentiating HS and STSA. In the tissue samples we selected that were previously diagnosed as either HS or STSA, there was no difference in IHC staining scores seen between the tumor types with CD204 or CD18. A significant difference was seen on IHC scores between the tumor types with Iba-1 and when the scores were combined. These findings suggest that the current clinical use of CD204 or Iba-1 alone for diagnosing HS may lead to a misdiagnosis. The result of this misdiagnosis could result in inappropriate prognostic information being relayed to an owner regarding their pet and suboptimal therapy being given. Our results suggest that a panel of markers would be more ideal for diagnosis of HS.

Treatment of dogs with HS continues to be unrewarding and no significant improvements have been made in new treatments for these dogs. Multidrug chemotherapy protocols are superior for treatment over single-agent protocols; however, treatment of dogs with HS still consists of using various drugs, most commonly lomustine, in a single agent protocol. Theoretically, a combination

protocol of two drugs which have shown effectiveness against HS, lomustine and vincristine or vinorelbine, should have improved effectiveness when combined. However, when lomustine was combined with various vinca alkaloids in vitro in this study, no additive effects were seen. Given the limitations and variability in the preliminary study performed here, additional in vitro studies are warranted and should be performed on additional histiocytic cell lines to determine the utility of combining these drugs into a useful treatment protocol for HS.

When evaluating the usefulness of biomarkers in dogs with HS and their correlation with response to treatment, no conclusions were able to be drawn due to the small sample size. Despite this, an increase in TK1 and neoplasia index were seen at baseline in dogs with HS showing that it has a potential role in screening predisposed breeds and other clinically healthy dogs, and if increased would support a recommendation for further testing. Additionally, a larger sample size may discover correlations between the biomarkers and response to treatment. Overall, the studies performed here provide important background data for future, larger studies to help better diagnose, monitor, and treat a disease with a devastating prognosis.

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