DISSERTATION

USE OF LIPOSOMAL BISPHOSPHONATES TO DEPLETE MACROPHAGES FOR CANCER IMMUNOTHERAPY

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ABSTRACT

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In order for continued growth, metastasis and evasion from immune surveillance, tumor cells are dependent on a complex matrix of supportive cells and tissues. These cells make up a significant percentage of the tumor mass and contribute to the hallmarks of malignancy. Of these, the tumor associated macrophage (TAM) has perhaps the most diverse role. In the majority of tumor types studied, increased percentages of these cells in the tumor correspond to a poorer prognosis for the patient. Macrophages are critical in wound healing, and as such provide a wide variety of factors that may be co-opted by the tumor to support its continued growth and metastasis.

Macrophages are capable of producing a wide variety of growth factors that directly promote tumor cell growth. These factors can also be used to induce tumor cell migration and invasion, which are critical steps in metastasis. They also produce matrix metalloproteinases that actively degrade basement membranes, further aiding in invasion and metastasis.

Macrophages also produce many factors that help induce angiogenesis, providing vital blood supply to the developing tumor. Through both direct and indirect mechanisms they are vital to providing new tumor blood vessels.

In addition to these direct tumor aiding effects, macrophages also play a critical role aiding in both local and global immunosuppresion in tumor patients, which allows the established tumor to continue to evade the immune system. Therefore, the targeting and killing of TAMs could potentially be a promising new adjunct to traditional cancer therapies, and may increase the efficacy of traditional therapeutics.

One potential drug for this purpose is liposomal clodronate. This drug is produced by encapsulating the bisphosphonate drug clodronate in a liposome. As a free drug, clodronate is very effective at inducing apoptosis of osteoclasts, a close relative to the macrophage. Encapsulation in a lipid bilayer prevents the dissemination of the drug to the bone matrix and instead allows for systemic distribution. However, only cells that phagocytize and degrade the lipsome are susceptible to killing by the enclosed clodronate. Liposomal clodronate has been used extensively to deplete macrophages in studies of autoimmune disease and more recently in tumor models. However the systemic depletion of tumor associated macrophages using liposomal clodronate (LC) has not been previously evaluated in clinical trials, and the effects of systemic LC administration on tumor growth have not been fully elucidated.

Studies presented here sought to further determine the role of tumor associated macrophages in tumor growth by studying the effects of their depletion. Specifically, in vitro studies were used to determine an optimal formulation of liposome to more effectively deliver the bisphosphonate drug to macrophages. Using multiple murine

macrophage cell lines and proliferation assays the most effective depleting liposome was determined. This formulation consisted of a net neutral charged phosphatidylcholine head group combined with an incorporated mannose group. These liposomes were then evaluated in vivo for their ability to deplete macrophages systemically. Once again, the modified liposome formulation was most effective. The drug was then evaluated for its ability to decrease tumor growth in a mouse fibrosarcoma model, using MCA 205 tumors subcutaneously implanted into C57BL/6 mice. The drug's ability to deplete tumor associated macrophages was also evaluated. Tumor growth rates and tumor associated macrophage numbers were significantly decreased in mice treated with liposomal clodronate as compared to untreated mice or those treated with liposomal PBS.

Additional studies were undertaken to determine if liposomal clodronate could be used as an effective cancer therapeutic in a spontaneous tumor model. The tumor evaluated was malignant histiocytosis (MH). This tumor was chosen as it is a tumor derived from macrophages or dendritic cells, and LC could potentially have both primary anti-tumor effects as well as efficacy due to depletion of TAMs. In vitro studies were undertaken which showed that LC was capable of effectively killing MH cells. Based on these results, a clinical trial was conducted for dogs with MH. Dogs were treated with 0.5 mL/kg of liposomal clodronate IV every other week for six treatments. A total of 12 dogs were treated in the study. Treated dogs were evaluated for tumor response, changes in circulating blood cells, and changes in circulating cytokines. We were able to observe a 40% biologic response rate (BRR). The development of a fever was positively correlated with response. Responding dogs also had an increase in neutrophils and a

decrease in monocytes while non-responding dogs did not. A significant reduction in serum Il-8 levels occurred post LC treatment.

As the clinical availability of LC is currently limited to experimental use additional studies were conducted to determine if combining free bisposphonates, which are readily available, with traditional chemotherapeutics could cause synergistic killing of MH cells in vitro. The combination of clodronate with vincristine or zoledronate with doxorubicin demonstrated synergistic killing in vitro. Further evaluation of these combinations will be necessary to determine if they have a similar effect in vivo.

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Chapter One

Literature Review and Project Rationale

Overview of inflammation and cancer

The study of the links between inflammation and cancer date back nearly as far as the study of cancer itself. In 1863, Rudolf Virchow first identified leukocytes in tumor tissues[1]. This initial observation led to the hypothesis that tumorgenesis and inflammation were somehow linked. This idea did not immediately catch on, and in fact for much of the next 150 years this observation was largely discounted or ignored. However, the study of inflammation and cancer has intensified significantly in recent years. This has led to significant gains in the understanding of the interaction between non transformed immune cells in the tumor stroma and the tumor cells. This interaction heavily involves cells of both the innate and acquired branches of the immune system. In fact a large amount of scientific evidence would now suggest that the interplay between immune cells and tumor cells in the tumor microenvironment is vital to the successful growth and spread of many types of tumors.

A role for inflammatory cells can also be found in each step of the tumor growth pathway. The targeting of these supportive cells in the tumor microenvironment alone or in addition to the direct targeting of tumor cells may therefore represent an intriguing new

avenue for cancer therapeutics. The following pages will specifically address the current understanding of these complex interactions between immune and tumor cells, and show how specific targeting of even one of these immune cell types could potentially lead to a new cancer treatment drug.

The association of inflammation with tumor initiation and promotion

The most recent studies in cancer epidemiology continue to demonstrate that the vast majority of tumors arise from somatic, and not germline mutations[2]. These mutations can be caused by a large number of environmental factors. Of these, many of the most common causes such as smoking (30% of cancer deaths), obesity and dietary (20%) and infectious agents (10-15%) have at least some of their tumorgenic potential due to chronic inflammation[3]. This link was perhaps first established in the study of chronic inflammatory diseases and an associated increased risk in tumors in the affected organ. The chronic inflammation associated with inflammatory bowel disease, ulcerative colitis, and Crohn's disease leads to an increased risk of colorectal cancer in affected patients[4-6]. By the same token, chronic obstructive pulmonary disease (COPD) can increase the risk of lung cancer[7].

Further evidence for the role of inflammation in tumor promotion has been extensively studied in the realm of infectious disease, where it is well established that the chronic inflammation associated with chronic infection can result in the development of a tumor in the infected organ system. Examples of this phenomenon include schistosomiasis causing transitional cell carcinoma of the bladder, human papillomavirus

causing cervical cancer, *Helicobacter pylori* causing both gastric carcinoma and MALT lymphoma, and hepatitis B and C virus causing hepatocellular carcinoma[1, 8, 9].

This inflammatory link can be seen even in disease processes where the inflammation is not directly due to an underlying pathogen or autoimmune condition. Obesity has been shown to be a proinflammatory process, and obesity is linked with an increased risk of developing cancer[10, 11]. At least one recent study has now shown a direct link between increased inflammation induced by obesity and the development of hepatocellar carcinoma in a mouse model[12]. Studies such as this suggest that there may be even more inflammatory links to chronic diseases and cancer development that have not yet been identified.

Inflammation can potentially influence tumorgenesis through each step of the tumor development pathway. The first step of the pathway is tumor initiation, the development of enough mutations for tumor cells to start dividing unchecked. This process likely requires at a minimum two mutations, and in most cases probably four or more in order to transform cells[13]. Therefore any process causing DNA damage could potentially increase the risk of this transformation. One of the major weapons of the immune system is the ability of cells to produce free radicals in order to kill pathogens. These include reactive oxygen and reactive nitrogen species. However in areas of chronic inflammation, both of these substances have the potential to cause DNA damage to the surrounding tissues and thereby may help promote initiation[14].

While induction of initiation is a potential mechanism for chronic inflammation's impact on tumorgenesis, more recent work has focused more on the role of the immune system in tumor promotion. This is the process whereby a transformed cell is able to

survive and proliferate, thus forming the early cancerous tumor. This may be an area where an inflammatory environment may be particularly beneficial for the developing tumor.

For example, while smoking contains multiple carcinogens that contribute to tumor initiation, recent work suggests that the promotion phase of tumor development caused by cigarette smoking is induced primarily by the chronic inflammation induced by repeated exposure to these inhaled irritants. The myeloid cells present in these inflammatory areas are able to directly stimulate and increase tumor cell proliferation[15]. This may be the case in chronic infections as well. It has been shown that chronic damage to hepatocytes by hepatitis C virus stimulates Kupffer cells, the myeloid cells of the liver, to produce growth factors that stimulate hepatocyte proliferation[16]. It therefore seems possible that myeloid cells in chronically inflamed tissues are primarily involved in the promotion of transformed cells, which may pave the way for the establishment of a tumor. The mechanisms by which these cells may initiate tumor promotion will be detailed in the following pages.

Immunoediting of tumors

Even those tumors that are not directly induced by the immune system due to effects on initiation or promotion still likely had to at one point escape from immune surveillance. Like the hypothesis of immune involvement in tumor progression, this is another idea formulated by a great early biologist that initially fell out of favor, but has developed a recent resurgence. This particular theory was first proposed by Paul Ehrlich

in 1909, when he postulated that a large amount of carcinomas likely arise during a lifetime but nearly all are repressed by the immune system[17]. Others argued that only virally induced tumors would be subject to this immune surveillance, and that tumors that arise from other carcinogens would not be subject to recognition by the immune system.

Ehrlich appeared to be proven incorrect when in 1974 Osias Stutman and others were able to show that nude mice did not form more tumors or form tumors faster than wild type mice when exposed to the same carcinogen[18]. While this finding nearly led to the abandonment of the idea that tumors are under immune surveillance, it had some flaws which have only recently been exposed. Chief among these is the knowledge that while nude mice do lack thymic developed T cells, they do have some functional T cells as well as other cells such as NK cells and γδ T cells that potentially may be capable of anti-tumor immunity[19, 20]. Better mouse models were therefore necessary to truly test this hypothesis.

Those mouse models are now available, and they have shown that immunodeficiencies do lead to an increase in the incidence of tumors from non-viral sources. The first model to show this was using knockout mice, specifically those lacking the recombination activating gene 1 (RAG-1). Mice lacking this gene are completely deficient in B cells, T cells, and NK cells, which circumvents the problems encountered with nude mice[21]. Tumor studies in these mice have shown them to be three times more susceptible to tumor formation by carcinogens and that they have a higher rate of spontaneous tumor formation than wild type mice[17]. Similar results have been found in mice with specific knockouts or antibody ablation of specific immune

components responsible for anti-tumor cytotoxicity such as NK cells, interferon γ, and perforin among others[17].

While these findings from mouse studies suggest that a fully functional immune system is required to prevent spontaneous tumor formation, they are still only a model of tumorgenesis in humans. In order to fully validate this theory, evidence is required from human oncology as well. It does appear that either primary or acquired immunodeficiency leads to an increased risk in the development of cancer[17]. However, this phenomenon could potentially be due to an increased risk of acquiring an oncogenic virus and not due to the same immunosurveillance mechanisms studied in mice. This does occur, as AIDS patients and organ transplant recipients, both of whom are severely immunosuppressed, have a much higher rate of rare, virally induced tumors such as the herpesvirus induced Kaposi's sarcoma, Epstein-Barr virus induced lymphoma, and human papillomavirus induced carcinomas[22].

Studies in organ transplant patients have also shown an increased risk of solid tumors not associated with viral infection in these immunosuppressed patients. Heart transplant patients have a higher incidence of lung cancer and skin tumors than the general population[23]. Another study looking at all transplant patients showed a 100 fold increase in cancer risk in transplant patients, and spontaneous regression of some tumors with cessation of immunosuppressive therapy [24]. A large study of renal transplant patients was able to demonstrate a 2-5 fold increase in risk of development of colon, lung, bladder, and prostate cancer[25]. These studies would suggest that increased cancer risk is not only due to increased risk of viral infection in these patients but also due to an

underlying defect in the ability of their immune systems to detect and destroy early tumors.

As Dunn et. al. suggest there are likely three distinct phases to immunosurveillance. The majority of tumors that arise are likely subjected to recognition and complete elimination by the immune system. Some tumors are however able to progress to an equilibrium phase where they are constantly under attack from the immune system but are not completely cleared. This allows these tumors to continue to mutate and for surviving clones to be selected based on their ability to survive immune attack , perhaps acquiring the ability to produce anti-inflammatory cytokines or other immunosuppressive functions in a model of Darwinian evolution at the cellular level. This allows for the third phase, which is escape of the tumor from immune surveillance and subsequent rapid growth and spread of the tumor[17]. As any large tumor has achieved escape, any immunotherapy will be required to first reverse the inherent immunosuppresion present in the tumor to be effective.

Anti-tumor effects of the immune system

The majority of this text will focus on the escape phenomenon and the innate immune response present during this phase of tumor progression. However it is important to realize the critical role the immune system plays in tumor recognition and destruction during the elimination phase of tumor immunosurveillance as these cells could potentially be recruited to combat the tumor if the immunosuppression present in the escape phase of tumor development could be reversed.

The initial growth and invasion of tumor cells causes local tissue destruction which leads to recognition by innate immune cells such as gamma delta T cells NK T cells and NK cells[17]. These cells produce interferon gamma, which serves to activate the immune system via the production of pro-inflammatory cytokines and the recruitment of additional innate immune cells such as macrophages and dendritic cells in addition to additional NK cells[26]. The NK cells and macrophages then cross activate each other to produce more interferon gamma and more pro-inflammatory cytokines such as IL-12. These pro-inflammatory cytokines, as well as the interferon gamma itself in addition to tissue factors such as reactive oxygen species, reactive nitrogen species, and perforin that directly kill the tumor cells.[27, 28]

Finally, the debris from the dead tumor cells is phagocytized by the recruited dendritic cells[29]. These cells then migrate to the regional lymph node. There they activate CD4 T cells that in turn activate CD8 T cells[29]. Both populations of T cells then migrate to the tumor, where the CD8 cells can directly kill the tumor cells.[30]

Unfortunately, in order for the tumor to achieve escape this process has already been disrupted. In the actively growing tumor, these innate and acquired immune responses are co-opted by the tumor to aid in the growth, spread, and survival of the tumor cells instead of their recognition and destruction by the immune system. Cells of the acquired arm of the immune system play an important role, as T regulatory cells have been implicated as a major cause of the immunosuppresion of T cell responses against tumor cells. However it is the innate immune system that plays an even more diverse role in this process, contributing not only to immunosuppression but also directly aiding the growth and spread of tumors. The remainder of this work will focus on the innate

immune system, and specifically myeloid lineage cells, and the many ways that they can contribute to tumor progression.

Myeloid suppressor cells and generation of the tumor microenvironment

We will first look at the most immature subtype of immunosuppressive myeloid cells, a group of cells defined as myeloid suppressor cells (MSC). Myeloid suppressor cells constitute an immature population of cells that are normally found in the bone marrow, and in very small percentages in the blood and lymphoid organs such as peripheral lymph nodes and the spleen. However pathological conditions such as sepsis, parasitic infections, cancer and even vaccination can lead to very large increases in the percentages of these cells found in circulation and in lymphoid organs[31]. Through their presence in these secondary lymphoid organs, the MSCs are able to suppress T cell responses and contribute to an overall immunosuppressive phenotype. Not surprisingly, increased numbers of these cells have been shown to correlate with a poorer prognosis and higher tumor burden in human cancer patients [32, 33].

Myeloid suppressor cells are not a uniform population of cells, but instead a heterogenous mixture of both granulocytic and monocytic cells that both display the surface markers CD11b and GR-1. The subsets are further defined in mice by surface markers and morphology, with the granulocytic cells being CD11b+Ly6G+Ly6C^{low} and having a characteristic granulocytic nucleus, and the monocytic cells being CD11b+Ly6G-Ly6C^{high} and having a monocytic appearance [34]. Both subsets are equally immunosuppressive and derive their functionality by direct contact suppression of T

cells[35]. However, while the granulocytic cells primarily use reactive oxygen species to exert their effects, the monocytic population primarily uses nitric oxide to induce T cell anergy[35]. In humans, the surface markers are different, with MSCs generally being defined as Lin(-)HLA(-)CD11b(+)CD 33(+)[32].

There is another important difference as well. While the granulocytic MSCs do not appear to mature to other cell types, the monocytic MSCs can mature as normal monocytes do into macrophages and dendritic cells[36]. However, when they do mature they keep their immunosuppressive phenotype. Adoptive transfer experiements have shown that MSCs derived from the spleens of mice mature into tumor associated macrophages [37]. These cells may therefore serve as a rapidly available pool for recruitment and replenishment of the tumor associated macrophage, a critical cell type for tumor development and survival.

Tumor associated macrophages and prognosis

As myeloid suppressor cells mature into their terminally differentiated states, they are able to perform more functions to benefit the tumor. The granulocytic cells are already terminally differentiated, but the monocytic subgroup can mature into tumor associated macrophages or dendritic cells.

When looking at the immune system as a whole, the cell that plays perhaps the most diverse role in tumor progression is the tumor associated macrophage (TAM). These cells have been reported to comprise as much as 50% of the overall tumor mass[38]. In the majority of tumors studied, an increase in macrophage number in the tumor tissue

strongly correlates with a poor prognosis for the patient[39]. Specifically, in breast, prostate, cervical, lung, and bladder tumors an increase in tumor associated macrophages leads to a poor prognosis[40-46]. Recent studies have also shown that increased numbers of TAMs carries a poor prognosis for hematologic tumors such as lymphoma[47, 48].

While some studies are equivocal and a few show a positive prognosis with increased macrophages, over 80% of studies undertaken show a significant correlation between macrophage density and a poor prognosis[39]. This wealth of information directly linking macrophages with prognosis in human cancer patients has led to a large number of studies to determine the mechanism by which this specific subset of immune cells can so greatly affect patient outcome. Understanding of these cells could therefore help elucidate the overall role of inflammation in tumors. More importantly, these cells could present a therapeutic target, as depletion of these cells could lead to a decrease in tumor progression. In addition, the depletion of these cells could potentially decrease the immunosuppressive nature of tumors that have achieved escape from immune surveillance.

Macrophage polarization

In order to understand the role of macrophages in tumors, and how their functions can be co-opted in order to benefit tumor cells, it is first important to understand normal macrophage biology. The cell now identified as a classically activated macrophage was first identified by Elie Metchnikoff in 1905, and he observed at that time that they were adept at phagocytizing and killing bacteria[49]. Macrophages are derived from circulating

monocytes, which are released from the bone marrow[50]. Monocytes circulate in the bloodstream, and extravasate into tissues in response to stimuli such as monocyte chemotactic protein 1 (MCP-1). There are many types of specialized macrophages in tissues, including Kupffer cells in the liver, alveolar macrophages in the lung, and osteoclasts in the bone[50]. They perform multiple functions in support of the innate immune system, and as such are capable of phagocytosis and cytotoxicity as well as the secretion of a wide variety of growth factors, cytokines, and other substances that help shape the immune response[50].

It is exactly this multifunctionality that makes these cells particularly advantageous to tumor cells. Macrophages demonstrate a high degree of plasticity and can differentiate into classically activated inflammatory cells analogous to the TH1 T cell phenotype, or a more anti-inflammatory phenotype more analogous to TH2 T cells[51].

For classification purposes, these cells are then commonly referred to as classically activated M1 or alternatively activated M2 macrophages, however any individual cell can likely alternate along a spectrum from one extreme to the other depending on the cytokine or chemokine signals present[51]. The M1 macrophages, or classically activated macrophages, are activated in response to microbial products and IFN-γ. These cells subsequently produce pro-inflammatory cytokines such as interleukin 12(IL-12) and tumor necrosis factor alpha (TNF-α). They are also able to effectively present antigens, and produce reactive oxygen and nitrogen species for effective killing of microorganisms and other cells[51]. The M2 or alternatively activated macrophages on the other end of the spectrum are found in response to anti-inflammatory signals such as glucocorticoids, interleukins 4, 13, and 10 (IL-4, IL-13, and IL-10). These cells then

subsequently produce anti-inflammatory cytokines and factors such as IL-10, transforming growth factor beta (TGF-β) and arginase among others[51]. This anti-inflammatory function as well as the ability to promote angiogenesis and proliferation of surrounding tissues makes these macrophages essential for wound healing[52].

As first suggested by Dvorak, tumors can well be thought of as a wound that will not heal[53]. When thought of in this context it is easy to visualize how M2 macrophages may be co-opted to aid in tumor promotion. We will next examine some of the mechanisms by which this promotion may occur.

The mannose receptor

While the delineation of M2 macrophages is largely functional, via the production of anti-inflammatory cytokines and lack of production of pro-inflammatory cytokines, there have been some surface markers identified in these cells. Of these, the mannose receptor was one of the first identified as corresponding to alternative activation with IL-4 and IL-13[54, 55]. This receptor is nearly universally accepted as being present on M2 or tumor associated macrophages[49, 56-58]. The receptor itself is a member of the C-type lectin family of receptors[59]. This family is part of the larger group of pattern recognition receptors (PRR) that recognize foreign antigens. Specifically, the C-type lectins recognize carbohydrate groups on potential pathogens, primarily yeasts, protozoa, and some bacteria such as *Mycobacterium tuberculosis* [60, 61]. Once bound by its ligand, this receptor type can induce phagocytosis and endocytosis of pathogens[59, 61]. However, as

with most areas of the immune system these receptors are divided into both pro and anti-inflammatory groups[59, 62].

Not surprisingly, much of the recent evidence would suggest that mannose receptor stimulation leads to an anti-inflammatory phenotype. When macrophages are co-cultured with mesenchymal stem cells, they increase their mannose receptor expression and produce high levels of IL-10 and low levels of IL-12[63]. Direct stimulation of the mannose receptor with *Mycobacterium* specific mannose or anti-mannose receptor antibodies leads to a decrease in IL-12 production in dendritic cells[64]. Macrophages derived from human ovarian carcinoma samples showed very high expression of the mannose receptor[62]. When stimulated with anti-mannose receptor antibody or the tumor mucins TAG-72 or CA125, these cells produced high amounts of IL-10 and low levels of IL-12 in response to LPS stimulation[62]. These studies suggest that while being an important identification marker for immunosuppressive TAMs, the mannose receptor may be stimulated in the tumor microenvironment and may also play a role in contributing to the switch to an M2 phenotype in these cells.

Macrophage recruitment to tumors

In order to make an impact in the tumor microenvironment, macrophages must first be recruited to the site of the tumor. As with normal macrophage migration, this occurs via attraction of monocytes out of the bloodstream, which than extravasate into the tumor tissue. As discussed previously, monocytic myeloid suppressor cells may also be recruited in the same manner from the blood, spleen or other secondary lymphoid organs.

They do this primarily in response to the chemokine monocyte chemotactic protein 1 (MCP-1) which can be produced by many types of tumor cells[65, 66]. However, other tumor derived signals such as vascular endothelial growth factor (VEGF) and macrophage colony stimulating factor (M-CSF) can also recruit monocytes to tumors[67, 68]. It is not surprising then that increased levels of MCP-1 and M-CSF are associated with an increase in macrophage numbers in tumors and a poorer prognosis[57, 69]. In mouse models, increasing the MCP-1 production of tumor cells leads to increased tumor growth in a melanoma model[70]. The same holds true in a study of M-CSF, where overexpressing M-CSF in transgenic mice led to an acceleration in tumor growth, while mice with a null mutation of M-CSF showed decreased tumor progression and metastasis[67].

This recruitment of macrophage precursors could obviously be detrimental to the tumor, if these cells were allowed to polarize to an anti-tumor M1 phenotype. In those few studies showing an anti-tumor effect of macrophages this is likely the underlying mechanism. It is therefore crucial that for continued growth and immune escape of tumors that the tumor cells are able to force the differentiation of incoming cells to an anti-inflammatory, pro-tumor phenotype. It has been shown that the cytokine milieu of the tumor microenvironment is indeed capable of pushing incoming immature monocytes to mature into cells that are able to phagocytize effectively but can't perform effective antigen presentation[71]. Other studies have shown that carcinomas can produce anti-inflammatory cytokines such as transforming growth factor β (TGF-β) and interleukin 10 (IL-10) as well as certain prostaglandins that are also anti-inflammatory[56, 72].

As mentioned above, tumors are also abundant producers of M-CSF. This cytokine has also been shown to polarize macrophages to an M2 phenotype, which indicates that this may be the default pathway of macrophage maturation in the absence of pro-inflammatory signals[38, 72].

Alternatively activated macrophages are also capable of producing a wide variety of anti-inflammatory cytokines, chemokines, and prostaglandins[72]. Therefore, once the initial TAMs have been recruited and polarized, they can in turn produce more TGF-β, IL-10 and other anti-inflammatory cytokines and thus induce M2 polarization in newly arrived monocytes[38, 72]. This creates a self-sustaining mechanism whereby existing cells ensure continued immunosuppression in the evolving tumor microenvironment.

Tumor associated macrophages and cell proliferation

As previously discussed, TAMs have the potential to increase the proliferation of tumor cells which may play a role in tumor promotion[15, 16]. Again if one thinks of M2 macrophages in a wound healing context they would need the ability to stimulate growth and proliferation of surrounding tissues such as fibroblasts and epithelial cells to aid in wound healing. Not surprisingly then, macrophages are able to produce both fibroblast growth factor (FGF) and epidermal growth factor (EGF)[57]. In fact, it has been reported that TAMs may be the most significant source of EGF in carcinomas such as breast cancer[73]. This particular growth factor is capable of inducing tumor cell proliferation as well as migration[74]. In addition, these cells are capable of producing additional growth factors such as hepatocyte growth factor (HGF), platelet derived growth factor (PDGF),

interleukin-8 (IL-8) and transforming growth factor β (TGFβ)[57]. This increase in proliferation has been demonstrated clinically by an increased mitotic index in renal cell carcinoma histopathology that correlates with an increase in TAMs in this tumor type[75]. Given the wide variety of growth factors produced, TAMs likely have the ability to stimulate proliferation in many many different tumor types.

Tumor associated macrophages and angiogenesis

Another critical factor for wound healing is the ability to repair and regenerate damaged blood vessels. Tumors are also critically reliant on the development of a new blood supply in order to grow. In fact, a tumor can't grow to a diameter of larger than 2-3 mm without the advent of new blood vessels[76]. As well as being associated with a poorer prognosis, increased TAMs have also been shown to correlate with an increase in microvessel density in breast cancer, gliomas, and lung cancer[77-79]. Conversely, the depletion of macrophages in rodent tumor models leads to a decrease in angiogenesis and microvessel density[80-82].

Macrophages are able to directly produce a large number of pro-angiogenic factors, including critical factors for angiogenesis such as vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8)[79, 83]. In fact, the production of these factors by macrophages has been shown to be increased by co-culture with tumor cells in vitro[79, 83]. Myeloid cells can also directly counteract the effects of anti-VEGF therapy, leading to increases in angiogenesis despite this treatment[84].

While the increase in production of pro-angiogenic factors has the potential to then directly increase angiogenesis, the interplay between macrophages and blood vessels is likely more complex than a simple association with increased angiogenic factors. For example, TAMs produce a factor known as thymidine phosphorylase (TP). This factor stimulates endothelial cell migration in vitro, and increased levels have been correlated to a poor prognosis and increased tumor angiogenesis in vivo[85]. As TAMs accumulate in hypoxic areas of tumors, the production of TP could increase endothelial cell migration into these hypoxic areas and increase blood flow[58]. Further, the accumulation of TAM in hypoxic regions can lead to an increase in HIF-2α production by the macrophages, which further stimulates VEGF production by both the macrophages and surrounding tissues[57]. Via these mechanisms, TAMs can migrate into hypoxic areas and in turn stimulate blood vessel production in these areas. This not only increases angiogenesis, but increases it in the particular parts of the tumor where it may be most needed.

The role of tumor associated macrophages in invasion and metastasis

One of the hallmarks of cancer is invasion and metastasis[13]. From a prognostic standpoint this hallmark is clearly most important, as the vast majority of cancer patients die due to distant spread and metastases of their primary tumors[86]. The first step of this process is invasion, the ability of tumor cells to break through basement membranes and migrate through the extracellular matrix to gain access to blood or lymphatic vessels. Macrophages play a role in the normal migration and invasion of epithelial cells as developing ducts invade fat pad tissue in normal mammary development [87]. When

tissues become cancerous, they can again co-opt this normal macrophage function to facilitate invasion and migration through basement membranes[88]. First, macrophages are abundant producers of matrix metalloproteinases (MMPs) that are used to digest basement membranes and allow tumor cell invasion[89]. Macrophages have been shown to increase the invasiveness of tumor cells in vitro, and tumor cells in turn increase the production of MMPs by macrophages. The increased invasiveness was blocked by MMP blockade[90]. In vitro studies have also revealed that TAMs and breast carcinoma cells participate in a paracrine loop, whereby TAMs produce EGF that stimulates carcinoma migration and invasion and the carcinoma cells produce M-CSF which has the same effect on the TAMs[91]. The cells show increased invasiveness in co-culture as compared to either cell type alone, and this invasiveness is blocked by blockade of either M-CSF or EGF[91].

The role of macrophages in extravasation and distant metastasis are less well understood, but there is evidence to support their participation in these steps of the metastatic pathway as well. In vivo imaging studies have been able to show that perivascular macrophages are associated with intravasation of tumor cells into blood vessels[92]. Using M-CSF knockout mice, this group was also able to demonstrate that lack of M-CSF leads to a decrease in perivascular macrophages in the tumor and a subsequent decrease in circulating tumor cells[92].

After intravasation tumor cells must survive in the blood stream or lymphatics, extravasate, and survive in a new tissue environment to be truly metastatic. There is evidence to suggest that TAMs play a role in these final steps of metastasis as well. The selective depletion of macrophages in the peritoneal cavity or liver led to slower growing,

more differentiated tumors in these tissues when the cells were implanted in a model of metastatic colon cancer[93]. Lack of alveolar macrophages has also been shown to correlate with decreased lung metastasis in mouse models of breast cancer[67].

Therefore, it has been shown that TAMs potentially play a role in all steps of metastasis. Briefly this includes tissue invasion, migration, vessel intravasation, and survival in a distant site. An increase in any of these steps could potentially increase metastasis, but an increase in all of these steps almost certainly leads to an increase in metastasis.

Anti-immune effects of tumor associated macrophages

Finally, when viewed in a wound healing context, M2 macrophages must be able to dampen the immune response to stop ongoing tissue destruction and thus allow wound healing. This function is also critical in order for tumors to fully escape immune surveillance, and thus escape killing by effector immune cells such as T-cells, NK cells, and phagocytic cells such as macrophages. As previously discussed, M2 macrophages are induced by anti-inflammatory cytokines such as IL-4, IL-10 and IL-13, and anti-inflammatory substances such as glucocorticoids[49, 51, 57, 58]. This in turn causes these cells to produce anti-inflammatory cytokines such as IL-10 and TGF-β. They additionally produce the anti-inflammatory chemokines CCL17 and CCL22[38, 49]. It is the production of these substances that have effects on both innate and acquired immunity, leading to an overall immunosuppressive phenotype.

The first consequence of this anti-inflammatory cytokine and chemokine milieu is a direct effect on the macrophages themselves, and a polarization of incoming monocytes to an M2 phenotype. These alternatively activated macrophages have a decreased ability to produce reactive oxygen and nitrogen species, thus making them inefficient at cell killing[38, 51, 94]. They also downregulate MHC class II and are poor at antigen presentation[72]. As a consequence of producting anti-inflammatory cytokines, they also down regulate production of pro-inflammatory cytokines such as IL-12 and IL-23[38, 72].

While the decreased cytotoxic function of macrophages in the tumor is important to immunosuppression, a far greater immunosuppressive role is achieved by the blunting of cell mediated immune responses of NK cells and cytotoxic T cells[38]. The combination of decreased IL-12 and increased IL-10 induces decreased proliferation and cytotoxicity of NK cells and T cells[28]. As mentioned above, TAMs have low levels of MHC expression and are poor antigen presenting cells, limiting their ability to activate T cells[72]. The IL-10 produced by macrophages also induces T regulatory cells, which further induce effector T cell suppression and anergy[89]. TAMs also secrete chemokines such as CCL17 and CCL22 that preferentially attract T regulatory cells, and CCL18 which recruits naive T cells which are likely to become anergic in the absence of antigen stimulation[38]. Additionally, TAMs produce a specific matrix metalloproteinase, MMP-7, that is capable of cleaving FAS ligand from tumor cells[95]. This may directly protect these cells from killing by T cells and NK cells[89].

Therefore, macrophages have an immense impact on the overall local immune response in the tumor. These cells act in many ways to provide an overall

immunosuppressive, anti-inflammatory environment which allows the tumor to continue to escape immune surveillance and destruction by cytotoxic immune cells.

Tumor associated macrophages and chemoresistance

While not nearly established as many of the other pro-tumor mechanisms of TAMs, there is some evidence to suggest that these cells may be able to directly protect tumor cells from the effects of chemotherapy. As mentioned previously, TAMs have the ability to produce matrix metalloproteinase 7 (MMP-7). This protein has shown the ability to directly protect multiple types of tumor cells from the affects of doxorubicin by cleavage of FAS ligand[96]. Another group has shown that TAMs can directly protect multiple myeloma cells from apoptosis induced by melphalan, and that this protection requires direct contact[97]. While certainly not exhaustive, these studies provide at least some preliminary evidence that TAMs may be able to protect tumor cells from cytotoxic chemotherapy, potentially via multiple mechanisms.

Free clodronate induces osteoclast apoptosis

Based on the above evidence, the depletion of tumor associated macrophages could theoretically decrease tumor cell proliferation, decrease angiogenesis, decrease invasiveness and metastasis, decrease tumor immunosuppression, and increase sensitivity to chemotherapy. The depletion of these cells could therefore be a highly effective

therapeutic strategy. One such strategy is to employ the use of liposomal clodronate, a drug that can selectively deplete phagocytic cells[98].

Clodronate is a member of the bisphosphonate (BP) class of drugs, which are defined by their characteristic structure linking two phosphate groups to a central carbon group in a P-C-P configuration[99]. Interestingly, these drugs were initially synthesized to be used as water softeners in the late 19th century[99]. In the 1960's it was discovered that (BPs) in their mineral binding capacity could also prevent dissolution of hydroxyapatite of bone[99]. Since then, this class has been developed to treat a wide variety of diseases leading to abnormal bone resorbtion and destruction such as osteoporosis, metastatic bone neoplasia, hypercalcemia of malignancy and Paget's disease[100].

The bisphosphonates have since been developed into two classes, with different mechanisms of action[99]. Clodronate belongs to the earlier generation of drugs which do not contain nitrogen as part of their structure. As with all BPs, when injected or given orally clodronate is rapidly taken up into the hydroxyapatite of bone or cleared by the kidneys, leaving very little drug in the blood, other organs or tissues two hours after administration[99, 101].

The mechanism of action of clodronate has only recently been determined. This drug reaches very high concentrations in the mineral matrix of bones, particularly of the appendicular skeleton[99, 101-103]. Once present in the bone, clodronate is taken up by specialized macrophages of the bone called osteoclasts[104, 105]. Once inside the cells, clodronate is metabolized by the cells to a non-hydrolyzable ATP analogue[106]. In the case of clodronate this occurs because Class II aminoacetyl-tRNA synthetases, which are also responsible for synthesis of ATP from ADP in addition to their more defined roles in

protein synthesis, use clodronate instead of a phosphate group in the synthesis of ATP[106]. This results in the toxic accumulation intracellularly of a non-hydrolyzable ATP analogue[106, 107]. This consequently disrupts vital cellular functions such as metabolism, signaling, and protein synthesis[106]. This leads to apoptosis of the osteoclasts[105, 108, 109]. The net effect of this loss of osteoclasts in the bone is a decrease in bone resorbtion, which aids clinically in diseases of increased bone destruction[102, 104].

Development of liposomal clodronate as a selective macrophage depletion agent

Nico van Rooijen was the first to hypothesize the idea that as clodronate is effective at osteoclast depletion, it makes sense that it could potentially be effective as a depleting agent for other macrophages as well. The trick was to prevent the uptake of clodronate into the bone matrix, as the free drug is rapidly taken up by the bone and does not achieve effective concentrations in other tissues[102]. van Rooijen's group was the first to determine a way to achieve systemic levels of clodronate in order to deplete macrophages. He found that clodronate could be encapsulated in a liposomal bilayer, thus preventing its rapid transit to bone[98]. His group developed the liposomal clodronate approach for macrophage depletion in the early 80's. While they tried several different liposome encapsulated drugs, they have repeatedly found that clodronate is the best agent for selective macrophage depletion[106]. As clodronate is a highly charged substance, it is not able to easily pass through the lipid bilayer of the liposome. This leaves the drug encapsulated until the liposome is taken up by a macrophage or other phagocytic cell. Once taken up, the liposome is digested, which releases the free drug into the cytoplasm

of the affected cell and causing the cell to undergo apoptosis. Studies have since shown that liposome encapsulated clodronate is much more effective at macrophage killing in vitro than free clodronate[110, 111]. As with the free drug, liposome encapsulated clodronate induces apoptosis in the affected cells[106, 112]. Most importantly, van Rooijen has shown that liposomal clodronate (LC) is very effective at depleting macrophages in the spleen and other tissues[98, 110, 113]. Due to the properties of liposomal clodronate, it has been shown to be very selective and depletes only phagocytic cells while sparing all other tissues. Similarily, non phagocytic immune cell populations are also spared by treatment with either free or liposomal clodronate[109].

These findings have led to the widespread use of clodronate in many mouse models of macrophage mediated auto-immune and inflammatory diseases[114-117]. In all of these studies, LC administered via either intravenous or intratracheal routes was safe and effective at depleting macrophages in mouse models. A potential therapeutic route was identified in a mouse model of immune mediated hemolytic anemia (IMHA)[118]. In this disease macrophages are directly responsible for the effects of the disease. Macrophages phagocytize red blood cells coated with antibody due to abnormal immune activation. In so doing they cause the severe anemia that is the hallmark of this disease[118, 119]. Depletion of macrophages with LC was able to decrease the anemia in a mouse model of IMHA[118]. Subsequently, the drug was shown to be safe and effective in the clinical disease when evaluated in dogs afflicted with IMHA[119].

Use of liposomal clodronate in tumor models

Until recently, liposomal clodronate has not been used in tumor models. This is somewhat surprising given the large amount of evidence showing macrophage involvement in tumor progression. A study by Zeisberger et. al. in 2006 was the first to show that macrophage depletion with LC could decrease tumor growth and angiogenesis in rodent tumor models. They showed both in teratocarcinoma and rhabdomyosarcoma that intra-peritoneal injection with LC decreased tumor growth as much as 92% due to a drastic decrease in tumor vasculature. Free clodronate did not have an effect on tumor growth[81].

Since this initial study, multiple groups have used LC as an anti-tumor agent. Depletion of macrophages by intra-peritoneal injection has also been shown to decrease metastasis in an ovarian tumor model and in a model of malignant mesothelioma, and to decrease tumor growth and angiogenesis in an orthotopic rat prostate tumor model[75, 116,117]. The use of clodronate has also been shown to decrease metastasis and angiogenesis in a model of hepatic carcinoma, and to decrease bone metastasis in lung tumors[77, 118].

Many of these studies have shown a decrease in angiogenesis and tumor growth following macrophage depletion with LC[80, 97, 120]. Other studies have also shown decreases in tumor invasiveness and metastasis when using LC in addition to decreases in angiogenesis[93, 121, 122]. Studies looking at the combination of LC with anti-angiogenic factors or tyrosine kinase inhibitors have shown a synergistic effect of combination therapy[76, 82]. Studies such as these suggest that LC may potentially have a beneficial

effect as an adjunctive treatment for many different types of tumors, potentially when paired with traditional chemotherapy. While limited in scope, these initial studies are encouraging in that they show the potential for LC to limit many of the pro-tumor functions of TAMs, resulting in decreased tumor growth and metastasis.

One limitation of these studies is the lack of systemic administration of LC. These studies as a whole used either intra-tumoral or intra-peritoneal administration to locally deplete TAMs. These treatment strategies are not viable in the clinical setting, where the majority of treatments must be administered systemically and remain effective.

Anti-tumor effects of systemic liposomal clodronate administration

Our lab has worked extensively to develop a version of liposomal clodronate that can be safely administered systemically via the intravenous route in animals. Chaper two describes in detail the development of this drug and initial experiments showing anti-tumor activity with systemic administration. Work by myself, Dr. Amanda Guth, and Dr. Leah Mitchell in our lab has shown that systemic administration of liposomal clodronate can have systemic effects on the immune system via the depletion of myeloid suppressor cells in the spleen and peripheral blood. The result of this depletion of myeloid suppressor cells is an increase in anti-tumor activity of T-cells as demonstrated by in vitro cell killing assays and intracellular cytokine staining for interferon gamma. Therefore, systemic administration may work by an entirely different mechanism than local administration with primary effects mediated by reversal of global immunosuppression in addition to effects of TAM depletion.

Malignant histiocytosis in dogs

An interesting model in the study of drug targeting of TAMs is a tumor in dogs known as malignant histiocytosis (MH). This tumor is intriguing in the study of TAMs because it is a tumor that can arise from histiocytic cells[112]. Therefore, a drug that specifically kills this type of tumor may reasonably be expected to have an effect on TAMs. We have therefore sought to evaluate new treatment options for this tumor in order to benefit dogs with this disease as well as to potentially uncover new ways to deplete TAMs.

Malignant histiocytosis, which is also known in the literature as disseminated histiocytic sarcoma, is a highly aggressive, rapidly metastatic, and uniformly fatal neoplasm of dogs. As the name suggests, the tumor arises due to a malignant transformation of histiocytic cells, in particular macrophages or dendritic cells[123-127]. There is a breed predilection for this disease in dogs, with Bernese Mountain Dogs being particularly over-represented[128]. However, many other breeds such as Rottweilers, Flat Coated Retrievers, Golden Retrievers and mixed breed dogs can also be afflicted with this disease[129].

While a localized form of the disease is recognized, the majority of cases present with diffuse disease involving multiple organs such as the liver, spleen, lungs, lymph nodes and central nervous system[130]. The disseminated nature of the disease renders localized treatment modalities such as surgery or radiation therapy ineffective in most cases[131].

Therefore, the treatment of choice for this disease is chemotherapy. The most evaluated drug used for the treatment of this disease is lomustine (CCNU)[132]. This agent is effective in prolonging survival in truly localized cases of this disease[133]. Unfortunately, the vast majority of cases present with disseminated disease, and tumors in these dogs are refractive or only transiently responsive to treatment with chemotherapy[132]. Consequently, the median survival time for this disease even with treatment is less than 6 months[129, 132, 134, 135].

These findings suggest that new treatment options are necessary for this disease. As the tumor is derived from cells of the macrophage lineage, it is possible that it may be directly affected by liposomal clodronate or other chemotherapy combinations that directly target macrophages.

Langerhans cell histiocytosis in humans

A disease analogous to MH in dogs is found in humans. As with the canine disease, Langerhans Cell Histiocytosis (LCH) is a tumor of histiocytic cells[136]. In contrast to dogs, the disease in humans most often arises as local disease without distant metastasis[136]. However, there is a small subset of patients, particularly children, who present with disseminated and aggressive disease[137, 138]. These patients have involvement of multiple organs and tissues including lymph nodes, bones, lungs, liver and spleen[137, 138]. The treatment of choice for disseminated disease in humans is chemotherapy, with agents such as vincristine and vinblastine commonly used in combination with prednisone or other immune suppressive drugs[136, 137]. Zoledronate is also used to palliate

bone pain in cases with bony involvement[139]. Like the disease in dogs, those patients with disseminated disease are poorly responsive to chemotherapy and 20% of these patients will succumb to their disease despite treatment[136, 140]. As with the disseminated disease in dogs, new treatment approaches are necessary in treatment of chemotherapy resistant or refractive LCH.

Anti-tumor effects of aminobisphosphonates

As previously discussed, the bisphosphonate class of drugs is divided into two subgroups, nitrogen and non-nitrogen containing BPs. The newer class of BPs are the nitrogen containing drugs, or aminobisphosphonates. This class of drugs includes alendronate and pamidronate which contain a simple amine group, and zoledronate which contains an amine ring[102]. The mechanism of action of these drugs is different from that of the non-nitrogen BPs, in that these drugs directly inhibit the enzyme farnasyl diphosphate synthase[141]. This enzyme is an essential component of the mevalonate pathway, which is responsible for protein prenylation. With this pathway blocked by the drug, osteoclasts are unable to attach proteins to the membrane, in particular critical prenylated proteins such as GTPases[141, 142]. This leads to subsequent apoptosis of the treated cells.

The aminobisphosphonate zoledronate has recently been more closely evaluated for its anti-tumor effects, which occur as a result of its unique amine ring structure that separates it from the other aminobisphosphonates. Like the other BPs, it has long been used for palliation of bone pain associated with bony metastasis or primary bone

tumors[139, 143]. Both in vitro and mouse tumor studies of multiple tumor types have begun to show that zoledronate may show some primary anti-tumor activity as well, particularly when used in combination with traditional chemotherapeutics where synergistic interactions between zoledronate and chemotherapy drugs has been observed[144-146].

In addition to direct cytotoxicity against tumor cells, zoledronate may also be able to exert effects on the immune system. Free zoledronate has been used to deplete TAMs in a mouse model of hepatic carcinoma[82]. Other studies have shown that zoledronate treatment can change the polarization of macrophages, switching them to a more anti-tumor M1 phenotype[147]. Studies have also shown that zoledronate treatment of tumor cells targets these cells for destruction by γδ T cells, further inducing an anti-tumor immune response against these cells[148, 149].

Studies such as these suggest that zoledronate may be effective in combination with traditional chemotherapy for the treatment of multiple tumor types, via a number of different mechanisms.

Project Rationale

The innate immune system is clearly an important component of tumor progression through a variety of diverse mechanisms. One of the critical cells that mediate many of these processes is the tumor associated macrophage. Depletion of tumor associated macrophages may therefore represent a new therapeutic strategy to treat established tumors. This may be particularly true in combination with traditional chemotherapeutics which target rapidly dividing tumor cells but likely have little to no effect on the non-dividing macrophages present in the tumor stroma.

One strategy to deplete tumor associated macrophages has been to employ the use of liposomal clodronate, a drug long known to deplete macrophages that has recently been shown to be effective in depleting tumor associated macrophages[82, 98]. When used to study depletion of TAMs in rodent tumor models liposomal clodronate has never been evaluated for systemic use intravenously, which is how the drug would most effectively be administered if used therapeutically.

We therefore first sought to develop a formulation of liposomal clodronate that would be effective in depleting TAMs if administered systemically. In order to do this, we had to develop the most effective liposome formulation for effective killing of macrophages. We hypothesized that liposome modifications could be done to make LC more effective at macrophage depletion than using standard liposomes, and that the use of this modified liposome would be effective at depleting TAMs when administered intravenously. Chapter two details the experiments involved in developing this modified drug and showing that it could be safely and effectively administered intravenously in a

rodent tumor model. We were also able to show that the killing induced by these liposomes is dependent on phagocytosis, and that non-phagocytic cells are not affected by the liposomal drug.

We next sought to determine if this modified liposomal clodronate could be used against a spontaneously arising tumor in dogs. Chapter three details the initial in vitro and following clinical trial in dogs. For these studies we chose malignant histiocytosis, a tumor of macrophages. We chose this tumor type because new treatment options are sorely needed for this tumor in dogs. We also hypothesized that liposomal clodronate would have multiple effects leading to anti-tumor activity. In order to conduct these studies, we first evaluated whether liposomal clodronate would be effective at depleting MH cells in vitro. We then further sought to determine the underlying mechanisms that caused the killing of the MH cells.

With this information we felt we had enough pre-clinical information to attempt to treat canines afflicted with malignant histiocytosis in a preliminary clinical trial using mannosylated liposomal clodronate as a single agent. We were able to demonstrate anti-tumor effects with administration of LC. We were also able to see physiologic effects of the drug, particularly in responding patients. These included development of a fever, an increase in neutrophils, a decrease in monocytes, and a significant reduction in IL-8 which is an important pro-growth and pro-angiogenic cytokine produced by macrophages and tumor cells[150-153]. Most importantly, the drug was safe and effective in treated dogs, with few side effects.

While these preliminary results were encouraging, liposomal clodronate is still not widely available for use. We therefore next sought to see if there were traditional

chemotherapeutics that could be combined with bisphosphonates to show synergistic killing of MH cells in vitro. Chapter four describes the multiple in vitro experiments undertaken in an attempt to identify synergistic combinations of chemotherapy drugs that could be effective against MH. We hypothesized that there would be some synergistic interactions, as BPs have previously been shown to synergize with traditional chemotherapeutics against other tumor types. We evaluated many different chemotherapy drugs and bisphosphonate drugs in combination, and were able to determine that combining doxorubicin with zoledronate or clodronate with vincristine demonstrated synergistic killing of MH cells in vitro. While both combinations killed cells through an increase in apoptosis, the mechanism of the synergistic interaction appeared to be different between the two drug combinations. Based on the results of these in vitro experiments, clinical evaluation of these combinations may be warranted.

The unifying goal of this work was to determine therapeutics that could be used to deplete tumor associated macrophages clinically, in the hope of improving tumor response and prognosis of dogs with tumors. These studies have shown LC to be safe and effective against at least one tumor type when used as a single agent. The true value of this drug likely lies in combination studies, as it has the potential to induce a truly synergistic interaction by targeting the supporting tumor stroma directly while chemotherapy targets tumors directly. Further investigation will be required to determine if this is indeed the case. It is my hope that this work may someday be applied not only to canine tumors but to human cancer patients as well. The following chapters detail the work undertaken to achieve these goals.

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Chapter Two

Determination of the Optimal Liposomal Formulation for Macrophage Depletion Using Liposomal Clodronate

Abstract

Recent studies have increasingly shown that tumor associated macrophages (TAMs) play critical roles in tumor growth and metastasis. Consequently, increased numbers of these cells in the tumor stroma are associated with a poorer prognosis for patients with most tumor types. Therefore, depletion of these cells may provide another treatment option for patients with tumors. The lipid encapsulation of clodronate, a bisphosphonate drug used for depletion of osteoclasts, provides a targeted agent that selectively depletes macrophages in vivo. Multiple mouse studies of autoimmune disease have successfully employed this drug for macrophage depletion. More recently, several groups have used liposomal clodronate (LC) for depletion of tumor associated macrophages in a wide variety of tumor models. However, there have been no studies to determine if a more optimal liposomal delivery system could be used for macrophage depletion. We therefore sought to determine if there was a more effective liposomal formulation for systemic macrophage depletion. We were able to show that a net neutral charged liposome with the incorporation of a mannose group into the liposome was able

to most effectively deplete macrophages in vitro. There was no effect of LC on non phagocytic cells in vitro due to a lack of liposome uptake. This liposomal formulation was also most effective for macrophage depletion in vivo in a wide variety of tissues. When used in the MCA 205 fibrosarcoma model we were able to show that intravenous (iv) treatment with mannosylated LC was able to achieve a significant decrease in tumor growth via depletion of tumor associated macrophages. This effect was achieved despite very little local uptake of the drug in the tumor tissue as shown by the use of fluorescently labeled Bodipy liposomes. This indicates that the macrophage depletion is most likely due to systemic depletion of macrophage precursors that are recruited to the tumor. Mannosylated liposomal clodronate is more effective than other liposome formulations for macrophage depletion and is effective at depletion of tumor associated macrophages. Further studies of mannosylated LC are warranted to determine if this could be an effective cancer treatment.

Introduction

As the tumor stroma has been more closely examined in recent years, the critical and diverse role of tumor associated macrophages has become increasingly more understood[1-3]. In most tumor models these cells have been identified as a negative prognostic variable, with increased numbers of these cells generally associated with a worse prognosis in many tumor types[4, 5]. The reasons behind this phenomenon point to the multifunctional nature of these cells in many biologic processes, including tumors.

Macrophages are capable of producing a variety of pro-angiogenic factors, thus leading to an increased blood supply to tumor tissues[6-8]. They have a vital role in recruiting and supporting other stromal support tissues such as fibroblasts which make up the support structure of the tumor[9]. Tumor associated macrophages are capable of producing matrix metalloproteinases that degrade extracellular matrix and allow for easier migration of tumor cells[10-12]. Macrophages have also been shown to participate in a paracrine loop with tumor cells, whereby tumor cells are led to blood vessels by migrating tumor associated macrophages[10, 13]. Additionally these cells are capable of significant immunosuppresion locally, while their precursors are capable of the same immunosuppression systemically[14]. Taken together, these roles present a picture of the tumor associated macrophage (TAM) as vital to the support, growth, and invasion of tumor cells.

The depletion of macrophages has historically been vitally important to the study of many different auto-immune diseases[15-18]. In order to achieve this goal, a drug known as liposomal clodronate (LC) was developed[19]. Clodronate is a bisphosphonate drug that

was initially developed to specifically kill osteoclasts for the treatment of bone destroying diseases such as osteoporosis and metastatic neoplasia[20, 21]. When given systemically, the drug has rapid and significant uptake by the bone matrix[21, 22]. When this matrix is digested by osteoclasts, the cells also take up the clodronate. Once intracellular the drug induces apoptosis of these cells[23, 24]. Clodronate is a highly charged molecule, which accounts for its rapid uptake in the bone[25]. When encapsulated in a small lipid bilayer, or lipsome, the drug is unable to cross that membrane. This liposomal clodronate may then be given locally or systemically where it is removed from circulation by phagocytic cells. When the lipsome is broken down in the lysosome of these cells, the free drug is released and it induces apoptosis just as in osteoclasts[26, 27].

Liposomal clodronate has been used extensively for the depletion of macrophages in many mouse models of immune disease[17, 19, 28, 29]. More recently it has been used in many tumor models where it has been shown to decrease tumor growth and tumor angiogenesis when administered locally to the tumor[30-35]. However, there have been no studies looking at a more clinically relevant model of systemic intravenous administration. We therefore set out to produce a drug that would be capable of being administered systemically and sought to determine if systemically administered LC could decrease tumor growth. To accomplish these studies we made modifications to the liposome itself to increase the effectiveness of the drug when administered intravenously. We then used this new drug, mannosylated liposomal clodronate, to study depletion of macrophages systemically and in a mouse fibrosarcoma tumor model. We show here for the first time that liposomal modifications can increase the effectiveness of LC, and that this modified drug is capable of anti-tumor effects when administered systemically.

Materials and Methods

Cell lines and mice.

Three murine macrophage cell lines AMJ.2 (ATCC CRL 2455), Raw 264.7 (ATCC TIB71), J774 (ATCC TIB67) and the mouse fibrosarcoma line MCA 205 were obtained from the American Type Tissue Collection (Manassas, VA). All cell lines were maintained in MEM (minimal essential medium, Invitrogen, Grand Island, NY USA) supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT), non-essential amino acids, L-glutamine, sodium bicarbonate, penicillin and streptomycin. The cell lines were maintained at 37° C in a humidified atmosphere containing 5% CO_2. Specific-pathogen-free, 6-8 week old female C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were housed in the laboratory animal resources facility at Colorado State University Veterinary Teaching Hospital and provided sterile water and food *ad libitum*. All research involving animals in these studies was conducted in accordance with guidelines and animal protocols approved by the Animal Care and Use Committee at Colorado State University.

Preparation of liposomal clodronate and liposomal PBS

Liposomal clodronate was prepared as previously described with the following modifications[36]. Phosphatidylcholine and cholesterol (both purchased from Avanti Polar Lipids, Alabaster AL, USA) were dissolved in chloroform and combined at a 5:1 molar ratio in a glass round bottom sterile tube. The lipids were partially dried using nitrogen gas in a fume hood and dried to completeness overnight in a vacuum lyophilizer (VirTis,

Gardiner, NY, USA). To prepare mannose-containing liposomes, p-amino phenyl mannopyranoside (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in methanol and added at 1.75 mg per 25 mg of phosphatidylcholine and dried down together with the phosphatidylcholine and cholesterol lipids. Liposomes were prepared by rehydration in a concentrated solution of clodronate (Sigma-Aldrich). Liposomal PBS (L-PBS) was prepared using the same liposome preparation techniques but by using a 1.5 M stock of PBS in lieu of clodronate.

For the preparation of fluorescent liposomes, 0.5 mL of a 1 mM solution of BODIPY cholesterol (Invitrogen, Eugene, OR) was added to the phopshatidylcholine and cholesterol lipid solution and dried down prior to rehydration. All of the lipids were stored in sterile HEPES buffer under Argon at 4˚ C.

Cell viability by MTT assay

For in vitro assessment of cell viability, the MTT assay wasu used[37]. In these analyses, MTT (thiazolyl blue tetrazolium bromide, Sigma-Aldrich. St Louis, MO) was added to wells containing live cells and incubated for 2 hours at 37˚ C. The cells were then dissolved in a 0.1N HCl solution in isopropanol and the absorbance was determined using an ELISA plate reader (Multiscan Ascent, Thermo Labsystems, Cambridge, MA). Cell viability was calculated as the mean percent absorbance of the treated wells compared to the mean absorbance of the untreated control wells, with the inverse of this value representing the percentage killing. The percentage specific killing was determined as the difference between the percentage killing elicited by LC and the percentage killing elicited by PBS liposomes.

In vitro liposomal clodronate cell killing assays

Cells used in the analysis were trypsinized, washed, and were pipetted into quadruplicate wells of a 96-well flat bottomed plate at a cell density of 4 X 10^3 cells/well. This was the cell number used through all of the *in vitro* assays. Each of the cell lines were given 24 hours to adhere and then were treated with LC or L-PBS at volumes of 1%, 2.5%, or 5% v/v in complete tissue culture medium. These concentrations were determined in serial dilution experiments using all 3 cell lines which demonstrated minimal specific killing at LC concentrations less than 1% v/v with a plateau of specific killing achieved at 5% v/v (data not shown). All of the cells were incubated with LC or PBS liposomes for 72 hours, as previously described.

Liposome uptake measurement using fluorescent liposomes and flow cytometry

Fluorescent BODIPY-labeled liposomes and flow cytometry were used to quantitate liposome uptake both by cell lines in vitro and by cells and tissues in vivo. Cells were trypsinized and re-suspended at a concentration of 5.0 x 10^5 cells/mL. The cells were then incubated with serial dilutions of BODIPY-labeled PBS liposomes in complete medium for 4 hours at 37˚C. The cells were washed twice to remove unbound liposomes. Next, incubation with trypan blue was used to quench the fluorescence emitted by surface bound but non-internalized liposomes prior to analysis by flow cytometry. Trypan blue quenching was accomplished by incubating samples with trypan blue (50 uL of a 0.008% solution of Trypan Blue (Sigma-Aldrich, St. Louis) diluted in 1 mL of cells) in PBS for 15 minutes. Flow cytometry was done using a Cyan-ADP flow cytometry(Beckman-Coulter, Ft Collins, CO) and BODIPY positive cells were defined

as those in the FITC channel. Analysis of the data was done using Summit software (Beckman-Coulter).

Mouse MCA 205 fibrosarcoma model

In order to generate a tumor fibrosarcoma model, C57BL/6 mice were injected *s.c.* with 2.5 x 10^5 MCA-205 fibrosarcoma cells. For these analyses, the mice were then randomized to n=5 mice per group (LC treated, L-PBS treated, or untreated control mice). Treated mice were injected with 200 µL of LC or L-PBS intravenously (iv) via the lateral tail vein three days after tumor injection and once per week after the initial injection. Tumor growth was monitored using calipers and recorded as mean tumor size in mm^2 (sum of the longest diameter and the diameter 90˚ to the first measurement). Tumor growth was measured twice per week.

Cell isolation and flow cytometry.

In order to prepare single cell suspensions, spleen, lymph node, and liver tissues were screened using a 10 µm cell strainer. Lung and tumor tissues were isolated into single cell suspensions using collagenase digestion. The collagenase digestion for lung and tumor tissues was achieved as follows. The tissues were minced using a #15 scalpel blade and incubated at 37C for 20 minutes in 2-3 mL of collagenase solution. Once in suspension, all tissues were treated with ACK solution (150 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA) for five minutes in order to lyse red blood cells. Cells were then washed using FACS buffer (PBS with 2% fetal bovine serum and 0.05% sodium azide). The cells were resuspended in FACS buffer and 10 µL of normal mouse serum to block non-specific binding prior to antibody staining. The following antibodies were used: anti-mouse F4/80 (clone BM8), anti-mouse CD11b (clone M1/70), anti-mouse Ly6G (clone 1A8), anti-mouse CD11c (clone N418), anti-mouse B220 (clone RA3-6B2), anti-

mouse Gr1 (clone RB6-8C5) and anti-mouse CD3 (clone eBio500A2). Antibodies were purchased from eBioscience (San Diego, CA).

In vivo liposome tracking experiments.

Mice were injected with MCA 205 cells subcutaneously as described above and tumors were allowed to grow for 2 weeks. The mice were then injected iv with 200 μL Bodipy labeled liposomes which were prepared as described above for in vitro studies. The mice were then sacrificed 6 hours later. Tumor, spleen, blood and draining lymph node tissues were then prepared and antibody stained as described above. The cells were then analyzed via flow cytometry and the percentages and mean fluorescence intensities (MFI) of Bodipy (FITC) positive cells were determined.

Statistical analysis.

Statistical analyses were done using GraphPad Prism software (San Diego, CA). Differences between two groups were compared using the Student's T test. Differences between more than two groups were determined using one-way ANOVA followed by Tukey's multiple comparison tests. Differences in tumor growth were evaluated using repeated measures ANOVA followed by Bonferroni's post-test. For all analyses, a *p*-value of < 0.05 was considered statistically significant.

Results

Determination of an optimal liposomal formulation

In order to determine an effective liposomal formulation for macrophage depletion we first used in vitro assays to determine macrophage killing. We first looked at the effect of charge and cholesterol content on the effectiveness of killing with LC. Liposomes were prepared using different charged head groups to create a net negative charge (DPPS), a net positive charge (DOTIM), or a net neutral charge (PC) to the overall lipid bilayer. The cholesterol concentration of the liposomes was also altered in order to change the stability of the overall liposome. Each liposome was rehydrated in either clodronate or PBS. Four different murine macrophage cell lines were treated with either the clodronate or PBS containing liposomes for each different charge or cholesterol formulation. After 72 hours the cells were analyzed using the MTT assay to determine cell viability. The viability of the cells treated with the clodronate containing liposomes was compared to the PBS containing liposomes in order to determine the percent specific killing achieved by the clodronate and removing any cytotoxic effect of the liposome alone as this effect was unlikely to be reproducible in vivo.

A net neutral charged liposome (PC) was the most effective in cell killing, followed by the negative charged liposome (DPPS). The positively charged lipsomes were not effective at cell depletion, and doubling the liposomal cholesterol concentration completely abrogated cell killing (**Figure 2.1)**. We then took the best liposome formulation from these initial experiments and modified it further. As tumor associated macrophages have been shown to express a mannose receptor, we sought to further

optimize the liposomes by incorporating a mannose subgroup into the liposomes. The addition of mannose to the liposomes led to significantly ($p < 0.05$) increased killing of Raw 264.7 (Raw) cells when compared to the PC liposomes alone (**Figure 2.1).** These experiments were repeated in 3 additional mouse macrophage cell lines with similar results. Data shown are representative of 3 independent experiments.

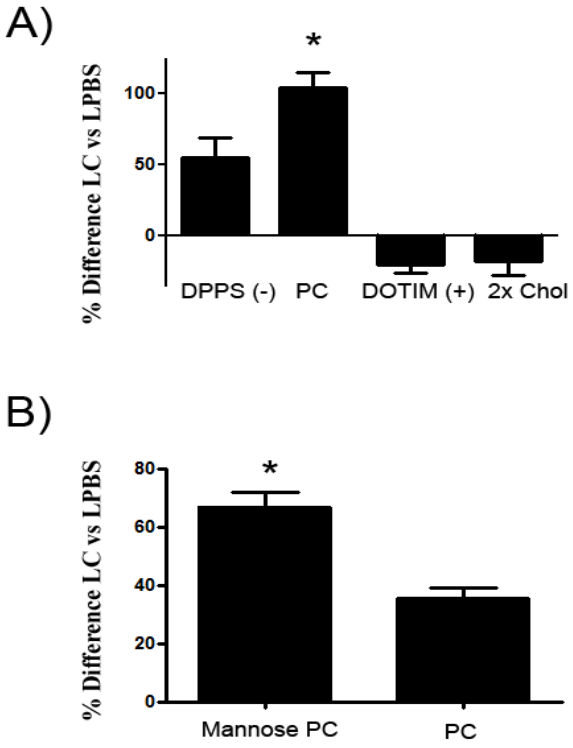

Figure 2.1: Alteration of the charge and surface properties of liposomes increases cell killing. A) Raw cells were incubated with liposomes containing positive (DOTIM), negative (DPPS) or neutral surface charge (PC). PC liposomes demonstrated significantly ($p < 0.01$) increased cell killing as measured by 1 way ANOVA. B) Raw cells were treated with PC liposomes with or without a mannose group. Mannosylated PC liposomes showed significantly ($p < 0.05$) increased killing as measured by Student's T test.

Mannosylated liposomes specifically kill phagocytic cells

We next sought to determine if adding the mannose targeting ligand changed the specificity of the liposomal clodronate as changing the liposome properties could potentially lead to an increase in non specific leakage of clodronate from the liposome, leading to increased cell death even in cells that do not actively phagocytize the

liposomes. If this were indeed the case these liposomes would not be as effective in vivo as the active drug would likely leak into circulation before it could be phagocytized by the target cells. In order to exclude this possibility, both the phagocytic Raw macrophage cells and MCA 205 fibrosarcoma cells, which are not phagocytic, were incubated with mannosylated LC. After 72 hours the cells were analyzed via the MTT assay. We were able to show that while mannosylated LC was again able to effectively inhibit the growth of Raw cells, the drug had no effect on the MCA 205 cells (**Figure 2.2)**. To further demonstrate that this effect was due to the ability of the cells to phagocytize the liposomes, we created fluorescent liposomes using the green fluorescent cholesterol Bodipy in lieu of standard cholesterol. PBS containing liposomes were then created using the Bodipy cholesterol so that cells that took up the liposomes would not be killed prior to analysis. The cells were then incubated with the liposomes for 6 hours and analyzed via flow cytometery to determine the number of FITC positive cells. We were able to demonstrate that the Raw cells were able to very efficiently take up the liposomes, with >90% of the cells staining FITC positive. The MCA 205 cells showed significantly ($p < 0.05$) decreased uptake of the fluorescent liposomes, further showing that phagocytosis is required for cell killing (**Figure 2.2)**

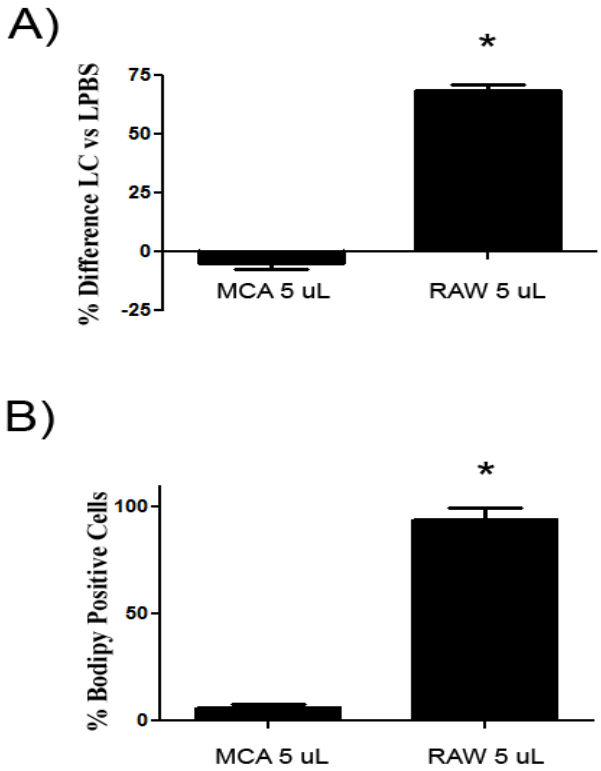

Figure 2.2: Phagocytosis is required for cell killing. A) Raw and MCA 205 cells were treated with mannosylated LC for 72 hours and analyzed via the MTT assay. Raw cells were killed at a signficantly ($p < 0.05$) higher rate than MCA 205 cells. B) Raw and MCA 205 cells were treated with fluorescent liposomes for 6 hours and analyzed via flow cytometry. Raw cells showed significantly ($p < 0.05$) increased liposome uptake as compared with MCA 205 cells. Results are representative of 3 independent experiments. Student's T test used for analysis.

Mannosylated liposomal clodronate is most effective at depleting macrophages in vivo

We next sought to determine if the in vitro results obtained against mouse macrophage cell lines would correlate to activity of the liposomes in vivo. In order to do this, ICR mice (n = 5 per group) were injected iv with 200 µL of clodronate containing liposomes. The top three liposome formulations from the in vitro studies were used for this experiment. Therefore, mice were injected with either DPPS (net negative charge), PC (net neutral charge), or mannosylated PC liposomes. Twenty four hours later the mice were sacrificed along with 5 untreated control mice. Splenic cells were isolated and

stained for flow cytometry. For these analyses, macrophages were defined as $Ly6G^-$, $F4/80^+$, and $CD11b^+$. We found that just as in the in vitro studies, mannosylated liposomal clodronate was most effective at depleting macrophages in vivo. We were able to see a significant ($p < 0.05$) decrease in macrophages in all treated groups as compared to control. However, the mannosylated liposomal clodronate group showed the most effective depletion, and was significantly ($p < 0.05$) decreased as compared to both the control and all other groups as analyzed by 1 way ANOVA with Tukey's post test (**Figure 2.3)**.

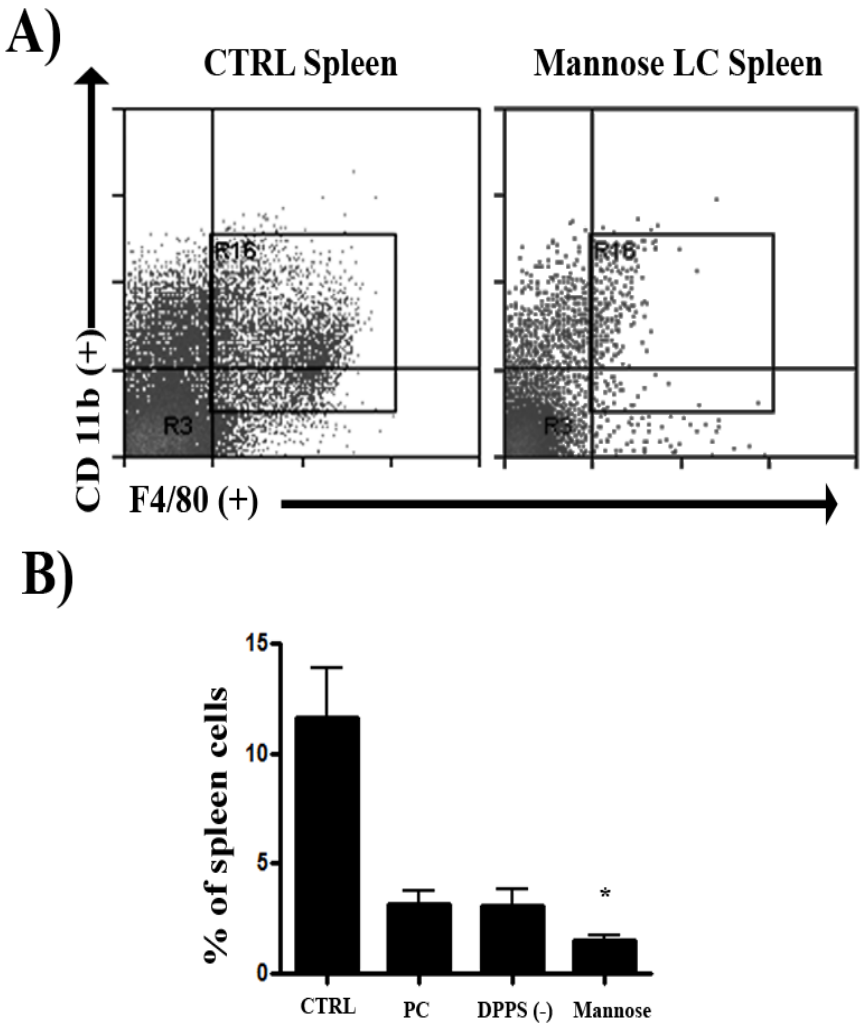

Figure 2.3: Mannosylated LC is most effective at depleting macrophages in vivo. ICR mice (n = 5 per group) were injected iv with 200 uL of either PC, DPPS, or mannosylated LC. 24 hours later the mice were sacrificed along with 5 untreated control mice and spleen cells were harvested and analyzed via flow cytometry. A) FACS plot showing depletion of CD 11b and F4/80 double positive cells with mannosylated LC. B) Bar graph showing macrophage depletion using each of the 3 liposome groups. Mannosylated LC showed significantly ($p < 0.05$) more depletion as measured by 1 way ANOVA.

In order to determine if multiple tissue types are affected by mannosylated LC, a second experiment was performed using LC injected IV into ICR mice. As previously, the mice were injected with 200 µL of mannosylated LC IV and sacrificed after 24 hours. The lungs, livers, and spleens of both treated mice (n=5) and untreated control (n=5) mice were harvested, stained, and analyzed via flow cytometry. We were able to show

that not only splenic macrophages, but also alveolar macrophages and Kupffer cells are significantly ($p < 0.05$) depleted by mannosylated LC (**Figure 2.4).**

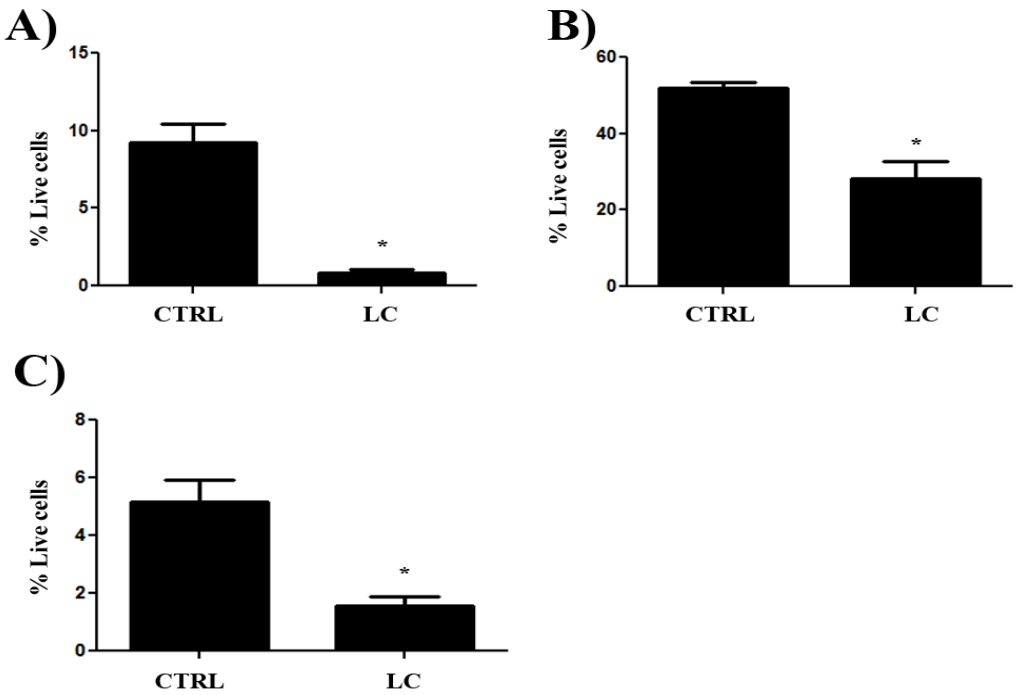

Figure 2.4: Macrophages are depleted in multiple tissue types. ICR mice (n=5 per group) were treated with 200 uL of mannosylated LC iv. 24 hours later the mice were sacrificed and spleen, liver, and lung tissues were analyzed for depletion of macrophages. A) Splenic macrophages are significantly ($p < 0.05$) depleted by LC as compared to control. B) Kupffer cells in the liver are signficantly ($p < 0.05$) depleted by LC. C) Alveolar macrophages in the lung are significantly ($p < 0.05$) depleted by LC. All results are analyzed by Student's T test.

Mannosylated LC is effective at decreasing tumor growth in vivo

We next sought to determine if mannosylated LC would be effective at decreasing tumor growth. We chose the MCA 205 tumor model, as we had already demonstrated that LC does not have any direct effects on the tumor cells as they do not phagocytize the liposomes (**Figure 2.2).** Therefore any anti-tumor effects would be due effects other than direct tumor cell killing by the drug. In these experiments, C57Bl\6 mice (n=5 per group) were injected subcutaneously in the right flank with MCA 205 tumor cells. Three days later, the mice were injected with 200 µL of mannosylated LC or mannosylated

liposomal PBS IV. A third group of untreated mice were used as a control. The mice were then injected once per week thereafter with the same volumes of liposomes IV. Tumor measurements were performed twice per week to monitor tumor growth. All mice were sacrificed when any mouse reached a tumor size of 1.5 cm. Fine needle aspirations of the tumors were performed at weekly intervals and stained for flow cytometry. As previously, macrophages were defined as $Ly6G^-$, $F4/80^+$, and $CD11b^+$. We were able to show that liposomal clodronate caused a significant decrease ($p < 0.05$ as measured by 2 way ANOVA) in tumor growth as compared to either liposomal PBS or untreated control mice (**Figure 2.5**). We were also able to demonstrate a significant decrease ($p < 0.05$ as measured by 1 way ANOVA) in tumor associated macrophages in the liposomal clodronate treated group as compared with either the liposomal PBS group or the untreated control group in fine needle aspirations performed at day 20 (**Figure 2.5)**.

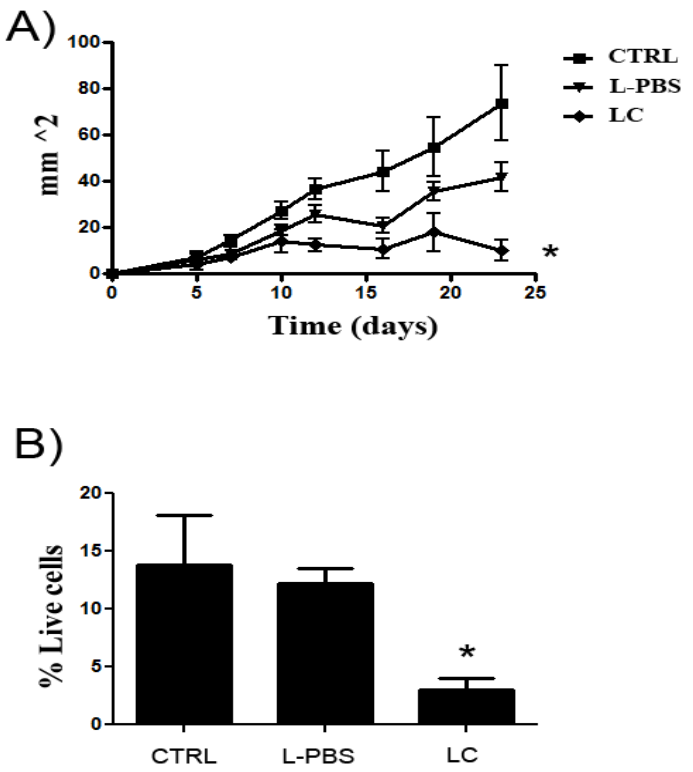

Figure 2.5: Mannosylated LC causes a significant decrease in tumor growth by depletion of TAMs. A) Tumor growth curve showing a significant ($p < 0.05$) decrease in tumor growth in LC treated mice as compared to L-PBS or control mice. B) Bar graph showing a significant ($p < 0.05$) decrease in TAMs in LC treated mice as compared to L-PBS treated mice or control mice. Results are representative of two independent experiments.

TAM depletion occurs despite minimal uptake of liposomes in the tumor tissue

We next sought to determine if depletion of TAMs occurred due to the uptake of liposomes by these macrophages directly. In order to test this fluorescently labeled lipsomes were prepared using Bodipy cholesterol and were rehydrated in PBS. C57Bl\6 mice (n=5 per group) were injected with MCA 205 tumors subcutaneously. After 2 weeks of tumor growth, the mice were injected with 200 µL of the fluorescent PBS liposomes IV. After 6 hours the mice were sacrificed and spleen, blood, tumor draining lymph node (TDLN), and tumor tissues were analyzed by flow cytometry. We were able to show a significant ($p < 0.05$ as measured by 1 way ANOVA) amount of liposome

uptake in the spleen as compared to other tissues. There was minimal uptake of liposomes in the TDLN, blood, or the tumor (**Figure 2.6**).

When cell populations in the spleen were further analyzed, there was a large amount of liposome uptake by macrophages and myeloid suppressor cells (MSC) in the spleen. Both of these cell types are depleted by liposomal clodronate. There was minimal uptake by T cells and dendritic cells (DC) which are not affected by liposomal clodronate. Interestingly, there was a large amount of liposome uptake by B cells, which are also not affected by liposomal clodronate (**Figure 2.6**). In order to determine the discrepancy between the percentage of positive cells and the lack of cellular depletion, we then looked at mean fluorescence intensity (MFI) of the cellular populations. We were able to see that the macrophages had a significantly ($p < 0.05$ as measured by Student's T test) higher MFI than B cells, indicating that macrophages take up more liposomes per cell than B cells. This may explain why macrophages are killed by liposomal clodronate why B cells are unaffected (**Figure 2.6**).

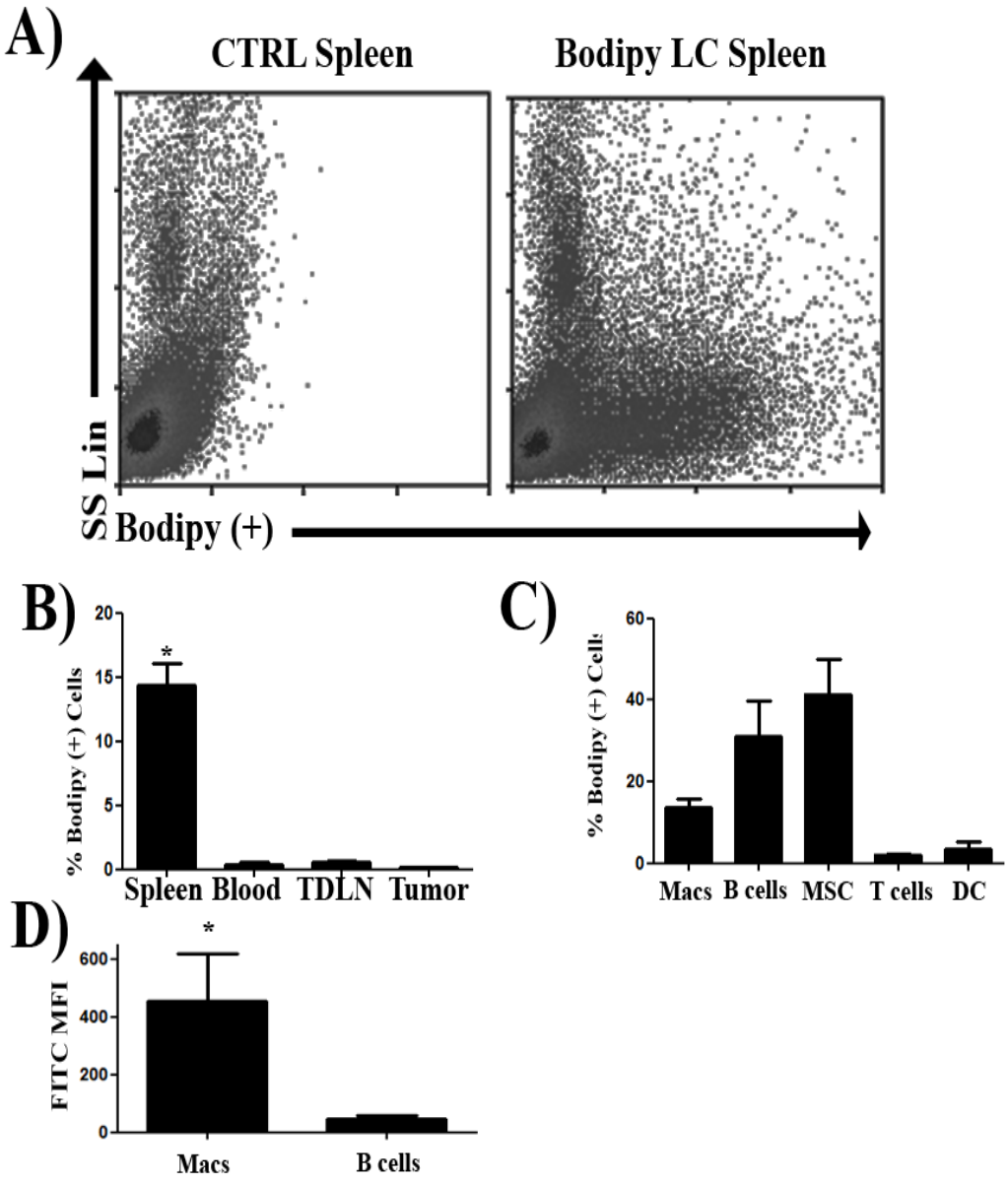

Figure 2.6: TAM depletion occurs despite lack of liposome uptake by tumor tissues. Tumor bearing mice were injected with Bodipy fluorescent PBS liposomes. After six hours the mice were sacrificed and tissues were analyzed by flow cytometry. A) FACS plots showing an increase in Bodipy + cells in the spleens of treatem mice as compared to untreated control. B) Bar graph showing a signficant ($p < 0.05$) increase in Bodipy + cells in the spleen compared to other tissues. C) Bar graph showing uptake by cellular populations in the spleen. B cells make up a significant percentage of Bodipy + cells though they are not depleted. D) Bar graph showing a significant ($p < 0.05$) increase in mean fluorescence intensity (MFI) between macrophages and B cells. Results are representative of two independent experiments.

Discussion

Liposomal clodronate has been used extensively in studies of autoimmune disease, and has recently showed promise as an anti-tumor agent[18, 28, 30-32, 38]. However, in order to be effective as a systemic agent, the drug will have to attain maximum macrophage depletion. We report here for the first time that altering the liposome properties can lead to more efficient macrophage killing. The addition of a mannose group to a net neutral charged liposome was more effective than any other liposome tested. We were able to observe a significant increase in macrophage killing across all four cell lines tested. We also saw a significant decrease in macrophage percentage in the spleen using the mannosylated liposomal clodronate as compared to either negatively charged or neutral charged liposomes without the mannose subgroup. These liposomes were able to effectively deplete macrophages in the spleen, lungs, and liver. They were also to cause a significant decrease in tumor growth using the MCA 205 fibrosarcoma model, and this decreased growth was associated by a decrease in tumor associated macrophages in the tumor tissue. However, when we used fluorescent liposomes to track uptake of the liposomes in vivo, we did not see significant liposomal uptake in the tumor tissues.

Altering the overall charge of the liposome had a significant impact on the ability of the liposome to kill macrophages. It is unclear why using a net positive or a net negatively charged liposome had negative effects on the ability of LC to kill macrophages. However, as clodronate is a highly charged molecule[21], it is possible that these changes in liposome charge changed the ability of the liposomes to incorporate

clodronate, particularly using a positive headgroup as this could potentially repel the clodronate and prevent efficient incorporation into the liposome. The reverse could be true with the negatively charged lipids, as tight binding to the clodronate could prevent release into the cytoplasm and subsequent cell killing once ingested. Further studies will be necessary to determine if changes in charge change the incorporation kinetics of clodronate into liposomes, however this data would suggest that alterations in charge are not recommended for producing effective liposomal clodronate.

Increasing the cholesterol content of the liposomes also had a negative effect on the ability of LC to kill cells. Again it is unclear why this would be the case. However, since increased cholesterol content increases the stability of cells it is possible that the liposomes become more difficult to break down in the lysosome. This would make it harder for the cell to release the free drug which would lead to a decrease in cell killing.

We were able to show that changing the surface properties of the liposome by adding a targeting ligand was able to increase the efficacy of cell killing by LC. We chose the mannose group specifically because it is expressed by macrophages, particularly M2 polarized macrophages which are most commonly implicated in playing a positive role for the tumor cells in the tumor microenvironment[3, 39, 40]. This compound is expected to integrate into the liposome and is relatively inexpensive to include in the liposome production process. Interestingly, this modification appeared to increase macrophage killing in all of the cell lines tested as well as against many different macrophage types in vivo. This indicates that the effect is likely not specific to M2 macrophages, and as such may not be specific to cells that express the mannose receptor. Further studies could be undertaken to determine if cells that express the mannose

receptor demonstrate a higher uptake of the mannosylated liposomes. There may be other targeting ligands that would also increase the efficacy of LC, however this has yet to be determined.

Another strategy may be to increase the specificity of the lipsomes to target specifically M2 macrophages or other immunosuppressive cells. However, this approach must be taken with some caution as our results indicate that systemic administration of liposomal clodronate does not result in direct uptake of the liposomes by cells already present in the tumor stroma. While we see a reduction in TAMs that is associated with a decrease in tumor growth, studies with fluorescent liposomes failed to see significant liposome uptake in the tumor. The secondary immune effects of systemic administration may therefore be the primary mechanism of action. This also suggests that it is the non specific killing of TAM precursors that depletes the pool of macrophage precursors available for recruitment to the tumor. Therefore increased specificity of the liposomes might in fact decrease the efficacy of the drug when given systemically by decreasing efficacy against these immunosuppressive precursors. Further studies, in particular the effect of LC against precursor cells such as myeloid suppressor cells, will be required to fully elucidate the mechanism of macrophage depletion despite lack of local uptake.

As the treatment of cancer continues to evolve, true multimodal treatment regimes that target not only the tumor cells specifically but also their stromal support tissues will likely be important to increasing the effectiveness of therapy. Further studies will be necessary to determine if TAM depletion by mannosylated liposomal clodronate can be combined with traditional chemotherapeutics to increase the effectiveness of both agents.

These results suggest that mannosylated liposomal clodronate should be used in future studies in order to better understand the role of macrophages in tumors. Given its anti-tumor activity as a single agent it should also be further evaluated as a potential cancer therapeutic drug as well.

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Chapter Three

Evaluation of Liposomal Clodronate for Treatment of Malignant Histiocytosis in Dogs

Abstract

Malignant histiocytosis (MH) is an aggressive cancer derived from myeloid lineage cells in both dogs and humans. In dogs, the tumor is characterized by the rapid development of metastatic tumors in multiple sites, including especially the lungs and lymph nodes. Humans develop an analogous disease known as Langerhans cell histiocytosis, which primarily affects children and young adults. Because these tumors are often resistant to conventional chemotherapy, there is a need for newer therapeutic approaches. Systemic administration of liposomal clodronate (LC) has been shown to effectively deplete phagocytic cells (eg, macrophages and dendritic cells) in mice. We investigated therefore whether LC could also be used to treat naturally-occurring MH in dogs. First, the susceptibility of canine MH cells to LC-mediated inhibition was assessed in vitro. Then the clinical safety and effectiveness of LC as a treatment for MH was assessed in a pilot study in 5 pet dogs with spontaneous MH. We found that canine MH cells were very susceptible to LC-induced apoptotic cell death, whereas other tumor cell lines were

resistant to inhibition by LC. Studies using labeled liposomes demonstrated that susceptibility to LC inhibition was directly related to the efficiency of liposome uptake. These findings suggest that liposomal delivery of clodronate and possibly other bisphosphonates may offer an effective new approach to treatment of histiocytic neoplasms in dogs and humans.

Introduction

With our success in developing a version of liposomal clodronate that could be administred effectively systemically, we next sought to test this drug in dogs with spontaneous tumors. We chose malignant histiocytosis, as this is a tumor comprised o macrophages and thus the drug could have primary anti-tumor effects as well as secondary effects by depletion of TAMs. This tumor also needs new treatment options. Malignant histiocytosis (MH) in dogs (also known as histiocytic sarcoma) is a neoplasm of histiocytic cell origin, which is thought to arise from either macrophage or dendritic cell (DC) precursors [1-4]. A similar malignancy known as Langerhans cell histiocytosis also develops in humans [5-7]. Malignant histiocytosis in dogs is rapidly metastatic, and often involves multiple organs including lung parenchyma, bone marrow, spleen, liver, and lymph nodes[8].

Thus, MH in dogs serves as a valuable spontaneous tumor model for Langerhans cell histiocytosis in humans[2, 5, 9]. Malignant histiocytosis in dogs also progresses very rapidly and is uniformly fatal, with reported median survival times of 2 to 4 months [3, 8, 10] The rapid and aggressive metastases that develop in dogs with MH disease often render conventional treatment modalities such as radiation therapy and surgery ineffective. Chemotherapy has also been typically unrewarding for treatment of MH in dogs, with treatment responses to corticosteroids and lomustine often transient and incomplete[3]. Given the generally aggressive nature of histiocytic malignancies in both dogs and humans, new approaches to treatment are needed.

Liposomal clodronate (LC) has been used extensively to deplete macrophages in mice for investigation of normal and pathological immune responses in animal models of infection, vaccination, and autoimmune diseases [11-14]. A large body of literature indicates that systemic administration of LC in mice can elicit rapid and effective macrophage depletion [11, 12, 15-17]. Clodronate is a bisphosphonate drug that kills osteoclasts and other macrophages via induction of apoptosis, possibly mediated by competition with ATP as a substrate for intracellular ATPase [18-21]. When clodronate is incorporated within liposomes, uptake by phagocytic cells such as macrophages is greatly enhanced, resulting in selective targeting of macrophages for inhibition [11, 13, 22].

Liposomal clodronate has also been used successfully to treat autoimmune hemolytic anemia (AIHA) in a rodent model and more recently in dogs with spontaneous AIHA [16, 23]. In the study in dogs, LC was safely administered i.v. to dogs and induced functional depletion of macrophages that was sufficient to block destruction of opsonized erythrocytes *in vivo*. Liposomal clodronate also induced in vitro inhibition of splenic macrophages from dogs[23].

Since MH in dogs is a tumor of macrophage and DC origin, we wondered if LC would be an effective agent for inhibition MH cells, and also whether the drug could be administered systemically to treat dogs with spontaneous MH. Therefore, we investigated the ability of LC to kill canine MH cells *in vitro* and assessed mechanisms of cell inhibition. We also conducted a pilot study to assess the safety and efficacy of LC therapy for treatment of 5 dogs with advanced MH tumors. We found that LC effectively killed canine MH cells in vitro, primarily via delayed induction of apoptotic cell death.

We conclude that LC has promise as a novel agent for treatment of histiocytic tumors in humans and dogs.

Materials and Methods

Cell culture and tumor cell lines

The canine MH tumor cell line DH82 and the canine osteosarcoma cell line D17 were both obtained from the American Type Tissue Collection (Gaithersburg, MD)[1]. Two other MH cell lines were established from primary cultures of biopsies obtained from dogs with MH and were a kind gift of Dr. Peter Moore (College of Veterinary Medicine, University of California-Davis, Davis, CA). The canine thyroid carcinoma cell line CTAC was a kind gift of Dr. Stuart Helfand (School of Veterinary Medicine, University of Wisconsin). The canine melanoma cell line Mel-J was derived from primary culture of a dog with malignant melanoma[24]. All cell lines were maintained in MEM (minimal essential medium, Invitrogen, Grand Island, NY USA) supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT), non-essential amino acids, L-glutamine, sodium bicarbonate, penicillin and streptomycin. The cell lines were maintained at 37° C in a humidified atmosphere containing 5% CO_2.

Preparation of liposomal clodronate and liposomal PBS

Liposomal clodronate (LC) was prepared as previously described[23]. Briefly, phosphatidylcholine and cholesterol (both purchased from Avanti Polar Lipids, Alabaster AL, USA) were dissolved in chloroform and combined at a 5:1 molar ratio in a glass round bottom tube and dried to completeness overnight in a vacuum lyophilizer (VirTis,

Gardiner, NY, USA). To prepare mannose-containing liposomes, p-amino phenyl mannopyranoside (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in methanol and added at 1.75 mg per 25 mg of phosphatidylcholine and dried down together with the phosphatidylcholine and cholesterol lipids. Liposomes were prepared by rehydration in a concentrated solution of clodronate (Sigma-Aldrich) as described previously[23]. Liposomal PBS was prepared in a similar fashion, using a 1.5M stock of PBS instead of clodronate to rehydrate the liposomes.

For the preparation of fluorescent liposomes, 0.5 mL of a 1mM solution of BODIPY cholesterol (Invitrogen, Eugene, OR) was added to the phopshatidylcholine and cholesterol lipid solution and dried down prior to rehydration.

MTT assay for cell viability

Cell viability was assessed using the MTT assay, as described previously[25]. Briefly, MTT (thiazolyl blue tetrazolium bromide, Sigma-Aldrich. St Louis, MO) was added to wells containing live cells and incubated for 2 hours at 37C. The cells were then dissolved in a 0.1N HCl solution in isopropanol and the absorbance was determined using an ELISA plate reader (Multiscan Ascent, Thermo Labsystems, Cambridge, MA). Cell viability was calculated as the mean percent absorbance of the treated wells compared to the mean absorbance of the untreated control wells, with the inverse of this value representing the percentage inhibition. The percentage specific inhibition was determined as the difference between the percentage inhibition elicited by LC and the percentage inhibition elicited by PBS liposomes.

In vitro assessment of MH cell inhibition

Cells were pipetted into quadruplicate wells of a 96-well flat bottomed plate at a cell density of 4 X 10^3 cells/well and this cell density was used throughout the *in vitro* assays. Cells were allowed to adhere for 24 hours and were then treated with LC or L-PBS at volumes of 1%, 2.5%, or 5% v/v in complete tissue culture medium. The LC concentrations used were determined in serial dilution experiments which demonstrated minimal specific inhibition at LC concentrations less than 1% v/v, as well as no additional increase in activity at LC concentrations greater than 5% v/v (data not shown). Malignant histiocytosis cells were incubated with LC or PBS liposomes for 72 hours, as previously described for inhibition of mouse macrophages with LC in vitro[13]. In some experiments, MH cells and non-phagocytic tumor cells (carcinoma [CTAC], melanoma [Mel-J], and osteosarcoma [D17]) were also incubated with free clodronate at varying concentrations. Finally, DH82 cells were incubated with either free clodronate or liposomal clodronate at equivalent concentrations, and analyzed via the MTT assay after 72 hours of incubation. A concentration of 1.7 mM of free clodronate was calculated to be equivalent to the amount of clodronate contained in 5 uL of LC, based on a previous study done using ^{99m}Tc labeled clodronate [11].

Generation of canine monocyte-derived macrophages

Blood monocytes were obtained by plastic adherence from blood of normal healthy dogs after separation of peripheral blood mononuclear cells by Ficoll density separation. The cells were incubated for 3 hours at 37°C and then all non-adherent cells were discarded. The cells were cultured in DMEM (Dulbecoo's modified eagle medium, Invitrogen, Grand Island, NY USA) supplemented with 1% glutamate, 10% heat inactivated fetal bovine serum, non-essential amino acids, L-glutamine, sodium bicarbonate, penicillin and streptomycin, with the addition of 10% v/v of L929 cell conditioned medium, as described previously [26]. Cells were cultured in medium for 10 days prior to treatment with LC and assessment of cell inhibition.

Measurement of liposome uptake by flow cytometry

BODIPY-labeled liposomes and flow cytometry were used to quantitate liposome uptake by MH and other tumor cell lines. Cells were re-suspended at a concentration of 5.0×10^5 cells/mL and incubated with serial dilutions of Bodipy-labeled PBS liposomes in complete medium for 4 hours at 37˚C, with periodic shaking to assure even distribution and uptake of liposomes. The cells were washed twice to remove unbound liposomes. In most experiments, incubation with trypan blue was used to quench the fluorescence emitted by surface bound but non-internalized liposomes prior to analysis by flow cytometry. Briefly, trypan blue quenching was accomplished by incubating samples with trypan blue (50 ul of a 0.008% solution of Trypan Blue (Sigma-Aldrich, St.

Louis) diluted in 1 ml of cells) in PBS for 15 minutes. The percentages of internalized and surface-bound liposomes were calculated by analyzing samples before and after blue quenching. Flow cytometry was done using a Cyan-ADP flow cytometry (Beckman-Coulter, Ft Collins, CO) and analysis was done using Summit software (Beckman-Coulter).

Determination of apoptosis by Annexin V and propidium iodide and flow cytometry

Detection of apoptotic cells was done using an Annexin V and propidium iodide (PI) assay and flow cytometry, as previously described[27, 28]. Briefly, cells in triplicate wells were treated with the indicated volume of liposomes for periods of 12 to 72 hours. A positive control for apoptosis was included with each experiment and consisted of cells incubated for 6 hours with a 4.5 μM solution of camptothecin (Sigma-Aldrich). After incubation with LC or L-PBS, cells were detached and washed and then stained with FITC-conjugated Annexin V, according to manufacturer's directions (BD Biosciences, San Jose CA). Immediately prior to analysis by flow cytometry, PI was added to the cells to identify dead cells. Early apoptotic cells were defined as $Annexin^{+}$ and PI^{-}, while cells in mid-apoptosis were defined as $Annexin^{+}$ and PI^{+}, and dead cells were defined as $Annexin^{-}$ and PI^{+}.

Investigation of LC treatment in dogs with MH

A study of LC therapy for treatment of pet dogs with MH was conducted in 5 dogs with biopsy-confirmed tumors. The LC treatment study was approved by the

Institutional Care and Use Committee at Colorado State University. A pre-treatment complete blood count (CBC), chemistry panel and urinalysis were performed in all animals and serum and plasma were frozen for further analysis. The dogs were not allowed to be on NSAID, steroid, or any other anti-inflammatory medication during the duration of the study, however there was no washout period prior to enrollment. Dogs were treated by i.v. administration of LC at a dose of 0.5 mL/kg, over a 60-minute period, as described previously. The treatment was repeated every 2 weeks until a total of 6 treatments or until determination of progressive disease. Dogs were monitored for the first 24 hours after treatment for changes in body temperature and heart rate and respiratory rate. Twenty-four hour post treatment a recheck CBC was run and serum and plasma were collected and frozen at -80° C. A CBC was checked prior to each of the additional treatments.

Statistical analysis

Comparison between two treatment groups was done by Student's paired t-test. For comparison of multiple treatment groups, ANOVA was used, followed by Bonferroni's multiple means comparison test. Analysis was done using GraphPad Prism software (GaphPad, San Diego, CA). Differences were considered statistically significant for p values less than 0.05.

Results

Assessment of MH cell susceptibility to LC-induced inhibition

Previous studies have shown that administration of LC effectively depletes macrophages in mice, both in vitro and in vivo[11-13, 15-17]. Therefore, we conducted in vitro assays using 3 different canine MH cell lines (DH82, MH-1, and MH-2) to determine their susceptibility to LC-induced inhibition. These cell lines were all derived from dogs with spontaneous MH malignancies and all 3 have been shown to possess characteristics typical of both DC and macrophages[1, 10, 29]. Liposomal clodronate was prepared for these studies using phosphatidylcholine liposomes, as described previously[23]. However, in our studies the PC liposomes were also modified by the addition of a mannosylated aminophenyl group**,** since we found that this modification increased uptake and inhibition of murine macrophages and canine MH cells (data not shown). Cells were incubated with indicated dilutions of LC for 72 hours and cell viability was assessed using MTT assay[25]. Non-specific cytotoxic effects of liposomes were controlled for by using liposomes prepared using concentrated PBS instead of clodronate.

We found that the DH82 cell line was the most susceptible to growth inhibition by LC, compared to the other two MH cell lines (**Figure 3.1**). For example, incubation with 5% LC induced a 69% loss in cell viability, whereas incubation with 5% L-PBS induced only a 2.5% loss in cell viability. In addition, LC also elicited significant specific

inhibition of the other two MH cell lines, also in a dose-dependent fashion. These data indicated therefore that canine MH cells were highly susceptible to LC growth inhibition.

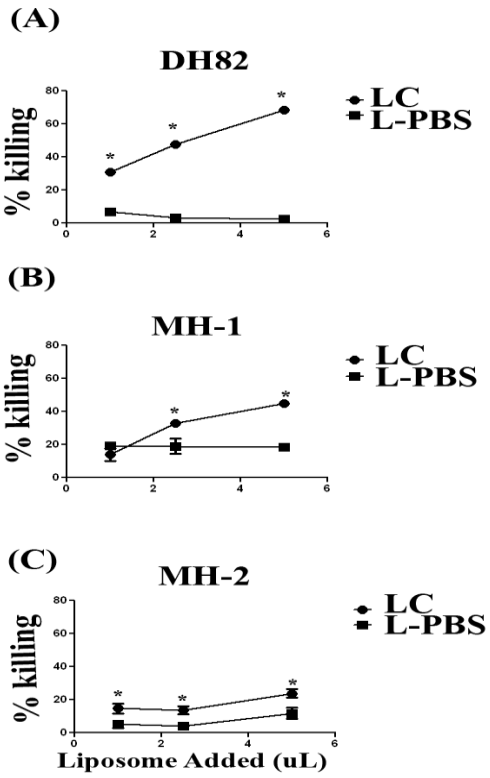

Figure 3.1: Assessment of LC-induced killing of canine MH cells in vitro. Quadruplicate wells containing 4 9 103 MH cells per well were treated with the indicated concentration of liposomal clodronate (LC) or PBS liposomes (L-PBS), incubated for 72 h, then cell viability was assessed by MTT assay, as described in ''Methods''. Results are reported as the mean (±SEM) percentage killing. In a–c, the doseresponsiveness of DH82 cells, MH-1 cells, and MH-2 cells, respectively, to incubation with LC and with L-PBS is depicted. Differences in the percentage of killing elicited by treatment with LC and L-PBS were compared at each time point using Student's t test. (* denotes statistically significant differences of p\0.05). These data are representative of three independent experiments

Susceptibility of non-phagocytic tumor cells and MH cells to inhibition by free clodronate

Free clodronate has been shown to kill osteoclasts, which is thought to be the mechanism by which clodronate reduces bone pain associated with skeletal metastases[30-32]. Moreover, it is also known that free clodronate and other bisphosphonates can elicit cytotoxicity against certain tumor cells [19, 33, 34]. Therefore, we assessed and compared the susceptibility of a variety of non-phagocytic tumor cell lines and MH cell lines to varying concentrations of free clodronate. We also evaluated the susceptibility of MH cells to inhibition by free clodronate versus LC. For these experiments, we used a free clodronate concentration (1.7 mM) that was calculated to be equivalent to the total amount of clodronate contained in 5 uL of LC, based on determinations from a previous study[11].

We found that all the tumor cell lines evaluated were susceptible to inhibition by free clodronate in a dose dependent manner (**Figure 3.2**). There were no significant differences in the dose response curves among the different cell lines evaluated. Interstingly, two out of the three non-phagocytic cell lines showed significantly more susceptibility to free clodronate than DH82 cells at high concentrations (**Figure 3.2)**. Similar results were obtained for the other two MH cell lines evaluated (MH-1 and MH-2, data not shown).

We next compared the degree of inhibition achieved in MH cells between free clodronate and liposomal clodronate. The degree of inhibition elicited in MH cells by free clodronate was significantly lower than that generated by LC treatment, when

adjusted for addition of equivalent amounts of clodronate. In addition, given the rapid distribution of free clodronate into bone following systemic administration, it is very unlikely that such a high, sustained dose of clodronate or other bisphosphonate drug could be attained *in vivo* [35-37].

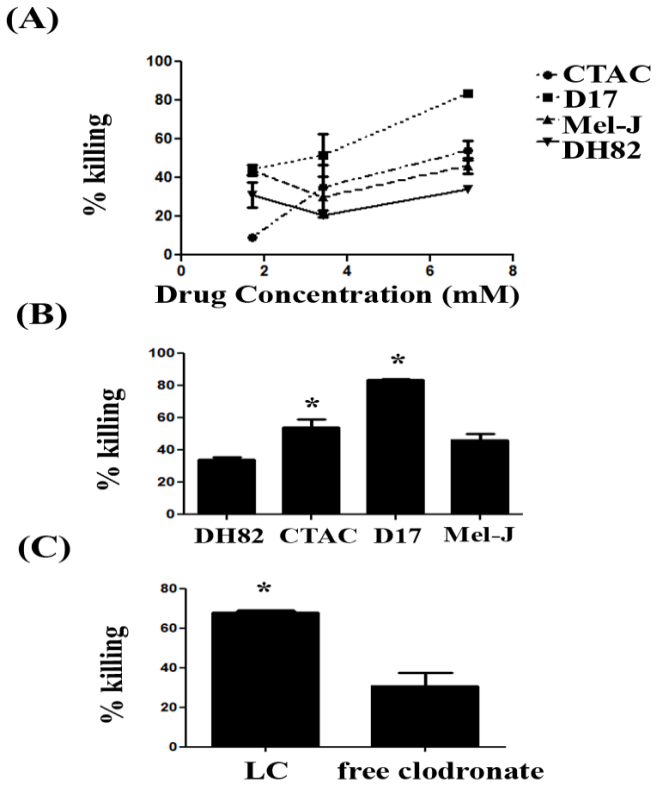

Figure 3.2: Susceptibility of tumor cells to killing by free clodronate versus liposomal clodronate. Killing of a canine MH cell line (DH82) was compared to killing of three non-phagocytic canine tumor cell lines (thyroid carcinoma {CTAC}, malignant melanoma {Mel-J} and osteosarcoma {D17}). The percentage of killing was determined after 72 h of incubation with varying concentrations of free clodronate (a) or at a maximal concentration of 6.7 mM free clodronate (b). Differences amongst the groups were assessed using ANOVA followed by Bonferroni's post-test. Although there were no significant differences between the dose response curves (a), there was significantly more killing (*, p\0.05) in the D17 and CTAC cell lines when compared to DH82 cells at the 6.7 mM concentration of clodronate. In (c), DH82 cells were incubated with a 5%solution of LC or with 1.7 mM solution of free clodronate for 72 h and cell viability was assessed by MTT assay.

Effects of LC treatment on non-phagocytic tumor cell lines

Experiments were conducted next to determine whether LC was capable of inhibiting non-phagocytic tumor cell lines as effectively as the phagocytic MH cell lines. Therefore, inhibition of 3 non-phagocytic canine tumor cell lines by LC, including carcinoma (CTAC cells), sarcoma (D17 cells) and melanoma (Mel-J cells) cell lines was compared to inhibition of MH cell lines.

We found that all 3 non-phagocytic cell lines were relatively refractory to inhibition by LC at all doses evaluated (**Figure 3.3**). However, when incubated with free clodronate, all three of these tumor cell lines were susceptible to cell inhibition by clodronate (data not shown). These results indicated therefore that the resistance of non-phagocytic cells to inhibition by LC was not mediated by inherent resistance to the cytotoxic effects of clodronate, but was instead more likely related to decreased uptake of LC.

We also compared the relative susceptibility of the 3 different canine MH cell lines to inhibition by LC and found that there were substantial differences between the 3 lines (**Figure 3.3**). For example, the DH82 cell line was significantly more susceptible to LC-mediated inhibition than either the MH-1 or MH-2 cell lines. These findings indicated that there might be substantial tumor-to-tumor heterogeneity in susceptibility to LC inhibition in dogs with MH tumors, or potentially in humans with Langerhans histiocytosis.

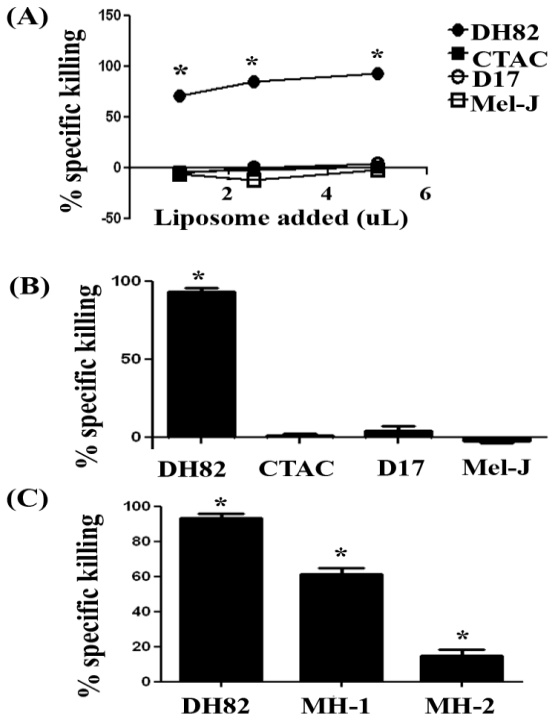

Figure 3.3: Susceptibility of phagocytic and non-phagocytic tumors to killing by LC. Killing of canine MH cell lines (DH82, MH-1, MH-2) by LC was compared to killing of three non-phagocytic canine tumor cell lines (thyroid carcinoma {CTAC}, malignant melanoma {Mel-J} and osteosarcoma {D17}). In (a), dose response curves of LC percentage specific killing were determined for the DH82 cell line and three nonphagocytic tumor cell lines (CTAC, D17, Mel-J). In (b), the percentage specific killing elicited by 5% LC was plotted for MH cells (DH82) and three non-MH tumor cell lines. Specific killing was significantly greater (*, p\0.05) for DH82 cells than for the other three tumor cell lines. In (c), **LC-specific killing of three different MH cell lines was compared, using LC at a concentration of 5%. Killing was significantly greater (*, p\0.05) for DH82 cells than for the other two MH cell lines.**

Susceptibility of canine monocyte-derived macrophages to inhibition by LC

The preceding experiments established that MH cells were very susceptible to growth inhibition by LC. However, we also wished to compare the relative LC susceptibility of malignant MH cells (derived from macrophages and/or DC) and non-transformed canine macrophages. Therefore, primary cultures of canine monocyte-derived macrophages were established and the cells were incubated with LC for 72 hours and cell inhibition was assessed. We found that LC induced significant specific inhibition of canine monocyte-derived macrophages (**Figure 3.4**). Interestingly, two of

the three canine MH cell lines were much more susceptible to LC inhibition than monocyte-derived macrophages. For example, at a 5% concentration of LC, 32% specific inhibition of monocyte-derived macrophages was observed, whereas there was 69% inhibition of DH82 cells and 45% inhibition of MH-1 cells. Thus, MH cells may be inherently more susceptible to LC inhibition than normal macrophages.

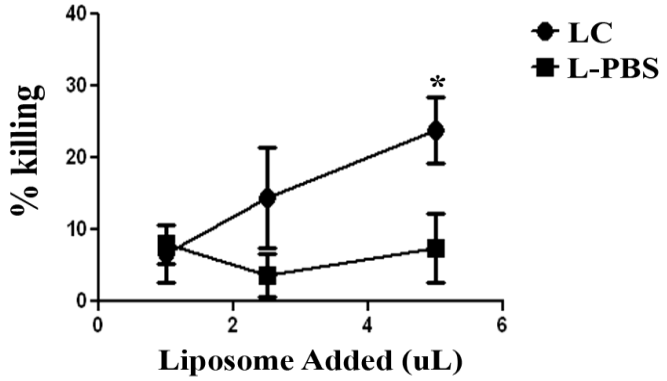

Figure 3.4: Killing of primary monocyte-derived macrophages by LC. Monocyte-derived macrophages (MDM) were generated from the blood of healthy dogs as described in "Methods". After 7 days in culture, the MDM were treated with LC or PBS liposomes (L-PBS) and cell viability was analyzed 72 h later by MTT assay. Cell viability following incubation with 5% LC or 5% L-PBS at each time point was statistically compared using Student's t test. Significant differences (p\0.05) were denoted by *.

Assessment of liposome uptake by MH and non-MH cells

In the preceding experiments, heterogeneity in MH cell susceptibility to inhibition by LC was observed (see **Figure 3.1**). In addition, we also found that the resistance of non-phagocytic cells to inhibition by LC was not due to inherent resistance to the cytotoxic effects of free clodronate (data not shown). Therefore, the observed differences in susceptibility to LC inhibition could likely be accounted for in part by differences in liposome uptake. To address this question, flourescently-labeled liposomes were used to compare liposome uptake by MH cell lines and by non-MH tumor cell lines.

Cells were incubated in suspension at a concentration of 5.0 x 10^5 cells/mL with PBS liposomes labeled with the fluorescent dye BODIPY, as described in Methods. After 4 hours of incubation, the cells were washed and analyzed for uptake of labeled liposomes by flow cytometry. To distinguish surface bound (i.e., non-internalized) liposomes from internalized liposomes, trypan blue was used to quench fluorescence by surface bound liposomes, as noted previously[38].

Liposome uptake by all 3 MH cells was significantly higher than uptake by the 3 non-MH cell lines (**Figure 3.5**). For example, the percentage of cells that contained internalized liposomes after 4h incubation was almost 90% in DH82 cells, whereas uptake by each of the 3 non-MH cell lines was less than 10% of the total cell population. These results suggested therefore that difference in susceptibility to LC-induced cell inhibition could be explained almost entirely by differences in the efficiency of phagocytosis and liposome uptake.

The efficiency of liposome uptake was also significantly different within the 3 MH cell lines. For example, the DH82 line exhibited significantly greater uptake of labeled liposomes than did either of the other 2 MH cell lines (**Figure 3.5**). The efficiency of liposome uptake also correlated directly with the efficiency of LC inhibition in the 3 MH cell lines (see **Figure 3.1**). The decreased susceptibility of the MH-1 and MH-2 cells to LC-inhibition was not however due to inherent resistance to the effects of free clodronate itself, as the MH1 and MH2 cell lines were actually more susceptible to free clodronate than the DH82 cells. Therefore, the relative ability to phagocytose liposomes appeared to be a primary determinant of the susceptibility of different MH cell lines to LC-mediated cell inhibition. This result suggests that highly phagocytic MH

tumors would be more susceptible to treatment with LC treatment than less phagocytic tumors.

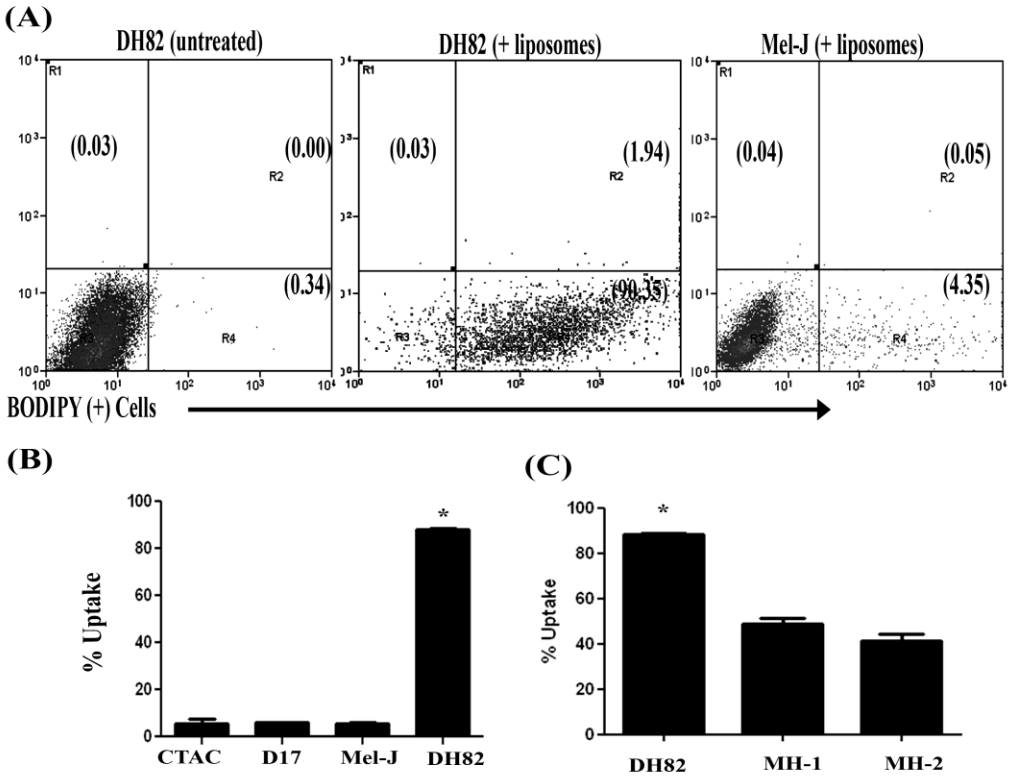

Figure 3.5: Comparison of liposome uptake by MH and non-MH tumor cell lines. In (a), representative dot plots of untreated DH82 cells (left panel), DH82 cells incubated with labeled liposomes (middle panel), and canine melanoma cells (Mel-J) incubated with liposomes (right panel) are shown. In (b), the mean percentages (±SEM) of liposome? tumor cells were plotted. The percentage of DH82 cells containing internalized liposomes was significantly higher than for either of the three canine non-MH tumor cell lines, as assessed by ANOVA with Bonferroni's post-test (*; p\0.05). In (c), the mean percentage of DH82 cells containing internalized liposomes was significantly higher than for the other two canine MH tumor cell lines (MH-1 and MH-2).

Mechanisms of cell death induced by LC treatment

Previous studies using murine macrophages have shown that LC-induced cell death was mediated primarily by induction of apoptosis, though with delayed kinetics relative to apoptosis induced by many chemotherapy agents[19, 39]. Therefore, we

investigated whether apoptosis might also account for LC-induced cell death in canine MH cells. For these assays, MH cells were incubated with LC for varying lengths of time, then stained with Annexin V and PI and analyzed via flow cytometry to identify apoptotic cells, as described in Methods.

Treatment with LC induced a significant increase in the percentage of early apoptotic and mid-apoptotic cells (**Figure 3.6**). For example, the percentage of early apoptotic cells increased from 3.5% (± 0.6%) of cells prior to treatment to 21.6 % (± 1.7%) of cells following 48 hours of incubation with LC. The MH-1 and MH-2 cell lines also underwent apoptosis following LC treatment, with similar kinetics as for DH82 cells (data not shown). Thus, the majority of cell death induced in MH cells by LC treatment appeared to be mediated by induction of apoptosis.

The kinetics of induction of apoptosis by LC in MH cells was examined next. These studies were prompted by the fact that induction of cell death (as assessed by cell viability assay) was relatively slow following incubation with LC and did not become apparent in the first 12 to 24 hours of incubation. For example, classical inducers of apoptosis in macrophages such as staurosporine or camptothecin induced large increases in apoptosis within 6 to 8 hours of incubation. In contrast, maximal induction of apoptosis in MH cells was not observed until 48 hours after treatment with LC (**Figure 3.6**). The delay in induction of apoptosis by LC in MH cells could be related the mechanism of action of clodronate, which involves competitive inhibition for ATP binding, a process that would be expected to induce cell death relatively slowly[32]. In addition, uptake and release of the contents of the clodronate containing liposomes into the MH cell cytoplasm may have also been a rate limiting factor.

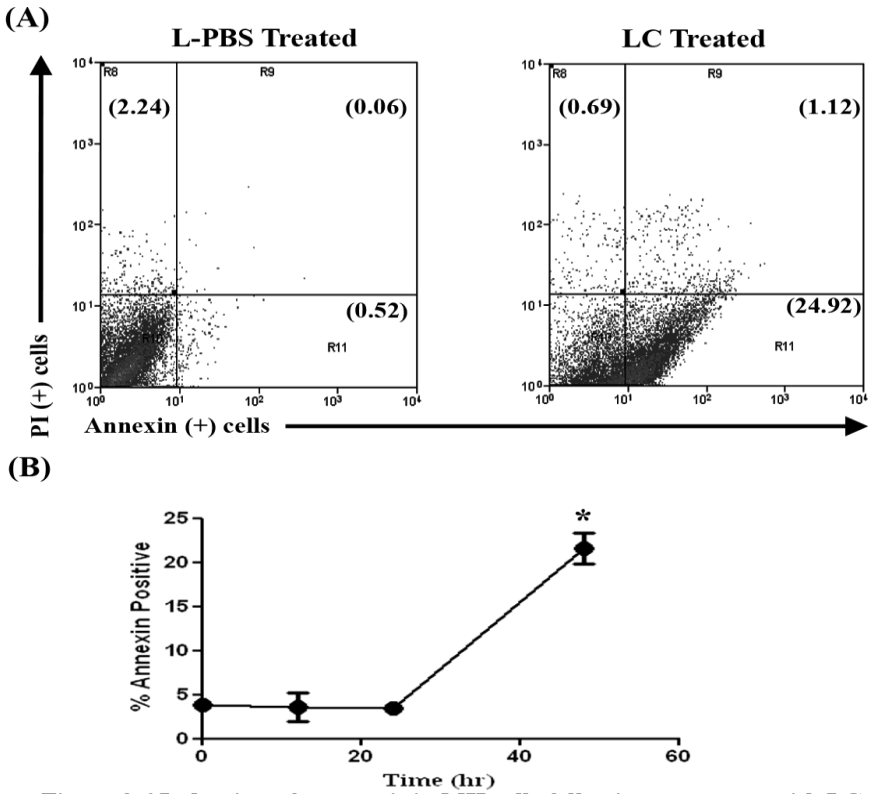

Figure 3.6 Induction of apoptosis in MH cells following treatment with LC. In (a),representative dot plot at 48 h of DH82 MH cells treated with LC or L-PBS. In (b), the time course of induction of apoptosis was assessed, using DH82 cells.The mean percentage ofAnnexin V positive cells (which included both early and midapoptosis cells) at varying time points after treatment with LC was determined. The percentage of apoptotic cells at each time point was compared by Student's t test with values forthe 0 h control; (*, p = 0.001).

Clinical evaluation of LC as a therapeutic for treatment of histiocytic cancer

The preceding experiments indicated that LC was an effective agent for inhibition MH cells *in vitro*. Clinically, MH in dogs is typically refractory or only moderately susceptible to treatment with conventional chemotherapy drugs [2, 4]. As a consequence, most dogs with MH are euthanized within weeks of diagnosis. Our *in vitro* studies indicated that LC had significant activity against MH cell lines. Thus, it was reasonable to propose that systemic administration of LC might be used therapeutically in dogs with spontaneous MH tumors. Fortunately, a safe and effective dose for i.v. administration of LC to healthy dogs and dogs with spontaneous autoimmune hemolytic anemia had been

established by our group recently[23]. Therefore, we conducted a pilot study to evaluate the safety and potential efficacy of LC administration as a new approach to treatment of MH and potentially other histiocytic neoplasms.

Five pet dogs MH were enrolled in a clinical trial to evaluate the use of LC for treatment of MH. All of the dogs had previously failed conventional chemotherapy, including treatment with predisone and lomustine. Dogs enrolled in the LC study were treated every other week by i.v. infusion of LC, using a dose of 0.5 mL/kg established in an earlier study[23]. The infusion was administered slowly over a 60-minute period through a peripheral intravenous catheter. Animals were monitored during the infusion for acute adverse effects (respiratory, heart rate, blood pressure) and for the next 8 hours for side-effects such as fever and respiratory and cardiovascular signs. Additional treatments were administered at 2-week intervals, using the same LC dose and delivery schedule.

Dog 1. A spayed female mixed breed dog was diagnosed with a large subcutaneous MH tumor on the shoulder. The tumor had not responded to two prior treatments with lomustine. The dog received two i.v. infusions of LC administered 2 weeks apart. The dog developed significant fever within 12 hours of administration of each of the two LC treatments and the febrile episodes lasted for approximately for 24 hours. Other adverse effects were not noted. The dog was evaluated for a period of a month following the 2 LC treatments, but no objective tumor response was noted.

Dog 2. A castrated, mixed-breed dog developed a cutaneous MH located on the flank. The tumor did not respond to prior treatment with prednisone. The dog received two i.v. infusions of LC, given two weeks apart, with no evidence of treatment related adverse effects. During the 4-week treatment period, objective tumor responses were not noted and treatment was therefore discontinued. However, when the dog was re-examined 10 months later, the previously noted cutaneous MH tumor was observed to have completely regressed. This dog also had a large solitary pulmonary mass that was present at the time of initial MH diagnosis, though the lung tumor was not biopsied. The lung tumor did not respond to treatment and continued to grow slowly. Based on the tumor location, solitary nature, and slow growth rate it was considered to most likely be a primary lung tumor, though this was not confirmed by histopathology.

Dog 3. A castrated male Golden Retriever dog developed metastatic MH involving the lungs, adrenal glands, and liver, which was noted on CT scan (**Figure 3.7**). Prior treatment with prednisone and lomustine had not produced objective tumor responses. The dog then received two LC infusions, two weeks apart. Adverse effects related to the LC infusions were not noted.

On follow-up CT scan taken 10 weeks after the last LC treatment, significant tumor regression at multiple sites was noted (**Figure 3.7**). Additional treatments were not administered and the dog was followed up with routine rechecks. Five months after completion of LC treatment, the dog developed fatal cardiac arrhythmias and was humanely euthanized. On post-mortem examination of the lungs and adrenal glands, there was no histologic evidence of the prior biopsy confirmed MH tumors. However,

MH was found in the left ventricle of the heart, which was presumed to be the cause of the fatal arrhythmia.

Dog 4. A spayed female Bernese Mountain Dog developed enlarged hilar lymph nodes, a caudal lung mass and a cranial mediastinal lung mass. Cytologic examination of aspirates of the masses were consistent with a diagnosis of MH. Prior treatment with carboplatin and prednisone had elicited only minimal tumor responses. The dog received two LC treatments, given two weeks apart. During the 4-week treatment period, adverse effects were not observed. However, at the end of this period, an objective tumor response was not noted and the treatment was therefore discontinued.

Dog 5. A castrated male Bernese Mountain Dog was diagnosed by thoracic radiographs and biopsy with pulmonary metastatic MH. Prior treatment with prednisone and lomustine had not produced a significant tumor response. The dog was therefore treated with two LC infusions, administered two weeks apart. During the 4-week treatment period, adverse effects were not observed. However, at the end of the treatment period, an objective tumor response was not noted on repeat thoracic radiographs and the treatment was therefore discontinued.

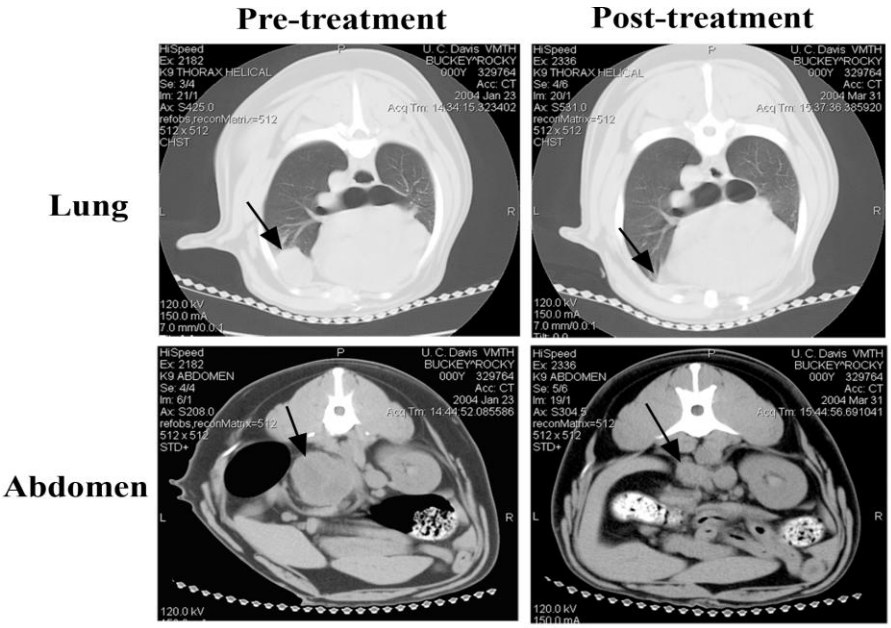

Figure 3.7: Tumor responses following LC treatment in a dog with malignant histiocytosis. A dog with metastatic MH was evaluated by CT imaging. Prior to treatment (left panels), there were large metastases in the lungs (upper panel, arrow) and the adrenal glands (bottom panel, arrow). The dog was then treated with two i.v. infusions of LC 2 weeks apart, as described in ‘‘Methods’’. When CT imaging was repeated 3 months later (right panels), there was marked reduction in the size of both lung and adrenal metastases. At necropsy5 months later, there was no evidence of MH in either the lung or adrenal sites.

Discussion

Previous studies have shown that LC is an effective macrophage depleting agent in rodents, following either systemic or local injection[11-14]. In addition, the clinical potential for LC treatment to be used as a treatment of autoimmune diseases has been evaluated in rodent models and in a study in pet dogs with autoimmune hemolytic anemia[16, 17, 23]. There are also several relatively recent reports evaluating the use of LC treatment in mouse tumor models[40, 41]. However, the current study is the first to our knowledge to investigate the potential for systemically administered LC therapy to be used for treatment of histiocytic neoplasms, particularly inasmuch as the studies were conducted in a large animal spontaneous tumor model.

Notably, we found that systemic LC therapy was well-tolerated in dogs with MH, even those with advanced disease and large tumor burdens. In addition, these preliminary studies demonstrated that LC administration was capable of inducing significant tumor responses in some treated animals. Thus, these results suggest that liposome-encapsulated bisphosphonate therapy warrants further evaluation as a potential treatment for histiocytic malignancies such as Langerhans cell histiocytosis in humans and dogs.

In vitro, LC was found to be very effective at inhibition canine MH cells. Liposome uptake studies also revealed that only phagocytic tumors were susceptible to the effects of LC-induced inhibition, even though non-phagocytic cells were in some cases more susceptible to the non-liposome encapsulated drug. Thus, selective targeting of MH cells for inhibition might explain in part the antitumor activity we observed in our

pilot study of LC therapy for treatment of MH in dogs. However, it should be noted that the antitumor activity we observed could also be attributed to indirect effects of LC therapy on the MH tumors. For example, recent studies have demonstrated that repeated LC administration is capable of depleting both tumor associated macrophages and myeloid suppressor cells[40-42]. We have also observed antitumor activity following i.v. administration of LC in several different non-histiocytic mouse tumor models (manuscript in preparation). Thus, the antitumor activity elicited following LC administration to dogs with MH may have been mediated by a combination of both direct and indirect tumor effects.

The variability in MH susceptibility to LC treatment observed in the 3 canine MH cells lines in our study also suggested that tumor heterogeneity may have an important impact on response to treatment. We have observed similar heterogeneity in responsiveness to LC in several different mouse macrophage cell lines, with more differentiated macrophages appearing to have greater susceptibility to LC inhibition (Hafeman, S, et al; unpublished data). The liposome uptake studies suggested that variability in susceptibility to LC inhibition could be largely attributed to variations in efficiency of liposome uptake.

The mechanism of LC-induced cell death in phagocytic cells is also relevant to the design of more effective clodronate analogues and to the safety of such drugs in vivo. Our results indicated that LC killed MH cells primarily by inducing apoptosis, but with unusual delayed kinetics. For example, evidence of apoptosis and cell death did not become evident until after 48 hours of incubation with LC (see **Figure 3.6**), which is much longer than required for induction of apoptosis by camptothecin (6 hours). Others

have also observed delayed macrophage apoptosis and cell death following LC treatment[39]. The reasons for this delay in *in vitro* apoptosis are not immediately apparent, but are probably related to the mechanisms of action of clodronate[43]. Curiously, administration of LC to mice elicits substantial elimination of splenic macrophages within 18 to 24 hours of administration[11, 12, 15, 23]. Thus, there are important and as yet unexplained differences between the *in vitro* and *in vivo* behavior of LC with respect to induction of macrophage apoptosis.

In summary, LC was found to be an effective agent for inducing cell death in histiocytic tumor cells of dogs. Preliminary pilot studies in dogs with spontaneous MH, including animals with advanced tumor metastases, also suggested *in vivo* efficacy of LC against histiocytic malignancies. We concluded therefore that additional studies for treatment of cancer were warranted and that liposomal delivery of bisphosphonate drugs may represent a promising approach to treatment of certain histiocytic neoplasms.

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Chapter Four

Determination of the hematologic and cytokine responses to liposomal clodronate treatment in dogs with malignant histiocytosis

Abstract

Malignant histiocytosis (MH) is a highly aggressive neoplasm that arises from macrophages and dendritic cells. This disease has a very poor prognosis due to generalized metastasis and poor response to chemotherapy. We previously reported the efficacy of liposomal clodronate against MH cells in vitro and in a pilot study of dogs with MH. The goal of this study was to expand our treatment population as well as monitor the physiologic effects of the drug. We were able to show an overall response rate of 40% and a median survival of 98 days. These values are comparable to those achieved with CCNU single agent therapy. There was a significant difference amongst responders and non responding dogs in development of fever, reduction in monocytes, and expansion of neutrophils. A significant decrease in IL-8 was also observed after 24 hours of LC treatment. Future studies are needed to determine if the combination of LC with standard chemotherapeutics will increase survival in dogs with MH.

Introduction

Malignant histiocytosis, a tumor also classified as histiocytic sarcoma, is a tumor of dogs that arises from cells of the histiocytic lineage[1-3]. Most commonly these include monocyte/macrophages and dendritic cells[2, 4]. The disease is overrepresented in many different breeds of dog, including Bernese Mountain Dogs, Flat Coated Retrievers, and Rottweilers[5, 6]. However, the disease has been reported in a large number of other breeds and in mixed breed dogs. The disease may manifest as either localized or disseminated disease[7-9]. However the majority of patients with initial localized disease will develop distant metastases as the tumor is highly aggressive and rapidly metastatic. Therefore, except in rare cases of localized disease traditional treatment modalities such as surgery or radiation therapy are largely ineffective. In cases where surgery is possible chemotherapy is necessary to delay disease recrudescence[8]. The use of single agent chemotherapy in the treatment of malignant histiocytosis has been largely unrewarding as the disease often has transient and refractory responses to most agents[5, 10]. Consequently multiple therapeutics have been used in an attempt to control the disease including prednisone, doxorubicin, lomustine (CCNU), and carboplatin[11, 12]. The disease is uniformLy fatal, with a median survival time of 2-4 months[5, 10, 12, 13].

The disseminated canine disease has its primary origins in the bone marrow, spleen, and lung. This clinical syndrome most closely resembles the human disease Langerhans cell histiocytosis which can also be multisystemic and refractory to single agent chemotherapy[14].

Liposomal clodronate has been studied extensively as an agent used to deplete macrophages[15-18]. Clodronate is a first generation bisphosphonate drug that is metabolized by osteoclasts and macrophages into a non-hydrolysable ATP analogue[19, 20]. The lack of ATP leads to mitochondrial dysfunction and subsequent apoptosis of the cell[21-23]. Liposomal clodronate has been used for efficient, systemic macrophage depletion in multiple rodent models[18, 24-26]. More recently it has been applied in multiple tumor models where it has been shown to be very effective in depleting tumor associated macrophages[27-31]. Our laboratory has shown that liposomal clodronate is able to kill MH cells in vitro and is a safe and effective treatment for MH in dogs in a preliminary study[32]. However, this study involved only 5 treated dogs and did not have a standardized protocol. Consequently, the physiological effects of LC treatment could not be studied in these animals.

In this study we treated a larger number of MH tumor bearing dogs with LC and primarily measured overall response. We were also able to document the physiologic effects of treatment in these animals and may have determined physiologic markers which predict response in this large animal model. We have been able to again demonstrate that LC is a safe treatment of MH in dogs and is comparable to the standard of care in terms of overall response and median survival time. We were also able to show a cytokine profile for MH dogs as compared with non tumor bearing dogs which may be able to serve as a biomarker of disease or response in these animals. We report here for the first time that treatment with liposomal clodronate is able to cause a significant decrease in serum IL-8 which may lead to anti-angiogenic effects in this and other tumor types.

Materials and Methods

Preparation of liposomal clodronate and liposomal PBS

Liposomal clodronate (LC) was prepared as previously described[32, 33]. Briefly, phosphatidylcholine and cholesterol (both purchased from Avanti Polar Lipids, Alabaster AL, USA) were dissolved in chloroform. These were combined (at a 5 :1 MW ratio) in a glass round bottom tube and dried to completeness overnight in a vacuum lyophilizer (VirTis, Gardiner, NY, USA). To prepare mannose-containing liposomes, p-amino phenyl mannopyranoside (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in methanol and added at 1.75 mg per 25 mg of phosphatidylcholine and dried down together with the phosphatidylcholine and cholesterol lipids. Liposomes were prepared by rehydration in a concentrated solution of clodronate (Sigma-Aldrich) as described previously. Liposomal PBS was prepared in a similar fashion, using a 1.5 M stock of PBS instead of clodronate to rehydrate the liposomes.

Investigation of LC treatment in dogs with MH

A study of LC therapy for treatment of pet dogs with MH was conducted in 10 dogs with biopsy-confirmed tumors. The LC treatment study was approved by the Institutional Care and Use Committee at Colorado State University. A pre-treatment CBC, chemistry panel and urinalysis were performed in all animals and serum and plasma were frozen for further analysis. The dogs were not allowed to be on NSAID, steroid, or any other anti-inflammatory medication during the duration of the study, however there was no washout period prior to enrollment. Dogs were treated by IV administration of LC at a dose of 0.5 mL/kg, over a 60-minute period, as described

previously[32, 33]. The treatment was repeated every 2 weeks until a total of 6 treatments or until determination of progressive disease. Dogs were monitored for the first 24 hours after treatment for changes in body temperature and heart rate and respiratory rate. Twenty-four hour post treatment a recheck CBC was run and serum and plasma were collected and frozen at -80° Celsius. A CBC was checked prior to each of the additional treatments.

Cytokine analysis

Pre and post treatment serum samples were stained for multiple cytokines using the Milliplex® MAP cytokine bead kit as per the manufacturer's directions (Millipore, Billerica MA, USA) and analyzed using the Luminex 100™ instrument. A population of values from non tumor bearing dogs was generously provided by Dr. Anne Avery. All values are reported as pg/mL of serum. Serum enzyme linked immunosorbent assays (ELISAs) were also run on the same serum samples. The MCP-1, IL-6, TGF-β and IFN-γ R&D Duoset® ELISAs (R&D Systems, Minneapolis MN, USA) were run according to the manufacturer's directions and analyzed using an ELISA plate reader. Standard curves were derived using Microsoft Excel® and results are reported in pg/mL of serum.

Statistical Analysis

In experiments where the mean of more than two groups was compared, one way ANOVA and Tukey's multiple means comparison test were used. For comparison between two groups the Student's T-test was used. For determination of correlation between events the Fisher's exact test was used. Statistical analyses were performed

using GraphPad Prism software (GraphPad, San Diego, CA). Differences were considered statistically significant for *p* values less than 0.05.

Results

Liposomal clodronate is safe and effective in the treatment of MH in dogs

Based on the positive results of the pilot study, we treated an additional 10 dogs that satisfied inclusion criteria into the current study. All of these dogs had disseminated disease. The patient characteristics mirror that of previous studies, with no sex predilection (50% male and 50% female patients) and a high number of Bernese Mountain Dogs (33.3%) and Rottweilers (25%). The median age of all study participants was 8.5 years old at presentation (range 6-14 years). All dogs had disseminated disease at the time of treatment. Of all treated animals, 50% failed at least one previous therapy regimen of doxorubicin, CCNU, or carboplatin. The remaining dogs did not have any previous treatment for their disease.

The standard response evaluation criteria in solid tumors (RECIST) system were used for evaluation of clinical response either by direct or radiographic measurement of the lesions. For the purposes of this study, stable disease was included as a positive response if maintained for greater than 30 days. Using these criteria, the overall response rate in this study was 50% (5/10). Of these, there was a 10% complete response (1/10), 10% partial response (1/10), and 30% stable disease (3/10). The median survival time for all treated patients in the study was 98 days.

Toxicity was evaluated using the Veterinary Comparative Oncology Group Common Toxicity Criteria for Adverse Events (VCOG-CTCAE). The treated dogs received a mean of 3 doses (range 1-12). All dogs that developed toxicity at the first

treatment developed the same toxicity with subsequent treatments, therefore results are summarized on a per patient basis. There were no dose limiting side effects, and no dose reductions were necessary. One dog developed a seizure 24 hours post treatment. Imaging was not allowed to determine whether the seizure was due to metastatic disease or a drug effect and the dog did not receive subsequent treatments. The most common side effect was the development of a fever (67%). Of these, 88% were high grade (3 or 4). All treated dogs had returned to a normal temperature as of 24 hours post treatment. None of these patients required medication to lower their body temperature. Many of these animals had corresponding grade I lethargy and 33% developed grade I GI signs. There were no additional adverse events associated with liposomal clodronate therapy as recorded in owner observation, physical exam, clinical signs or changes in blood work.

Physiologic differences exist between responders and non responders.

There were several interesting differences between responders and non responding dogs in the study. Although not significantly correlated, breed appeared to have an effect as no Bernese Mountain Dogs responded to treatment while all Labrador Retrievers and Rottweilers had at least some response to treatment. The development of a fever was significantly and positively correlated with response ($p = 0.007$, odds ratio = 39). We therefore sought to determine if there were any other differences between responding and non-responding patients.

All patients were evaluated with full pre treatment blood work as well as a CBC prior to discharge 24 hours after treatment. When evaluating the CBC data from all

patients, there were no significant differences in the numbers of neutrophils and monocytes pre and post treatment (**Figure 4.1)** However, when we looked at responding patients there was a significant decrease in pre and post treatment monocytes and a significant increase in pre and post treatment neutrophils (**Figure 4.1**). There were no significant differences in any other peripheral blood cell population, either overall or in the responding group. These results indicate that in responding dogs, LC is able to deplete monocytes as previously described. In addition to the fever observed in responding dogs, the increase in circulating neutrophils post treatment indicates that LC is able to elicit a pro-inflammatory response. These data also show a potential response profile that can be monitored in future trials.

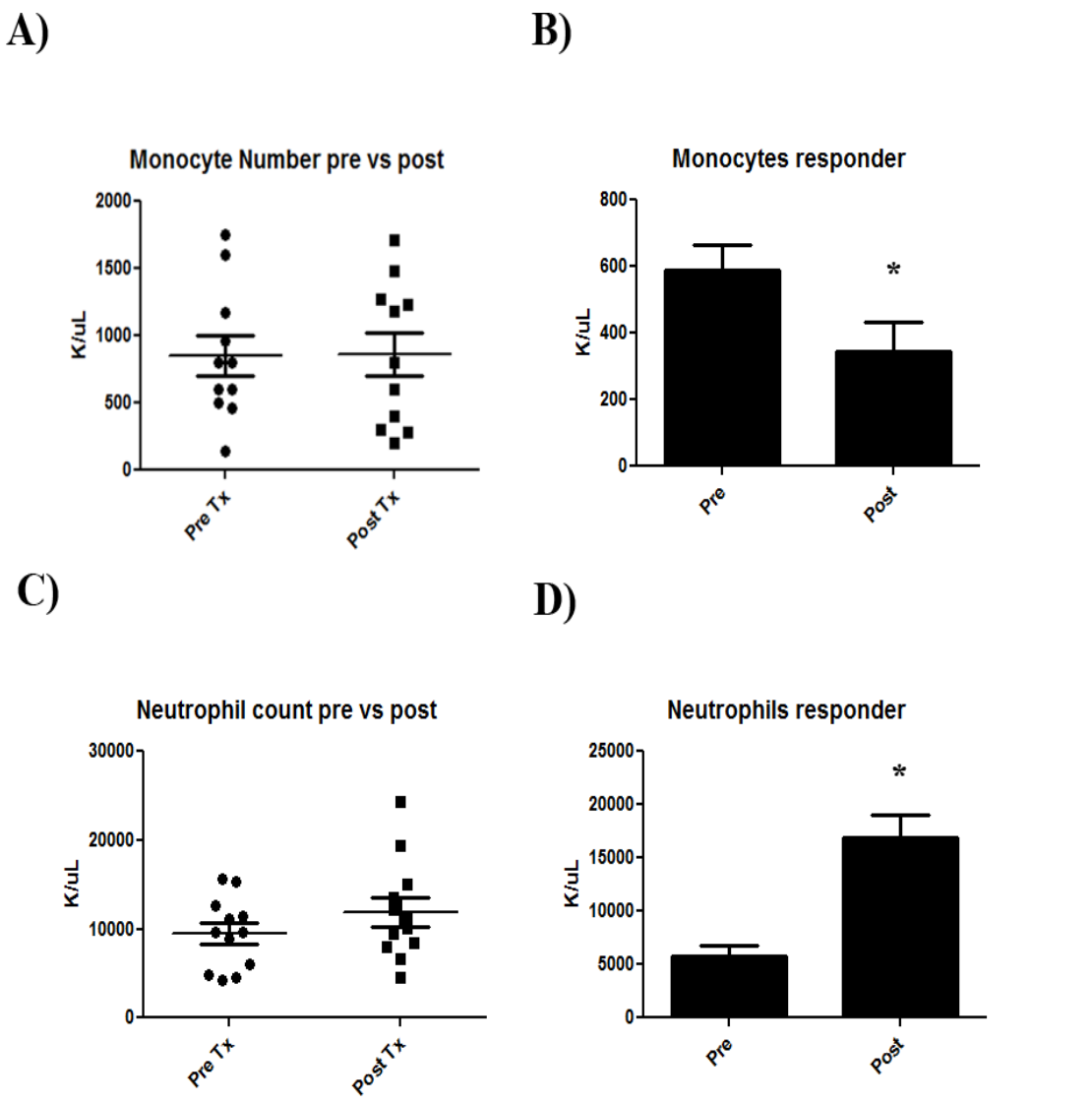

Figure 4.1: Liposomal clodronate affects circulating cell populations in responding dogs. (A) Total peripheral blood monocyte number pre and post treatment for all dogs. A significant difference was not observed. (B) Total monocyte number pre and post treatment for responding dogs showing a significant ($p < 0.05$) decrease in this population. (C) Total neutrophil count pre and post treatment for all dogs. A significant difference was not observed. (D) Total neutrophil count pre and post LC treatment for responding dogs showing a significant ($p < 0.05$) increase in neutrophil counts in this population.

Cytokine profile of MH tumor bearing dogs

We next sought to determine if there was a cytokine response associated with MH in dogs, and if liposomal clodronate induces changes in cytokines that might explain the inflammatory changes seen clinically. We elected to analyze all of the pre and 24 hour post treatment serum samples using the Millipore Luminex assay for canine cytokines. This assay included nine cytokines.

No significant changes were seen in IP-10, IL-2, IL-4, IL-6, TNF-α or IL-18 with MH compared with non tumor bearing dogs or pre compared with post treatment serum (data not shown). However, we did observe a significant increase in four tumor growth promoting cytokines in the serum of MH patients as compared to non tumor bearing dogs (**Figure 4.2**). All of the observed cytokines are produced by monocytes, macrophages or dendritic cells and are responsible for either increased proliferation (GM-CSF, IL-15), or increased angiogenesis and proliferation (KC which is also referred to as CXCL1, and IL-8 also called CXCL8)[35-41].

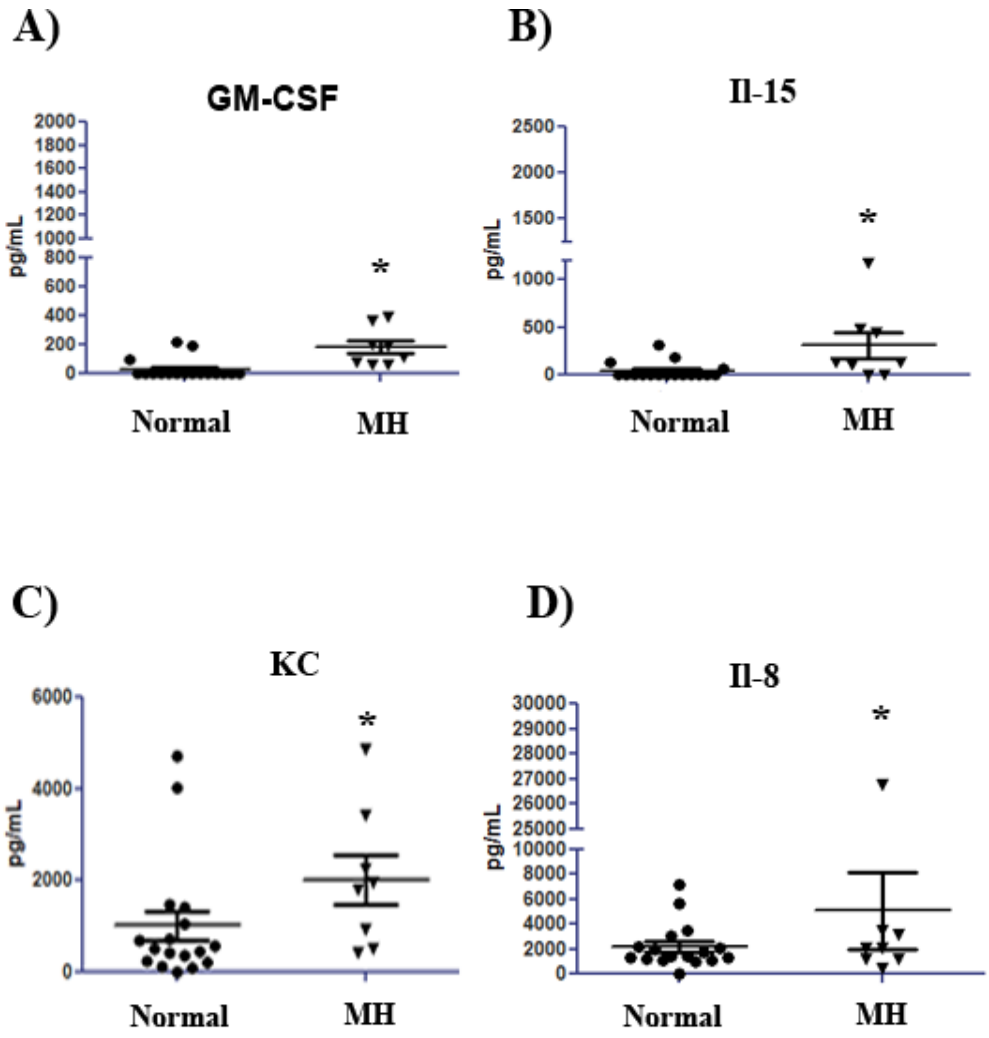

Figure 4.2: MH patients display changes in their cytokine profile.
Serum from MH tumor bearing dogs was analyzed via the Luminex assay for multiple cytokines and compared to historical non-tumor bearing dog serum. Significant ($p < 0.05$) increases in (A) GM-CSF (B) Il-15 (C) KC and (D) Il-8 were observed in MH dog serum demonstrating a pro-angiogenic and pro-growth cytokine profile for this tumor as compared to non-tumor bearing dogs.

When we compared the pre treatment serum to that of dogs 24 hours post treatment with LC, there was a significant decrease in IL-8 (**Figure 4.3**). None of the other cytokines measured showed significant changes after treatment although the overall levels of KC were also decreased (**Figure 4.3**).

In order to compliment the Luminex assay and further establish as cytokine profile in MH patients we also evaluated serum samples for MCP-1, Il-6, IFN-γ, and TGF-β both pre and post treatment. There were no significant changes in these cytokines as compared with normal dogs, however there was an overall increase in MCP-1 levels as compared to non-tumor bearing patients (data not shown). There were no significant differences in the pre and post treatment serum levels of any of these cytokines (data not shown).

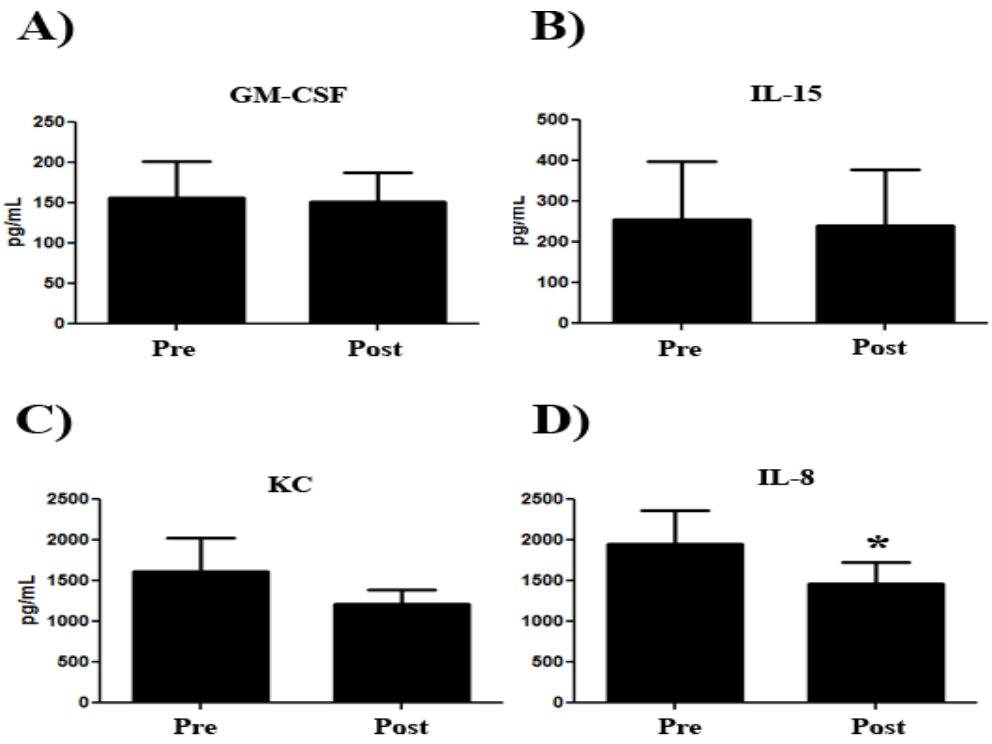

Figure 4.3: Liposomal clodronate therapy leads to a decrease in pro-angiogenic cytokines. (A) Pre and post LC treatment levels of GM-CSF in MH dogs. (B) Pre and post LC treatment levels of Il-15 in MH dogs. (C) Pre and post LC treatment levels of KC in MH dogs showing a decrease with LC treatment. (D) Pre and post LC treatment levels of Il-8 in MH dogs showing a significant ($p < 0.05$) decrease with LC treatment.

Discussion

The results of this study indicate that liposomal clodronate is safe and effective in the treatment of malignant histiocytosis in dogs. The drug has been shown to be well tolerated in previous canine studies[32, 33]. The current study again demonstrates that the drug has no long term side effects, and that treated animals display a transient fever, lethargy, and mild gastrointestinal signs as the primary short term side effects of therapy. A randomized, double blinded study would be required to determine if LC is comparable to CCNU, which is currently accepted as the standard of care.

We were able to determine a difference in responding dog as compared with non responding dogs. These changes included the development of a fever, a decrease in monoctye number, and an increase in neutrophil number in the peripheral blood. The lack of changes in non-responders could indicate breed specific differences in the immune response. However, although LC has been evaluated previously to determine a safe dose to use in dogs, a true phase I dose to toxicity trial has not been performed in a large animal model with this agent. Consequently, it is possible that non-responding patients were under-dosed using our standard 0.5 mL/kg LC dose. These results may make it possible to dose to effect prior to toxicity in future trials.

We were able to show here for the first time that MH patients have increases in four cytokines responsible for tumor growth and angiogenesis. By combining the canine Luminex assay with traditional ELISA analysis we were able to evaluate both non tumor bearing dogs and MH dogs for twelve cytokines commonly studied in tumor growth and immune surveillance. Of these, GM-CSF, Il-15, KC, and IL-8 were significantly

increased. Any of these 4 could potentially serve as biomarkers for initial diagnosis or to monitor response to therapy. Unfortunately as all dogs had disseminated disease at the time of blood draw for analysis we were not able to relate the levels of these cytokines to tumor burden.

Of these cytokines, IL-8 has been the most extensively studied for its role in tumor growth, angiogenesis and metastasis [35-37, 42]. It is produced by macrophages, endothelial cells, and tumor cells[36, 37, 42]. There are various functions of this cytokine involved in tumor growth in both an autocrine and paracrine fashion[35, 42]. It can also induce angiogenesis via increased endothelial cell recruitment, proliferation, and survival[35]. We were able to demonstrate a significant decrease in IL-8 in dogs treated with LC. This may be an important mechanism of action of this drug, and warrants further investigation.

The primary limitations of this study are a very small sample size and the lack of a control group treated with lomustine. Due to the lack of a clear demonstration of increased benefit when compared to lomustine in these preliminary patients, future studies in this tumor will most likely focus on the combination of CCNU and LC for the treatment of MH as our preliminary results with this combination have been very encouraging. The cytokine changes over the first several hours post treatment will also be evaluated in further studies, as we have not yet identified the cytokine changes responsible for the febrile response seen in the treated dogs.

In summary, we have been able to demonstrate that LC is safe and effective for the treatment of MH in dogs. There is a responding profile with LC treatment which may be exploited to determine an effective dose in future studies. MH patients have a

proliferative and pro-angiogenic cytokine profile as compared with non tumor bearing dogs, and treatment with LC is able to cause a significant decrease in IL-8 which is an important cytokine for tumor growth and angiogenesis. Therefore LC may be most effective in combination with other anti-angiogenic therapies and may be efficacious against multiple tumor types.

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Chapter Five

Bisphosphonates Significantly Increase the Activity of Doxorubicin or Vincristine Against Canine Malignant Histiocytosis Cells

Abstract

Canine malignant histiocytosis (MH) is an aggressive neoplasm of macrophages and dendritic cells. It carries a poor prognosis due to the development of widespread metastasis and poor sensitivity to chemotherapy. Thus, there is a large need for new treatments for MH. We hypothesized that bisphosphonates might be useful to increase the effectiveness of cytotoxic chemotherapy against MH. To address this question, we conducted in vitro screening studies using MH cell lines and a panel of 6 chemotherapy and 5 bisphosphonate drugs. The combination of clodronate with vincristine was found to elicit synergistic growth inhibition which was associated with a significant increase in cell cycle arrest. Second, zoledronate combined with doxorubicin also significantly increased cell growth inhibition. Zoledronate significantly increased the uptake of doxorubicin by MH cells. Based on these findings, we conclude that certain bisphosphonate drugs may increase the overall effectiveness of chemotherapy for MH in dogs.

Introduction

While the results of our experiments and clinical trial with liposomal clodronate for treatment of MH in dogs was encouraging, the drug is still only available on an experimental basis, and likely will be so for some time. Therefore, we sought to determine a more clinically available course of treatment for this disease. To our knowledge, this is the first pre-clinical study ever undertaken to determine the effectiveness of chemotherapy against this disease. Additionally, as MH is derived from canine macrophages, effectiveness against these cells may provide another treatment strategy for TAM depletion in dogs and humans.

Malignant histiocytosis in dogs, also known as histiocytic sarcoma, is a tumor that arises from cells of the histiocytic lineage, including monocytes and dendritic cells [1-6]. The disease is more common in certain breeds of dogs, including Bernese Mountain Dogs, Flat Coated Retrievers, and Rottweilers, suggesting a genetic component to disease susceptibility[3, 7, 8]. However, the disease also occurs sporadically in other breeds of dogs as well as in mixed breed animals. Malignant histiocytosis may develop as either a localized tumor, or may instead present as widely disseminated disease[4]. However, even animals with initially localized disease often develop distant metastases [7, 9]. Therefore, except in truly localized cases traditional tumor treatment modalities such as surgery or radiation therapy are largely ineffective for this neoplasm in dogs. Chemotherapy is usually administered to dogs with MH to help prevent local or systemic recurrence of tumor[10]. The use of single agent chemotherapy has been largely unrewarding for treatment of MH, as treatment responses are typically incomplete and/or short-lived [7, 11, 12]. Consequently, combined therapy is usually implemented for the initial treatment of MH, including various combinations of prednisone, doxorubicin, lomustine (CCNU), and carboplatin[10, 11, 13]. Unfortunately, even with aggressive treatment the disease is often

fatal. In cases with disseminated disease the median reported survival time is 2-4 months[2, 4, 7, 8, 12].

The disseminated canine disease has its primary origins in the bone marrow, spleen, and lung[4, 7-9, 14]. This clinical syndrome most closely resembles the human disease Langerhans cell histiocytosis which can also be multisystemic and refractory to single agent chemotherapy[15-17]. These patients are most often treated using vinca alkaloids in combination with multiple immunosuppressive agents[16{Egeler R., 2006 #80, 17, 18]. Zoledronate, an aminobisphosphonate, has also been used effectively in cases with bone involvement[19].

Bisphosphonates have been studied extensively for their ability to deplete macrophages[20-23]. Clodronate is a first generation, non aminobisphosphonate that is metabolized by osteoclasts and macrophages into a non-hydrolysable ATP analogue[24-26]. The lack of ATP leads to mitochondrial dysfunction and subsequent apoptosis of the cell[26-28]. Liposomal clodronate has been used for efficient, systemic macrophage depletion in multiple rodent models[29-33]. More recently it has been applied in multiple tumor models where it has been shown to be very effective in depleting tumor associated macrophages[34-38]. Our laboratory has shown that liposomal clodronate is able to kill MH cells in vitro and is a safe treatment that may be efficacious for treatment of MH in dogs[39, 40].

Newer generation bisphosphonates incorporate nitrogen into their structure and subsequently work via a different mechanism of action[41]. These drugs inhibit the enzyme farnesyl diphosphate synthase, which inhibits macrophages and tumor cells from protein prenylation. This stops the cells from being able to activate signaling GTPases such as Ras, which leads to subsequent apoptosis of the cell[41-43]. The amine ring containing bisphosphonate zoledronate has been used extensively in the palliative treatment of bone metastases in humans[44-46]. Recent work has shown that in addition to its effects on osteoclasts, zoledronate can work synergistically with doxorubicin to kill tumor cells in

vitro and decrease tumor growth in vivo in multiple tumor types[47-52]. This drug has also been shown to be very effective at growth inhibition tumor associated macrophages in mouse tumor models[38]. Zoledronate has also been shown to be safe for administration in dogs[53].

As MH is a tumor of macrophages and dendritic cells, we sought to determine if combining bisphosphonates, drugs specific for macrophage growth inhibition, with traditional cytotoxic chemotherapy would demonstrate synergistic growth inhibition of MH tumor cells.

Materials and Methods

Cell lines

The canine MH tumor cell line DH82 was obtained from the American Type Tissue Collection (Gaithersburg, MD). The other MH cell lines (designated MH-1 and MH-2) were established from primary cultures of biopsies obtained from dogs with MH and were a kind gift of Dr. Peter Moore (College of Veterinary Medicine, University of California-Davis). All cell lines were maintained in MEM (minimal essential medium, Invitrogen, Grand Island, NY USA) supplemented with 10% heat inactivated fetal bovine serum (Hyclone, Logan, UT), non-essential amino acids, L-glutamine, sodium bicarbonate, penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO). The cell lines were maintained at 37° C in a humidified atmosphere containing 5% CO_2.

Drug preparation, storage and initial screening

Stock solutions of all drugs were stored at -20˚C. Working concentrations of each drug were made by diluting stock solutions in sterile water. New working concentrations of each drug were made prior to each analysis. The following drugs were initially screened alone using the reported ranges for their effects on DH82 cell viability using the MTT assay as described below; dexamethasone (0.15-15 µg/mL), doxorubicin (0.002-2 µg/mL), chlorambucil (0.35-35 µg/mL), carboplatin (0.5-0.005 µg/mL), CCNU (0.15-1.5 µg/mL), vincristine (0.25-25 µg/mL), clodronate (0.5-50 µg/mL), zoledronate (0.02-2 µg/mL), pamidronate (0.02-2 µg/mL), alendronate (0.02-1 µg/mL), and etidronate (0.02-2 µg/mL). The dosages of these drugs that elicited 5-20% growth inhibition were used in subsequent experiments. All bisphosphonates were tested with all chemotherapy drugs using the optimized doses of each drug.

Cell viability assays

The cells previously diluted in MEM were pipetted into a 96 well flat bottomed plate using a final volume of 100 µL/well to give a final number of four thousand cells/well. The cell number plated per well was consistent for all cell lines used. The cells were allowed to adhere for 24 hours. After this time, the cells were treated with chemotherapeutics alone, bisphosphonates alone, or both in combination. Control cells were treated with sterile water at the same volume used for the diluted drugs. The cells were incubated with the drugs for 72 hours prior to MTT analysis.

Cell viability was assessed using the MTT assay, as described previously[54]. Briefly, MTT (Thiazolyl Blue Tetrazolium Bromide; Sigma-Aldrich) was added to wells containing live cells and incubated for 2 hours at 37C. The cells and resultant tetrazolium bromide crystals were then dissolved in a 0.1N HCl solution in isopropanol and the absorbance was determined using an ELISA plate reader (Thermo Lab Systems, Salem NH) at 570 nm. Cell viability was then calculated as the percent absorbance of the treated wells as compared to the average absorbance of the untreated control wells, with the inverse of this value representing the percent growth inhibition. To confirm the MTT results, a second set of cells was treated exactly as described previously, then trypsinized, stained with trypan blue to exclude dead cells, and counted using an electronic cell counter (Cellometer Nexcelcom Bioscience, Lawrence MA). All drugs were obtained from Sigma-Aldrich with the exception of zoledronate which was a kind gift from Novartis. All reported results are representative of at least three independent experiments. Similar results were obtained between the DH82, MH-1, and MH-2 cell lines.

Apoptosis assays

Induction of apoptosis was quantitated using Annexin V and propidium iodide (PI) staining and flow cytometry, as previously described[55]. Briefly, cells in triplicate wells were treated with the indicated concentrations of drugs, alone or in combination, for 48 hours. The negative control consisted of a population of untreated cells. A positive control included cells incubated for 6 hours with 50 µg/mL camptothecin (Sigma-Aldrich, St. Louis MO). Cells were then detached and washed prior to analysis of phosphatidylserine expression with Annexin V. Cells were incubated with FITC-conjugated Annexin V, according to manufacturer's directions (BD Biosciences, San Jose CA). Immediately prior to analysis by flow cytometry, PI was also added to the cells to assess cell membrane integrity. Cells were assessed using flow cytometry and data were analyzed using Summit software. The percentage of cells in early or late apoptosis or necrosis was calculated as noted previously[55]

As a second measure of apoptosis, after cells were treated with drug combinations for the indicated periods of time they were then treated using the SensoLyte® Homogenous AMC Caspase-3/7 Assay Kit (AnaSpec, San Jose, CA), which was performed according to manufacturers' recommendations. Briefly, MH cells were incubated in 0.2 µg/mL doxorubicin and 0.2 µg/mL zoledronate or 0.25 µg/mL vincristine and 5 µg/mL clodronate for 48h. Cells were then removed from the incubator, and 50 µL of a dual caspase 3/7 substrate and lysis solution was added to each well (AnaSpec). Reagents were mixed by shaking on a plate shaker for 180 min at 200 rpm. Fluorescence emission was determined at a wavelength of 360/460nm, using an optical density reader (BioTek, Winooski VT). Assay results were reported in relative fluorescence units. All reported results are representative of at least three independent experiments. Similar results were obtained between the DH82, MH-1, and MH-2 cell lines.

Cell Cycle Analysis and Doxorubicin Uptake

For determination of intracellular doxorubicin accumulation, cells were treated for 24 hours with doxorubicin at a concentration of 0.2 µg/mL, zoledronate at a concentration of 0.2 µg/mL, or with both doxorubicin and zoledronate at the above concentrations. The cells were trypsinized and analyzed via flow cytometry to determine the innate mean fluorescence intensity (MFI) of doxorubicin fluorescence, using flow cytometry.

For determination of cell cycle arrest, cells were grown in serum-free medium for 24 hours to initiate cell cycle synchronization. The cells were then switched to complete cell culture medium with 10% FBS and then either untreated or treated with vincristine at a concentration of 0.25 µg/mL, with clodronate at 5 µg/mL, or with both drugs in combination, for 48 hours. The cells were detached and washed twice and then resuspended in 70% ice cold EtOH and frozen overnight. The cells were then washed and resuspended in 250 µL extraction buffer and 100 µL PI-RNAse reagent (Sigma-Aldrich). The cells were then analyzed via flow cytometry and the data were analyzed using Summit software (Dako Colorado, Inc. Ft. Collins, CO) to determine cell cycle parameters. All reported results are representative of at least three independent experiments. Similar results were obtained between the DH82, MH-1, and MH-2 cell lines.

Statistical analyses

In experiments where the mean of more than two groups was compared, one way ANOVA was used, followed by Tukey's multiple means comparison test. For comparison between two groups, the Student's t-test was used. For synergy calculations treatment groups were compared using a 2-way ANOVA, as described previously[56].

Bliss analysis was also used in synergy calculations, as described previously[57]. For example, to determine whether the addition of bisphosphonates to chemotherapy drugs synergistically enhanced cell growth inhibition, the Bliss independence model was utilized. Briefly, Bliss synergy is derived by the following equation: $E(x,y) = E(x) + E(y) - E(x) \times E(y)$

For these comparisons E(x) is the fractional inhibition of bisphosphonates (clodronate (5 µg/mL) or zoledronate (0.2 µg/mL) respectively) (between 0 and 1), E(y) is the fractional inhibition of concentration *y* of vincristine (0.25 µg/mL) or doxorubicin (0.2 µg/mL) respectively, and E(x,y) is the combined inhibition. Theoretical growth inhibition curves were derived utilizing this equation, and standard deviations were estimated by error propagation of experimental SD. Differences between treatment groups (Bliss theoretical vs. experimental) were assessed using two-way ANOVA and Tukey's post-test. Statistical analyses were performed using Prism5 software (GraphPad, San Diego, CA). Differences were considered statistically significant for *p* values less than 0.05.

Results

Bisphosphonates synergize with cytotoxic chemotherapy to kill MH cells in vitro.

We conducted in vitro screens to determine whether aminobisphosphonates or non-aminobisphosphonate drugs increased the activity of 6 commonly used chemotherapy drugs against 3 different canine MH cell lines. The chemotherapy drugs were administered in vitro at concentrations that elicited only 5-20% cell growth inhibition in order to allow the detection of synergistic activity of the bisphosphonate-chemotherapy drug combinations. We found the following drugs had activity against canine MH cells at the following drug concentrations: dexamethasone (dex) (15 µg/mL), doxorubicin (dox) (0.2 µg/mL), lomustine (CCNU) (1.5 µg/mL) and vincristine (vinc) (0.25 µg/mL) (**Figure 4.1**).

Next, these 4 chemotherapy drugs were evaluated for enhanced activity when combined with pharmacologically relevant concentrations of clodronate (5 µg/mL) and zoledronate (0.2 µg/mL)[41, 58, 59]. The bisphosphonate drugs were also screened for activity alone against the MH cell lines (**Figures 4.1 and 4.2**). After incubation for 72 hours, the cells were analyzed for viability using the MTT assay. With clodronate, we detected a significant ($p < 0.05$) interaction in terms of increased cell growth inhibition when clodronate and vincristine were combined, while an interaction was not observed between clodronate and dexamethasone, doxorubicin, or lomustine (**Figure 4.1**). A significant interaction ($p < 0.05$) in terms of increased MH cell growth inhibition was also noted between zoledronate and doxorubicin, while no interaction between zoledronate and dexamethasone, vincristine, or lomustine was observed (**Figure 4.1**). Similar results were obtained using all three MH cell lines.

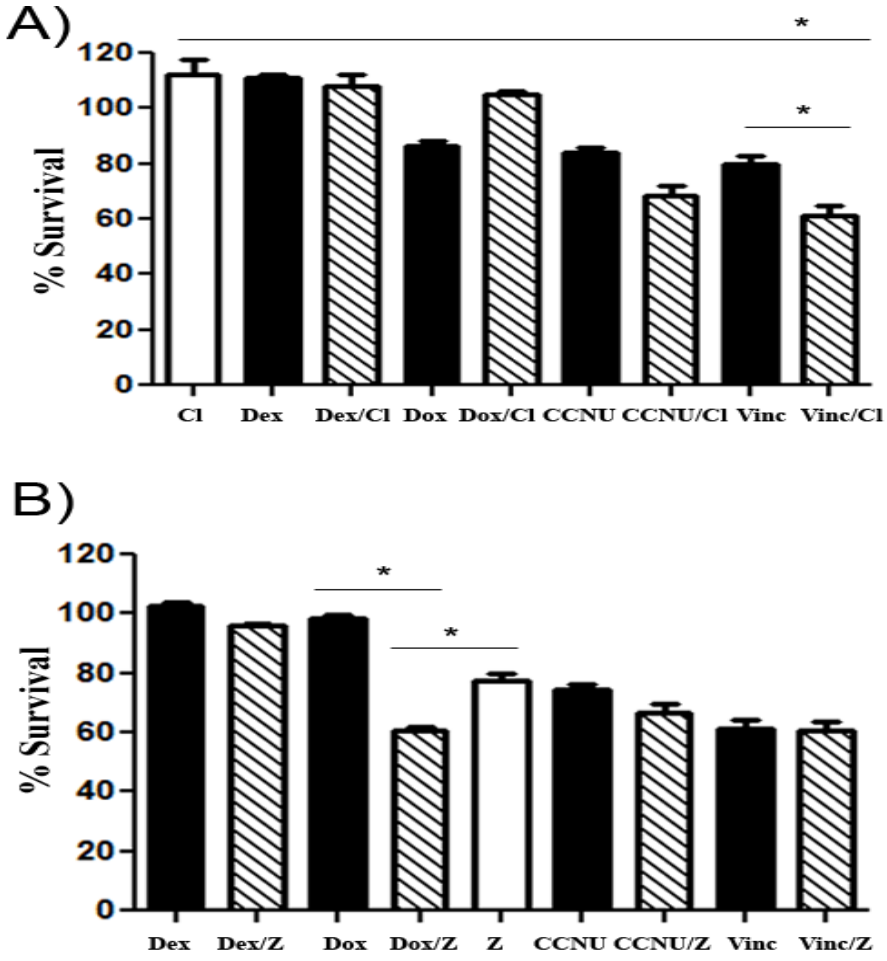

Figure 4.1: Certain bisphosphonate and chemotherapy combinations elicit significantly increased killing of canine MH cells in vitro. In (A), the effects of clodronate on chemotherapy-induced killing of canine DH82 MH cells was assessed, using an MTT assay to assess tumor cell viability. Cells were treated with chemotherapy drug alone (black), clodronate alone (white), or with the combination of clodronate and chemotherapy drug (cross-hatch). In these assays, only the combination of clodronate and vincristine demonstrated a significant positive interaction (* = $p < 0.05$), as assessed by 1 way ANOVA with Tukey's post test. In (B), the effects of zoledronate on MH-1 MH cell sensitivity to killing with chemotherapy drugs were assessed, using a similar approach as for (A). Cells were treated with chemotherapy drugs alone (black), zoledronate alone (white), or the two in combination (cross-hatch). The combination of zoledronate with doxorubicin showed a significant positive interaction(* = $p < 0.05$), as assessed by 1 way ANOVA with Tukey's post test . Cl = clodronate (5 µg/mL), Dex = dexamethasone (15 µg/mL), Dox = doxorubicin (0.2 µg/mL), CCNU = lomustine (1.5 µg/mL), vinc = vincristine (0.25 µg/mL), z = zoledronate (0.2 µg/mL).

Two additional aminobisphosphonates (alendronate and pamidronate) were screened for activity with doxorubicin and each showed a significant interaction ($p < 0.05$) (**Figure 4.2**). These experiments were also repeated using two additional canine MH cell lines, designated MH-1 and MH-2. In all experiments, similar results were

obtained with all three MH cell lines evaluated (data not shown). In addition, since the MTT assay does not differentiate between decreased metabolic activity and decreased cell number, we also assessed the effects of the bisphosphonate and chemotherapy drug combinations on cell numbers by direct counting of cells and confirmed that the results obtained using the MTT assay were indeed due to decreased cell numbers, with control and single agent treated cells having cell counts greater than 400,000 cells/mL and combination treated cells showing counts less than 200,000 cells /mL which was significantly ($p < 0.05$) reduced.

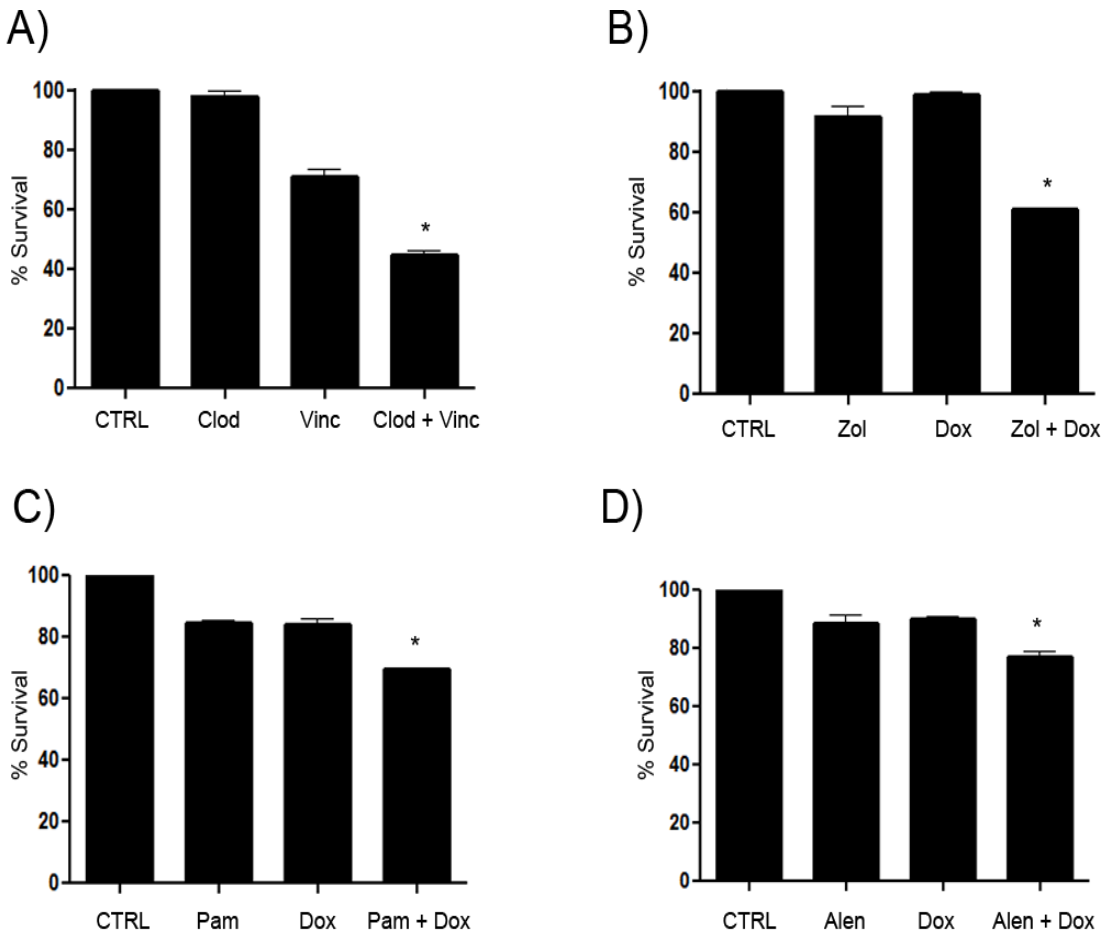

Figure 4.2: Synergistic enhancement of MH cell killing by combinations of bisphosphonates with vincristine or doxorubicin. Significant killing of MH cells was seen in A-D when bisphosphonates were combined with chemotherapy as determined via MTT assay (A), DH82 MH cells were either untreated (CTRL) or treated with clodronate (Clod) or vincristine (Vinc) or both in combination (Clod + Vinc) for 72 hours. In (B), the interaction between zoledronate (Zol) and doxorubicin (Dox) was assessed. Similar experiments were done in (C) for the combination of pamidronate (Pam) with doxorubicin and in (D) for the combination of alendronate (Alen) and doxorubicin. (* = $p < 0.05$).

We next sought to determine whether the interactions between bisphosphonates and cytotoxic chemotherapy drugs reflected truly synergistic interactions. To determine synergy, two different statistical analyses were used. First, the effects on MH cell viability of increasing concentrations of doxorubicin, with or without the addition of zoledronate (0.2 µg/mL), were evaluated. The results of the first analysis demonstrated a significant reduction ($p < 0.05$) in the IC50 concentration of doxorubicin when combined with zoledronate (**Figure 4.3**). In addition, the combination of drugs induced synergistic growth inhibition as described below. Similar experiments were done using increasing

concentrations of vincristine with clodronate (5 µg/mL). This combination also demonstrated a synergistic interaction ($p < 0.05$) (**Figure 4.3**).

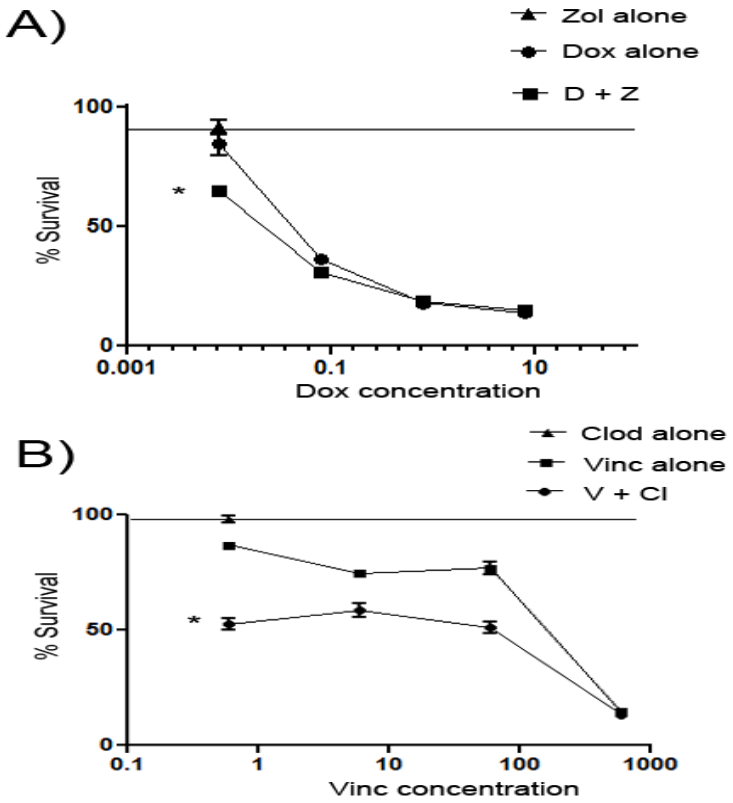

Figure 4.3. Dose response curves for determination of drug interactions between zoledronate and doxorubicin or between clodronate and vincristine. In (A), dose response curves were generated for DH82 cells treated with zoledronate, doxorubicin, or zoledronate plus doxorubicin in order to compare drug interactions via Bliss analysis. Cell viability was significantly reduced in cells treated with zoledronate (0.2 µg/mL) and increasing doses of doxorubicin, compared to cells treated with zoledronate alone (0.2 µg/mL) or increasing doses of doxorubicin alone, as assessed by Bliss analysis. In (B), a similar analysis was conducted using DH82 cells treated with clodronate (5 µg/mL) alone, with clodronate (5 µg/mL) plus increasing doses of vincristine, or with increasing doses of vincristine

As a second measure of synergistic interactions between bisphosphonates and chemotherapy drugs, Bliss analysis was conducted as described in Materials and Methods. This analysis also revealed a synergistic interaction ($p < 0.05$) between doxorubicin and zoledronate in combination, as well as between clodronate and vincristine in combination. The Bliss analysis however did not however support a synergistic interaction between pamidronate and doxorubicin or between alendronate and doxorubicin, despite the fact that these drugs exhibited significant interaction via one way

ANOVA ($p < 0.05$). To further validate the Bliss analysis, the data was also subjected to synergy calculations as described by Slinker, using two-way ANOVA[56]. This analysis also revealed a significant interaction ($p < 0.05$) between doxorubicin and zoledronate and between vincristine and clodronate. In contrast, an interaction between pamidronate and doxorubicin or between alendronate and doxorubicin was not identified using two-way ANOVA. Therefore, we concluded that based on multiple modeling approaches, there was strong evidence of synergistic interactions between these drugs.

Combined bisphosphonate and vincristine or doxorubicin treatment increases MH apoptosis.

Experiments were conducted next to elucidate the mechanism(s) by which bisphosphonate drugs increased growth inhibition of MH cells when combined with vincristine or doxorubicin. Canine DH82 MH cells were treated with clodronate alone (5 µg/mL) or vincristine alone (0.25 µg/mL), or both drugs together, and the effects on induction of apoptosis were assessed using Annexin V and propidium iodine staining and flow cytometry. Treatment with the combination of vincristine and clodronate induced a significant increase ($p < 0.05$) in the percentage of apoptotic cells (**Figure 4.4**). Similarly, a significant increase in MH cell apoptosis was also obtained following treatment with combined doxorubicin (0.2 µg/mL) and zoledronate (0.2 µg/mL) ($p < 0.05$) (**Figure 4.4**).

The effects of combined treatment on induction of activated caspase 3/7 activity (a measure of late apoptosis induction) was also assessed. When DH82 cells were treated with the above mentioned concentrations of vincristine and clodronate in combination for 48 hours, there was a significant increase in caspase 3/7 activity, compared to treatment with either drug alone ($p < 0.05$) (**Figure 4.4**). The combination of zoledronate with doxorubicin at the same concentrations as used in the Annexin V assay also generated a

significant increase in caspase 3/7 activity when compared to single drug treatment in DH82 cells ($p < 0.05$) (**Figure 4.4**). Similar results were also obtained using the MH-1 and MH-2 cell lines (data not shown).

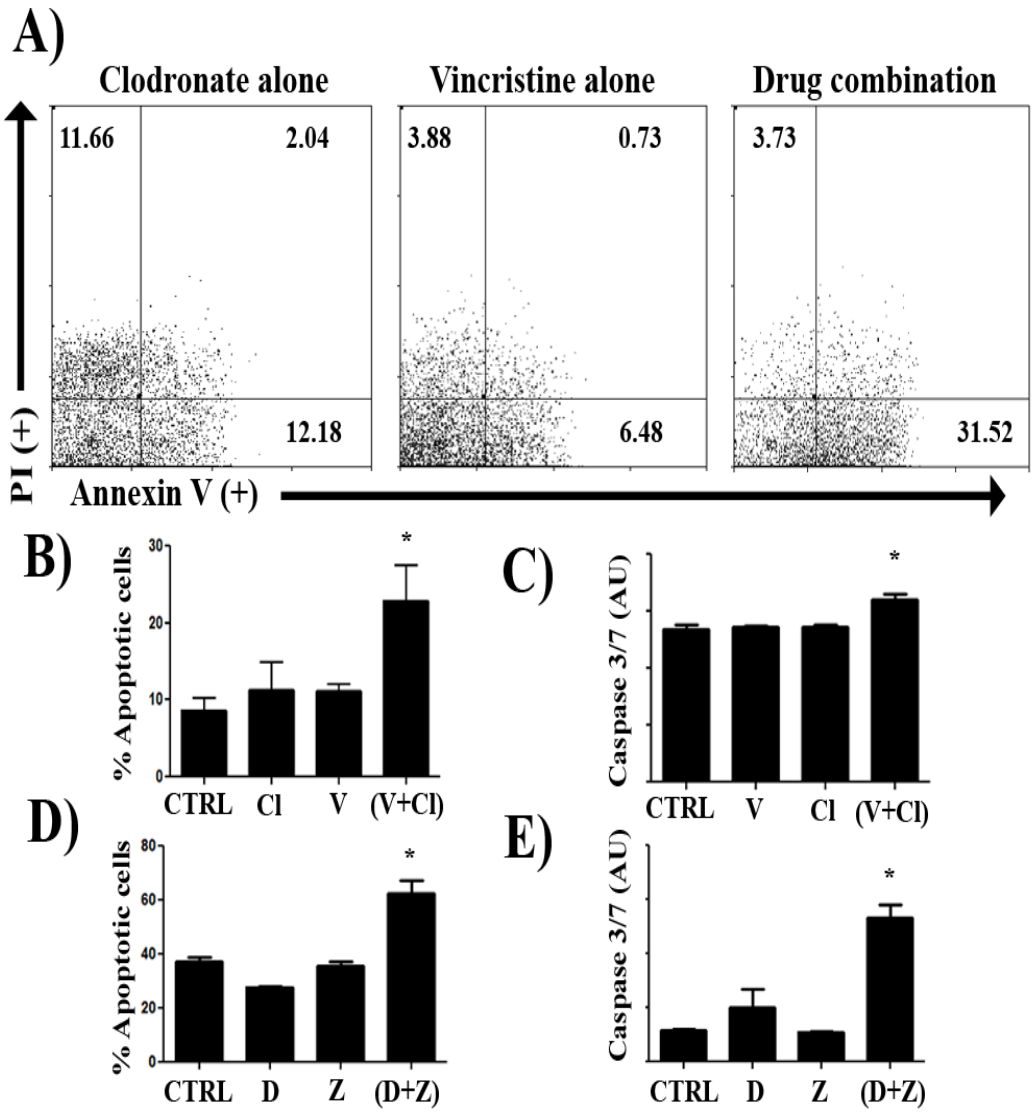

Figure 4.4: Combined treatment with zoledronate and doxorubicin and clodronate and vincristine results in increased MH cell apoptosis. In (A), an increased percentage of Annexin V+ cells were noted in cells treated with the combination of two drugs, as assessed by flow cytometry and depicted in these representative FACS plots. In (B), the mean percentage of apoptotic cells was compared between MH cells treated with clodronate or vincristine alone or in combination. There was a significant increase (* = $p < 0.05$) in the percentage of apoptotic cells in the combination treated group, as assessed by ANOVA and Tukey's multiple means comparison test. In (C), induction of apoptosis was also assessed by measuring induction of caspase 3 and 7 activity, as described in Methods. Treatment of DH82 cells with the combination of clodronate (5 µg/mL), vincristine (0.25 µg/mL), or both induced a significant increase (*= $p < 0.05$) in caspase 3/7 fluorescence (AU, arbitrary fluorescence units) as compared to untreated control cells or cells treated with one drug only, as assessed by ANOVA. In (D), combined treatment with zoledronate (0.2 µg/mL) and doxorubicin (0.2 µg/mL) induced a significant increase in the number of apoptotic MH cells, as assessed by Annexin V and propidium iodide staining. In (E), the combined treatment with zoledronate (0.2 µg/mL) and doxorubicin (0.2 µg/mL) resulted in a significant ($p < 0.05$) increase in caspase 3 and 7 activity in MH cells, compared to cells treated with either drug alone

Treatment with clodronate enhances G2 cell cycle arrest induced by vincristine

We hypothesized that clodronate may potentiate vincristine's effect on the cell cycle, leading to the observed synergistic interaction. Therefore, we assessed the effects of clodronate treatment on induction of cell cycle arrest by vincristine. We found that addition of clodronate (5 µg/mL) induced a significant increase in sub G1 and G2/M arrest in MH cells treated with vincristine (0.25 µg/mL) ($p < 0.05$) (**Figure 4.5**).

It therefore appears that the depletion of cellular ATP by clodronate leads to an increase in G2/M arrest when combined with vincristine.

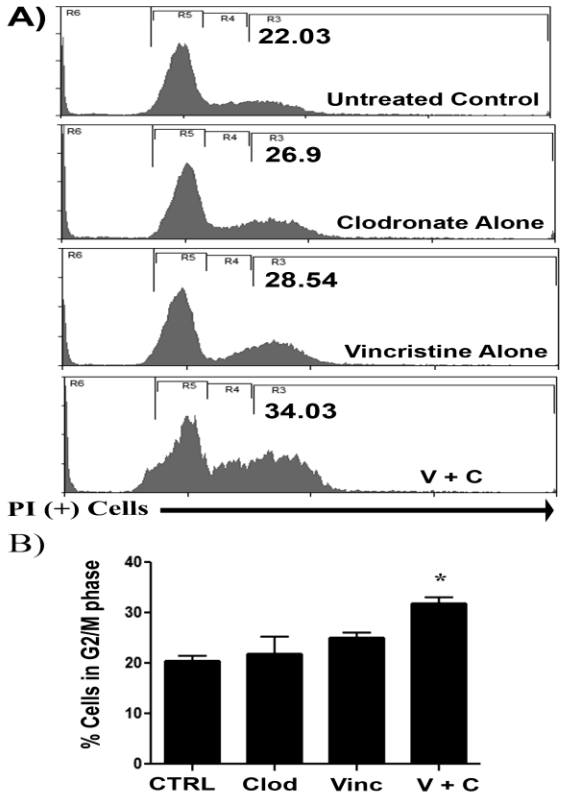

Figure 4.5: Addition of clodronate to vincristine treatment increased cell cycle arrest in canine MH cells. The effect of the addition of clodronate to vincristine treated DH82 cells was assessed using flow cytometry and cell cycle analysis. In (A), representative flow cytometry histograms are displayed for cells treated for 48 hours with clodronate (5 µg/mL), vincristine (0.2 µg/mL), or with both in combination. In cells treated with the combination of both drugs, there as a notable increase in the percentage of DH82 cells exhibiting G2/M arrest (R3 = G2/M). In (B), the mean percentages of cells in the G2/M stage of cell cycle progression were calculated for untreated cells or cells treated with clodronate or vincristine, alone or together. A significant increase (* = $p < 0.05$) in cells in the G2/M stage was observed in cells treated with clodronate plus vincristine.

Treatment with zoledronate leads to an increase in intracellular doxorubicin accumulation.

We did not see changes in the cell cycle when cells were treated with zoledronate in addition to doxorubicin. Therefore, we assessed the effects of zoledronate treatment on the permeability of MH cells to doxorubicin, using a fluorescence assay and flow cytometry. Cells were treated for 24 hours with doxorubicin at a concentration of 0.2 µg/mL, with zoledronate at a concentration of 0.2 µg/mL, or with both doxorubicin and zoledronate at the above concentrations. Intracellular doxorubicin was then evaluated via flow cytometry. We found that treatment with zoledronate resulted in a significant increase in doxorubicin uptake by MH cells, whereas treatment with other bisphosphonates did not ($p < 0.05$) (**Figure 4.6**). These results suggest that increased doxorubicin accumulation might account for the increase in MH cytotoxicity observed following treatment with both zoledronate and doxorubicin.

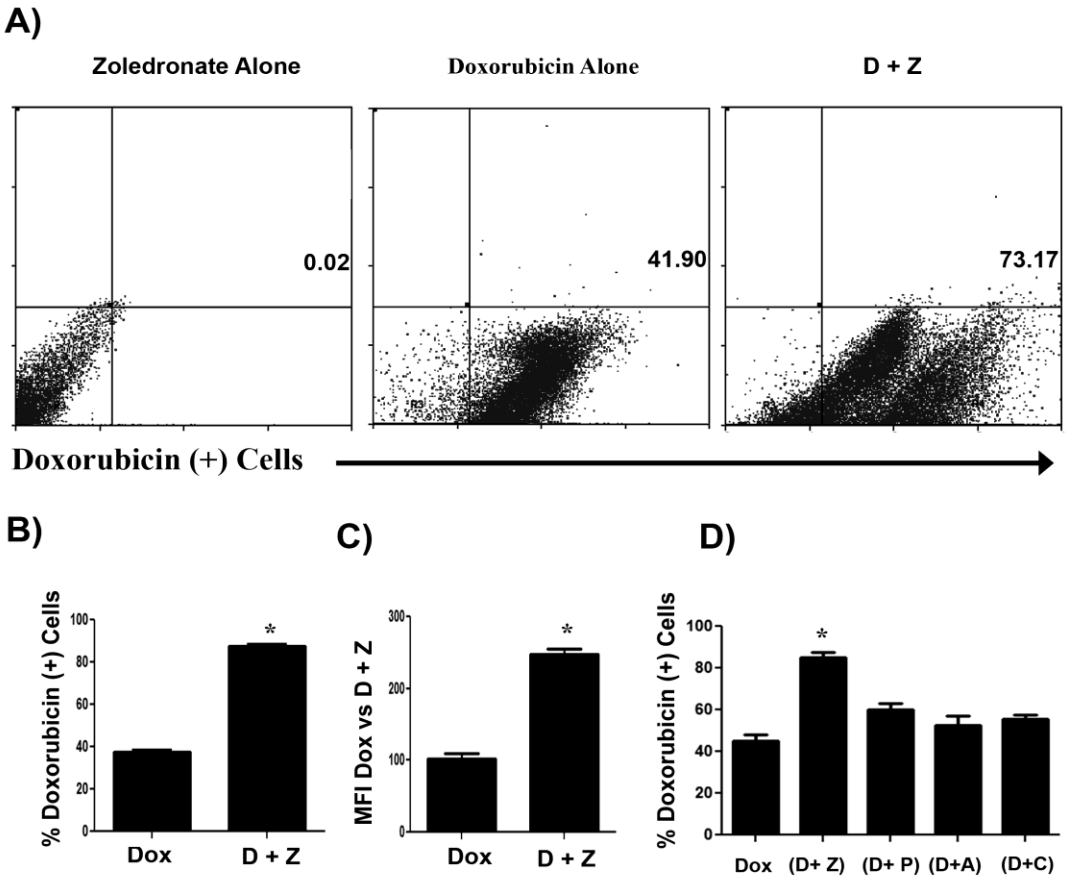

Figure 4.6: Treatment with zoledronate increases doxorubicin uptake by DH82 cells. DH82 cells were treated with doxorubicin alone (0.2 µg/mL), or with zoledronate (0.2 µg/mL) plus doxorubicin for 24 hours. The cells were then analyzed for intracellular concentrations of doxorubicin using flow cytometry. In (A), representative dot plots of doxorubicin fluorescence intensity for cells treated with zoledronate alone, doxorubicin alone, or zoledronate plus doxorubicin are depicted. In (B), the mean percentage of doxorubicin+ MH cells from triplicate wells treated with doxorubicin alone or doxorubicin plus zoledronate was plotted. The percentage of doxorubicin+ cells was significantly increased (* = $p < 0.05$) in the combination treated cells. In (C), the mean of the mean fluorescence intensities (MFI) of doxorubicin expression by cells treated with doxorubicin alone or doxorubicin plus zoledronate was plotted. The mean MFI of doxorubicin expression was significantly increased (* = $p < 0.05$) in the combination treated cells. In (D), MH cells in triplicate wells were treated doxorubicin alone or with zoledronate (Z), pamidronate (P), alendronate (A), or clodronate (C) plus doxorubicin for 24 hours and the mean percentage of doxorubicin+ cells was determined by flow cytometry. Only treatment with zoledronate produced a significant increase (* = $p < 0.05$) in the percentage of doxorubicin+ cells.

Discussion

Malignant histiocytosis (MH) is a devastating disease in dogs, with short survival times and poor response rates to treatment[7, 8, 11, 12]. These tumors often progress very rapidly in dogs and MH is often highly resistant to chemotherapy, a phenomenon that is also observed in humans with aggressive forms of a similar neoplasm known as Langerhans cell histiocytosis[16-18]. Treatment with vinca alkaloids and zoledronate can often be temporarily effective against chemoresistant Langerhans cell histiocytosis humans. These tumors can originate from the bone marrow in dogs and humans and the bone marrow may also serve as a site for tumor recrudescence[4, 7, 9, 14, 16]. Bisphosphonate drugs reach their highest concentrations in bone, which may allow this class of drugs to reach effective concentrations against MH tumors involving bone marrow [41, 53, 59, 60].

Our current study revealed that there were two novel drug combinations that might be expected to have significant *in vivo* activity against canine MH. The first effective combination was clodronate combined with vincristine, which induced a synergistic increase in apoptosis of MH cells, presumably by increasing cell cycle arrest. Such a combination might be particularly effective in dogs with bony involvement with MH. The combination of clodronate with vincristine has the additional advantage of being relatively inexpensive to use, although clodronate is not licensed for use in the United States and would thus have to be obtained from foreign sources for treatment of animals here.

While the exact mechanism of this synergistic interaction is not yet fully defined, we have been able to show that the addition of clodronate to vincristine potentiates the effects of vincristine on the cell cycle. We observed a higher percentage of cells in G2/M arrest when treated with the combination of the drugs than with either drug alone. This indicates that clodronate has a direct potentiating effect on the effects of vincristine, as this is the primary anti-tumor mechanism of vincristine via effects on microtubules. As

the primary effects of clodronate inhibit ATP usage by the cell, our hypothesis is that clodronate disrupts formation of actin filaments which are essential for successful cytokinesis. The combination of vincristine and clodronate may affectively block both microtubules and actin filaments, thus leading to an increase in G2/M arrest. Further studies are needed to confirm this proposed mechanism.

We also found that zoledronate increased the activity of doxorubicin against canine MH cells. The effects of zoledronate appeared to be mediated at least in part by increasing tumor cell permeability to doxorubicin. Studies in non myeloid tumor cell lines in humans and rodents have previously demonstrated a synergistic interaction between zoledronate and doxorubicin, though the effects of zoledronate on doxorubicin uptake were not examined in those studies [48-50]. The mechanism for this increased uptake remains uncertain. The primary effects of zoledronate on the cell are due to decreases in protein prenylation and subsequent inactivation of small GTPases such as Ras. Therefore, the increased drug accumulation could be due to decreased ability of the cells to excrete doxorubicin secondary to inhibition of these GTPases. Many tumors have upregulated Ras expression, and this increase has been shown to directly protect tumor cells from doxorubicin induced apoptosis[61]. Further studies will be needed to support this hypothesis.

More recent studies have shown that zoledronate has potent immunomodulatory effects in addition to direct effects on tumor cells[38, 51]. In particular, zoledronate partially depletes tumor associated macrophages (TAM), which in turn which leads to decreased tumor angiogenesis and increased activation of anti-tumor immunity[38, 51]. Since TAM have also been shown to decrease the sensitivity of tumor cells to chemotherapy, depletion of TAM using zoledronate could potentially augment the effectiveness of cytotoxic chemotherapeutics in vivo by a mechanism independent of direct drug-drug interactions[62]. Zoledronate is also much more potent than clodronate, so clinically achievable therapeutic drug levels are possible in viscera as well as bone[42, 45, 58].

Zoledronate has been administered previously to dogs with osteosarcoma for relief of malignant osteolysis[53, 58]. In addition, the combination of zoledronate and doxorubicin has been administered without apparent increased toxicity to dogs with advanced osteosarcoma metastases (Fan, TM; personal communication). Thus, combined treatment with zoledronate and doxorubicin is feasible in dogs and should be investigated further in dogs with MH.

The results of our in vitro studies reported here indicate that combined treatment with selected bisphosphonates may increase the effectiveness of either vincristine or doxorubicin for treatment of MH in dogs. In particular, the combination of clodronate with vincristine may be indicated for animals with MH bony involvement, as clodronate reaches high concentrations in bone, while zoledronate combined with doxorubicin may be beneficial for treatment of visceral tumor metastases due to greater non-osseous tissue concentrations achieved with zoledronate. In summary, our results provide the rationale behind additional clinical evaluation of combined bisphosphonate and vincristine or doxorubicin chemotherapy for treatment of dogs with advanced MH disease.

Acknowledgements

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Chapter Six

General Conclusions and Future Directions

General Conclusions

While initially identified as an important component of the overall cellular makeup of tumors, the cells that make up the surrounding tumor stroma were largely ignored in much of the subsequent research into tumor biology and treatment. Most initial treatments were developed and identified for their cytotoxic effects on rapidly dividing tumor cells. While these treatments will always be the cornerstone of chemotherapeutic treatment of tumors, it has recently been recognized that the support system of the tumor associated stroma may also represent an attractive target for anti-cancer chemotherapeutics. Of the stromal components, tumor blood vessels were the first to be identified and targeted as vitally important to tumor growth. Subsequently much work continues to be performed looking for effective anti-angiogenic treatments that can be used as an adjunct to cytotoxic chemotherapy. The goal of this project was to attempt to develop an effective treatment to target another crucial area of the tumor stroma, the tumor associated macrophage.

The ideal chemotherapeutic drug is one that targets multiple mechanisms, as tumors represent a moving target that is constantly evolving and changing characteristics.

A broad based therapy is better able to sustain effectiveness in this type of environment. From this perspective, the targeting of tumor associated macrophages potentially impacts most if not all of the stromal components in the tumor microenvironment. Such a drug could be anti-proliferative, anti-angiogenic, decrease invasion and metastasis, and increase immune response against the tumor cells. This could therefore represent a very effective anti-tumor treatment strategy.

In order to target tumor associated macrophages, we chose to use an established macrophage depleting drug. While liposomal clodronate has previously been evaluated and shown to be effective in murine tumor models, it has never been evaluated using a clinically relevant, intravenous route of administration. Our first goal was to therefore attempt to optimize the drug for better macrophage depletion. We have been able to show that the addition of a mannose ligand to the liposome structure allows more efficient killing of macrophages both in vitro and in vivo. This macrophage killing correlates with liposome uptake as cells that do not take up the liposomes, including tumor cells, are unaffected. When given intravenously, LC was able to deplete resident tissue macrophages in the spleen, liver, and lungs. When administered intravenously to mice in a cutaneous fibrosarcoma tumor model, this modified liposomal drug was able to induce anti-tumor effects despite a lack of direct effects on the tumor cells. We were able to demonstrate a decrease in tumor associated macrophages in treated mice.

Interestingly, this decrease was not due to local accumulation of liposomes in the tumor. This suggests that systemic administration of liposomal clodronate leads to the depletion of peripheral monocytes, which are the cells that are recruited into the tumor to become tumor associated macrophages. While this decrease in TAMs potentially has

several anti-tumor effects as previously discussed, the depletion of monocyte precursors systemically has some interesting potential therapeutic benefits as well. These cells make up a component of cells known as myeloid derived suppressor cells (MDSC). These cells are vitally important in maintaining a systemically immosuppressive phenotype in cancer patients, and unlike TAMs are not only present in tumor tissues but also circulate systemically. Therefore these cells would likely be largely unaffected by localized tumor treatment with liposomal clodronate, whereas they could potentially be depleted by systemic liposomal clodronate administration. Further work will be needed to determine if LC can effectively deplete these cells systemically, and if that depletion can reverse the immunosuppressive phenotype found in most tumor patients.

Given these initial successes in the mouse tumor model, we next sought to determine if LC would be effective in a naturally occurring tumor in an outbred animal species. We chose to treat malignant histiocytosis as this is a tumor that is in desperate need of new treatment options in canine patients. LC also has a potential for a dual benefit in these tumors, both via direct anti-tumor effects and due to effects on non transformed macrophages present in the stroma of these tumors. We were able to show that LC had a direct cytotoxic effect on three MH cell lines evaluated. However, these effects did vary greatly between the cell lines. It was the phagocytic capability of the tumor cells that dictated their susceptibility to the drug, which correlates with the findings in the murine cell lines.

Importantly, there were no serious side effects of treatment other than fever development and corresponding lethargy and decreased appetite in some dogs. These side effects were transient, and resolved in all patients within 24 hours with no or

minimal supportive care. We were able to determine a physiologic profile of fever development, increased neutrophils, and decreased monocytes which correlated with tumor response. We were also able to identify potential biomarkers of MH, as there were 4 cytokines that were significantly elevated over normal dogs in the MH dogs. One of these, interleukin 8, was significantly decreased 24 hours after treatment with LC in these dogs. This could potentially hold promise both from an anti-proliferative and anti-angiogenic standpoint in these dogs.

Unfortunately, we are unsure of why some patients had a good response and some did not. We did not do a phase I study prior to initiation of treatment, and it may therefore be necessary to increase the clodronate dose in dogs that do not develop the physiologic signs of response. As MH is caused both by DCs and macrophages, it is likely that the underlying phenotype of the tumor will determine tumor susceptibility in vivo, as was seen with the difference in cell line susceptibility in vitro. Further studies would be needed to further characterize the underlying phenotype of tumors prior to initiation of treatment, and determine if a more phagocytic tumor would be more responsive to LC treatment or if dose adjustments are necessary to initiate a tumor response.

We were also unable to determine tumor stromal effects of treatment with LC. This is due to the fact that the majority of our treated patients had visceral disease which did not make their tumors amenable to repeated biopsies. We are currently evaluating LC in treatment of soft tissue sarcomas in dogs. We are therefore able to perform repeated biopsies on these tumors, and measure response as well as the potential for macrophage depletion in these tumors. Another important endpoint will be evaluation of

IHC for decreases in angiogenesis to see if the decreased IL-8 levels identified in these patients will correlate to an overall decrease in angiogenesis in the tumors.

While the safety and efficacy achieved with LC were encouraging, even in a best case scenario this drug would not be available on a routine clinical basis for many years. Preclinical studies of drug therapy in MH and Langerhans Cell Histiocytosis are sorely lacking, and as such we next sought to determine if there were any chemotherapy combinations that could show efficacy against MH in vitro. We chose to combine traditional chemotherapy with bisphosphonates due to the well established capability of BPs to kill macrophages as single agents, the use of BPs clinically in human LCH with bone metastasis, and recent evidence suggesting synergistic interactions between BPs and cytotoxic chemotherapy agents in other tumor models.

We were able to show that the combination of clodronate with vincristine, or doxorubicin with zoledronate is capable of killing MH cells synergistically in vitro. The combination of clodronate and vincristine is interesting in that it is a relatively inexpensive treatment, and potentially holds promise using the liposomal formulation of clodronate as well. The major disadvantages of these drugs clinically are the difficulty in obtaining clodronate in the United States, and uncertainty as to whether clodronate will reach high enough levels systemically to synergize with vincristine. Unfortunately, using free drug this combination may be relegated only to cases with bone involvement where surgery is not possible.

Future Directions

The combination of zoledronate and doxorubicin holds more promise clinically. This combination has the disadvantage of being far more expensive than vincristine and clodronate, or the current standard of care CCNU. However zoledronate should come down in price in the coming years, making this treatment more cost effective. More importantly, the drug levels evaluated in vitro can be achieved in vivo with zoledronate, making this drug combination more likely to be effective against visceral disease.

The fact that we were able to see synergistic interactions at these low levels also makes this drug combination potentially attractive against other tumor types as well. Tumors of bone or with bone metastasis such as osteosarcoma, multiple myeloma, breast cancer and prostate cancer for example could potentially show a benefit of this drug combination. Further in vitro studies should be undertaken to determine if this combination shows efficacy against other tumor types in vitro. If they do, this could potentially open the doors for a phase I clinical trial in multiple tumor types to determine if this drug combination is safe and effective clinically.

As MH cells are tumors derived from histiocytic cells, there is the additional possibility that this drug combination could have an effect against tumor associated macrophages as well as direct anti-tumor effects. Further studies will be needed to determine if this is indeed a possibility.

It is in the field of combination therapy that liposomal clodronate holds the most promise. With the exception of MH, LC is not expected to have primary anti-tumor cell activity. Therefore, as a single agent it can only be expected to decrease tumor

progression, not eliminate a tumor completely. It will be imperative that liposomal clodronate or any macrophage depletion agent be used in combination with standard chemotherapy to achieve maximum benefits. By combining direct tumorcidal activity with the benefits of LC against stromal support, true synergistic interactions may be possible. LC has the added benefit of a wide safety profile that is unlikely to add to the toxicity of standard chemotherapy. Our initial clinical evaluations have not demonstrated any additive toxicities when combining LC with chemotherapy. We have safely used the combinations of clodronate with CCNU and clodronate with vincristine in small numbers of dogs. We have seen some dramatic responses using liposomal clodronate with CCNU, and are currently evaluating this combination in an MH clinical trial.

Another avenue that holds great promise when combined with liposomal clodronate in cancer treatment is the use of anti-cancer vaccines. If LC can reverse tumor immunosuppression, treatment with an immune stimulant or an anti-cancer vaccine could have greatly increased effectiveness. Studies are currently under way looking at the possibility of combining therapeutic cancer vaccines and liposomal clodronate. If the global immunosuppression found in tumor patients can be reversed, it may re-open the door to effective immune therapies for cancer treatment.

The treatment of cancer, particularly aggressive and malignant neoplasia, continues to be extremely challenging. True gains in long term survival or cures in this setting have continued to be difficult to come by. A tumor is a complex, always changing conglomeration of transformed tumor cells, blood vessels, support tissues, lymphatics and immune cells. The ideal therapy then will likely be less a magic bullet and more a shotgun shell composed of a multitude of treatments. Further study will be required to

determine if true multi-modal chemotherapy, aimed at targeting each of these support systems in addition to the tumor cells themselves, is indeed possible. If so, it opens up an exciting new avenue for the development of cancer therapeutics.