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Liposomal Clodronate Treatment For Tumor Macrophage Depletion in Dogs with Soft Tissue Sarcoma

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Abstract

Increased numbers of tumor-associated macrophages correlate with rapid tumor growth and metastasis in tumors. Thus, macrophage depletion has potential as a novel cancer therapy and positive responses have been reported in rodent tumor models. To investigate the effectiveness of this approach in dogs with cancer, we evaluated the effects of the macrophage depleting agent liposomal clodronate (LC) in dogs with soft tissue sarcoma (STS). To this end, we conducted a clinical trial of LC therapy in thirteen dogs with soft tissue sarcoma. Repeated LC administration was well tolerated clinically. Preliminary examination of tumor biopsy sets from 5 of the 13 dogs demonstrated that the density of CD11b⁺ macrophages was significantly decreased after LC treatment. Circulating concentrations of IL-8 were also significantly reduced. These preliminary studies are the first to suggest that LC can be used as a systemic macrophage-depleting agent in dogs to reduce numbers of tumor-associated macrophages.

Keywords

Macrophage; cancer; immune; canine; myeloid

Introduction

Increased numbers of tumor-associated macrophages (TAMs) in tumor tissues are associated with increased tumor growth and metastasis in several mouse models of cancer.[1,2,3] In addition, the correlation of increased TAM density with reduced overall survival times has been noted in humans with a variety of different tumor types.[4,5,6,7,8,9,10] Recently, it was also shown that increased TAM density correlated positively with decreased survival times in dogs with mammary adenocarcinoma.[11,12]

TAMs are thought to promote tumor growth via multiple, non-redundant mechanisms. For example, TAMs promote tumor angiogenesis by producing pro-angiogenic growth factors such as VEGF and IL-8.[13] In addition, TAMs support tumor invasion and metastasis via the local elaboration of matrix metalloproteinases such as MMP-9.[14] Moreover, TAMs exert potent immune suppressive activities, including interference with T cell signaling and proliferation as well as inhibition of antigen presenting cell function.

[15,16,17,18,19,20,21,22,23,24] Therefore, there are multiple reasons to believe that depletion of TAMs would lead to more effective control of tumor growth and metastasis.

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Conflict of interest: S.W.D. is an author on a submitted patent relating to the use of liposomal clodronate for cancer treatment.

The use of liposome-encapsulated clodronate (LC), a first generation bisphosphonate drug encapsulated in a liposome, to deplete macrophages in animals with cancer holds considerable appeal. First, there is a large body of evidence in mice indicating that systemic administration of LC efficiently depletes macrophages in a variety of body sites. [1,2,3,25,26,27,28,29,30,31,32] Introduction of clodronate into the cytoplasm of macrophages appears to induce rapid onset of apoptosis via competition for ATP binding to cellular substrates.[30] Secondly, several studies now indicate that systemic LC administration can induce significant antitumor activity in multiple mouse tumor models. [1,2,3,25,26,27,28,29,33,34] The antitumor activity of LC administration has been primarily attributed to depletion of TAMs and secondarily to inhibition of tumor angiogenesis. [1,2,3,25,26] Third, our group has pioneered the use of LC therapy in dogs, initially as a means of depleting macrophages for control of autoimmune hemolytic anemia.[35] More recently, we reported that LC administration controlled tumor growth in dogs with malignant histiocytosis (MH), presumably due to direct killing of phagocytic MH cells by LC.[36]

We were interested in determining whether LC administration also exerted antitumor activity in dogs with tumors, such as soft-tissue sarcomas (STS), that were resistant to the direct tumor killing effects of LC.[36] To address this question, we used the canine STS model that, because of its cutaneous location and relatively slow growth, is well-suited to studies requiring repeated tumor sampling. To this end, 13 dogs with STS were enrolled and treated with LC administered on a weekly or a bi-weekly basis. The primary study endpoints were effects of LC treatment on TAM density and tumor microvessel density (MVD). Secondary endpoints included effects of LC administration on circulating leukocyte numbers and cytokine concentrations and on tumor growth rates.

Materials and Methods

Patient population

Client-owned dogs with biopsy confirmed soft-tissue sarcomas were recruited into the study between September 2008 and July 2011. The study protocol was approved by the Institutional Animal Care and Use Committee at Colorado State University. Exclusion criteria included dogs with significant other medical diseases, dogs with tumors > 15 cm in diameter, and dogs that had received local radiation therapy to the tumor site within the past 3 months. Dogs that developed progressive disease during the treatment period, defined as a greater than 20% increase in longest tumor diameter, were removed from the study before the end of the anticipated study period.

Treatment protocol

Liposomal clodronate was prepared as described previously.[35,36] Dogs were treated by *i.v.* administration of 2.5 mg/kg liposome-encapsulated clodronate at a volume of 0.5 ml/kg over a 60-minute period via an indwelling venous catheter, as previously described.[35,36] Dogs were either treated once weekly (n = 11) or once every two weeks (n = 2) for a total of six or four treatments, respectively. Body temperature, heart rate, and respiratory rate were monitored for 24 hours after the initial LC infusion. CBC values were obtained prior to and 24 hours after the first infusion and then immediately prior to each subsequent LC treatment. Adverse events were recorded and reported using VCOG scoring.[37] Tumor punch biopsies were collected prior to the initial treatment and again at the conclusion of the study. Tumor volume was determined at each treatment. Tumor progressive disease was defined by RECIST criteria.[38] Specifically, progressive disease was defined as a greater than 20% increase in size of the longest diameter of the tumor.

Tumor immunohistochemistry

Tumor tissue biopsies were embedded in OCT media (Sakura Fine-Tek, Torrance, CA) and frozen at -80°C . Tissues were cryosectioned into $4\text{ }\mu\text{m}$ sections and placed on glass slides (Superfrost, VWR, Radnor, PA) and stored at -80°C prior to immunostaining. Sections were fixed in ice-cold acetone and air-dried, then rehydrated in PBS and treated with hydrogen peroxide to block endogenous peroxidase activity and blocked in normal goat serum prior to antibody staining. A mouse anti-dog CD11b antibody (Clone CA16.3E10, Serotech, Raleigh, NC) was used to quantitate TAM, while a mouse anti-MCAM (Clone PIH12, Millipore, Billerica, MA) antibody was used to quantitate tumor microvessels, as reported previously.[39] Tissue samples were incubated with primary antibodies at 4°C overnight. After washing with PBS, biotin-conjugated goat anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA) was used as the secondary antibody followed by Vectastain ABC kit, with AEC used as the detection substrate (Vector Laboratories, Burlingame, CA). Slides were counter-stained with hematoxylin. For enumeration of TAM or microvessel endothelial cells, three independent, random 100X or 400X field images from each slide were obtained using a Carl Zeiss Axioplan 2 imaging scope coupled with an AxioCam HRC Carl Zeiss Camera (Carl Zeiss, Thornwood, NY). Numbers of CD11b⁺ cells (400X) and CD146⁺ cells (100X) were quantitated by examining 3 separate 400X or 100X fields and averaging the cell density between these fields using Axio Vision 4.3 software (Carl Zeiss). Analysis of three fields for both TAMs and angiogenesis was chosen given the small size of the samples obtained.

Serum cytokine analysis

Serum samples collected immediately prior to the first LC administration and again 24 hours later were analyzed for concentrations of 12 cytokines (IL-2, IL-4, IL-6, IL-8, IL-10, IL-15, IL-18, IP-10, TNF, GM-CSF and KC) using the Canine Milliplex MAP cytokine bead array kit (Millipore, Billerica, MA), following the manufacturer's directions. Cytokine concentrations were determined using a Luminex 100 instrument (Luminex, Austin, TX).

Statistical analyses

In experiments where mean values were compared between 2 groups, a nonparametric *t*-test (Mann-Whitney) was used. In experiments where mean numbers of CD11b⁺ or CD146⁺ cells in tumor tissues were compared between day 0 and day 21 of the study, a one-way, paired *t*-test was performed, given that our null hypothesis was that liposomal clodronate treatment would decrease CD11b⁺ cells and CD146 staining. Statistical analyses were performed using GraphPad Prism software (version 5.0, GraphPad, San Diego, CA). A *p*-value of > 0.05 was considered to be statistically significant.

Results

Study population characteristics

Thirteen dogs were enrolled in the study between September 2008 and July 2011. Breeds represented included 2 Golden Retrievers, 2 mixed-breed dogs and one Irish Wolfhound, one Cocker Spaniel, one Beagle, one Pomeranian, one English Setter, one Labrador Retriever, one Miniature Schnauzer, one Shiba Inu, and one German Short-haired Pointer. Characteristics of enrolled dogs are displayed in Table 1.

LC treatment

Dogs were treated with 2.5 mg/kg liposome encapsulated clodronate at a volume of 0.5 ml/kg administered slowly over one hour *i.v.* once weekly ($n = 11$ dogs) or every other week ($n = 2$ dogs) for a total of 6 treatments. The initial 2 dogs enrolled in the study were treated

every two weeks with LC and displayed no side effects. At this time, we also obtained data from ongoing mouse studies that indicated that more frequent dosing with LC increased treatment efficacy. Therefore, the remaining 11 dogs enrolled in the study were placed on weekly treatment with LC. With the exception of one dog developing a Grade I febrile response (VCOG score, body temperature of 103.5F) for 4–6 hours after receiving one treatment with LC, none of the other treated animals developed any treatment-related adverse events.

Short-term hematological changes associated with administration of LC

Prior studies in mice have indicated that *i.v.* LC administration induced transient monocytopenia.[40,41] Therefore, we examined leukocyte values prior to and 24 hours after administration of LC in 12 of the 13 study dogs. We found that LC administration did not induce monocytopenia in any of the study dogs (Table 2). However, there was a significant increase in numbers ($p = 0.002$) and percentages ($p = 0.003$) of neutrophils 24 hours following LC treatment. The elevation in neutrophil numbers returned to baseline values by 7 days after LC administration (data not shown). Numbers and percentages of eosinophils, basophils and lymphocytes remained unchanged following LC treatment (data not shown). We also observed a significant decrease ($p = 0.0002$) in platelet numbers 24 hours after LC infusion (Table 2), which returned to normal values by one week after treatment (data not shown). Four of the thirteen treated dogs developed a Grade I thrombocytopenia. However, clinical evidence of coagulopathy (*eg*, spontaneous petechiation) did not develop in any of these 4 dogs. Thus, the dose of LC administered in our study was not sufficient to induce significant depletion of circulating monocytes, but did induce transient neutrophilia and reduction in baseline platelet counts.

Effects of LC treatment on TAM density

The effects of LC administration on TAM density was also examined based on prior evidence that LC administration could reduce TAM density in mice. [1,2,3,25,26,27,28,29,33,34,35] For this study, pre- and post-treatment tumor biopsies were judged to be adequate for immunohistochemical evaluation from 5 of the original 13 enrolled animals. In the other nine study animals, tumor biopsies were either too small for evaluation or did not contain actual tumor tissue. All of these five animals were in the once weekly treatment group. We observed a significant ($p < 0.01$) decline in the density of TAMs in post-treatment tumor biopsies compared to pre-treatment biopsies (Figure 1). The mean TAM density in pre-treatment biopsies was 51 cells per high-powered field, while the mean post-treatment TAM density was 35 cells per high-powered field.

Effects of LC administration on tumor angiogenesis

Tumor sections were also evaluated by IHC for quantitation of tumor angiogenesis (MVD). The mean tumor MVD was 38 vessels per high-powered field (HPF) in pre-treatment biopsies, whereas the mean tumor MVD was 27 vessels per HPF in post-treatment biopsies. While we observed a significant decrease ($p = 0.05$) in tumor MVD when pre-treatment and post-treatment MVD values were compared (Figure 2), an obvious decrease in vessel density was only observed in 3 of the 5 dog tumor sample sets. Hence, our small sample size is only suggestive that treatment with LC leads to decreased angiogenesis in dogs with STSs, and further studies with a larger number of dogs are warranted before any conclusive statements can be made in regards to LC and angiogenesis.

Effects of LC administration on circulating cytokine concentrations

In our prior published study of LC administration in dogs with MH, we observed the development of fever in several of the treated animals.[36] In addition, we noted transient

fever development in one of the dogs in the present study. Therefore, we chose to evaluate the effects of LC administration on circulating cytokine concentrations in the first 24 hours following treatment in dogs in the present study. To this end, serum concentrations of 12 canine cytokines (IL-2, IL-4, IL-6, IL-8, IL-10, IL-15, IL-18, IP-10, TNF, GM-CSF and KC) were compared between pre-treatment and 24 hour post-treatment serum samples in dogs treated with LC, using multiplexed cytokine detection assays.[42] Serum concentrations of IL-2, IL-4, IL-6, IL-10, IL-15, IL-18, IP-10, TNF, GM-CSF and KC were not significantly different when pre- and post-treatment serum samples were compared. However, we did note a significant decline in serum IL-8 concentrations 24 hours after LC administration (Figure 3).

Effects of LC administration on tumor growth rates

The average tumor diameter for dogs in this study was 6.7 cm (range of 1.5–13 cm). Of the 13 animals that began the LC study, only 4 animals completed the entire study. Nine dogs completed half of the study (3 LC treatments), withdrawing after day 21–28. The following are reasons for withdrawal from the study: 5 dogs developed PD, including 2 dogs that developed tumor ulceration. Three dogs were removed from the study due to tumor ulceration, which could be considered a marker of PD. One dog was withdrawn from the study due to progressive glaucoma that was judged not treatment related. Of the five samples collected for TAM analysis, 2/5 dogs showed PD despite having a decrease in TAMs. Of the 4 dogs that showed an obvious decrease in TAMs, one had PD. Of these same five samples collected for the vessel density analysis, 2/3 of the dogs that showed an obvious decrease in angiogenesis demonstrated PD as defined by increase in tumor burden and/or ulceration. Interestingly, 2/3 dogs that showed decreases in TAMs also showed a decrease in tumor vessel density. Thus, for STS, a decrease in TAMs and/or angiogenesis did not necessarily correlate with decreased tumor burden

Discussion

In the present study, we found in five dogs with STS that a series of infusions of LC were capable of depleting TAM and suppressing circulating IL-8 concentrations. While LC treatment was not associated with clinically significant tumor growth responses, these studies nevertheless demonstrated the potential for macrophage-depleting therapeutics to be employed in animals with spontaneous cancer, including dogs and potentially humans.

Importantly, repeated infusions of LC did not cause acute or cumulative toxicity in the dogs in the present study. The transient neutrophilia noted following LC administration could not be fully explained although it has been noted previously in rodent studies that charged liposomes such as the phosphatidylcholine liposomes used in the present study are capable of causing immune activation.[46] However, we have observed in numerous rodent studies that liposomes which contain PBS instead of clodronate solution do not elicit antitumor activity following *i.v.* administration (data not shown). This result suggests that macrophage depletion is the primary mode of LC action. The failure of LC administration to induce monocytopenia in dogs in the present study was unexpected, based on the significant monocytopenia that has been noted in rodent studies. [40,41] The most likely explanation for this is insufficient LC dosing, as much higher LC doses on a body weight-basis have been used in mouse studies compared to the doses used in dogs in the present study. For example, mice are typically treated with 5–6 ml/kg LC, whereas dogs in this study received roughly 1/10th the mouse dose (0.5 ml/kg LC).[1,25,26,27,29,36] Thus, it might be expected that higher doses of LC could induce monocyte depletion in dogs, along with possibly greater antitumor activity. However, higher doses of LC might also be associated with greater risk of toxicity, as was noted in the original LC studies in dogs.[35]

The relatively high prevalence of tumor ulceration (4 of 13 dogs) noted in the present study suggests that this could be an LC-related event. However, it should also be pointed out that of the 4 tumors that ulcerated, the tumor sizes were relatively large (8.2 cm, 7.3 cm, 4.6 cm, and 4.3 cm in diameter) and 3 of the tumors were located in regions with high skin tension (1 on stifle, 2 on forelimbs). It should also be noted that tumor ulceration has not been observed in mice with cutaneous sarcomas treated with LC (Guth, A; manuscript submitted). Therefore, based on the small sample size and the presence of potentially confounding variables, no firm link between LC treatment and tumor ulceration could be established in the present study.

The suppression of serum IL-8 concentrations following LC administration was also unexpected and not fully explicable. The rapidity of the effect suggests that it may have been mediated by rapid depletion of myeloid cells secreting IL-8, including possibly TAMs, as has been observed in mouse tumor models treated with LC (manuscript submitted). [13,47] However, due to the current inability to directly detect specific subpopulations of myeloid cells (i.e. myeloid-derived suppressor cells) in dogs, further investigation into this hypothesis proves difficult. It is also interesting to speculate that suppression of IL-8 production might also account for the decrease in angiogenesis observed in 3 of the 5 tumor samples, given that IL-8 is a potent pro-angiogenic cytokine.[47] However, at present we do not know whether the reduction in serum IL-8 concentrations noted in the first 24 hours following LC administration was transient or sustained.

The failure to observe significant antitumor activity in the present study is not completely unexpected, given that dosing protocols have not been fully optimized for administration of LC in dogs. For example, the first 2 dogs enrolled in the study were treated at 2-week intervals, while the next 11 animals were treated at weekly intervals. This change was prompted by observations in our ongoing mouse studies that suggested closer treatment intervals were more effective in inducing antitumor activity (unpublished data). In addition, as mentioned above, the LC doses administered to mice in our studies are ten-fold higher than that administered to the dogs. Thus, higher doses of LC may be required to induce significant tumor regression in dogs. Moreover, most of the STS patients examined in this study had more slowly growing grade I and II tumors, and the duration of this study was not long enough to determine whether stable disease was truly achieved in the treated dogs.

However, it is also important to note that we believe the primary utility of LC treatment is unlikely to be as a single agent cancer therapeutic. Rather, we propose that LC, or similar macrophage depleting agents, could be most effectively employed in combination with other treatment modalities, including conventional chemotherapy, tumor immunotherapy, or radiation therapy. Since the scope of effects of TAMs appears to be quite broad, depletion of these cells may have profound and unexpected effects when combined with treatments with synergistic mechanisms of activity. Indeed, we have noted significantly enhanced effects of tumor immunotherapy and vaccination when combined with macrophage depletion therapy (Dow, S; unpublished data).

In summary, in the present study we found that significant depletion of TAMs could be achieved following weekly administration of LC. In the future, we propose that the use of LC combined with other tumor treatment modalities should be explored, as macrophage depletion therapy represents a novel approach to tumor immunotherapy.

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Figure 1.

Effects of LC treatment on TAMs. Tumor tissue biopsies (pre-treatment and day 21 of treatment) were obtained from 5 dogs enrolled in the study and analyzed for CD11b expression by IHC. A) Quantitation of number of CD11b+ TAMs per 400X highpowered field from 3 independent fields. TAMs were significantly reduced on day 21 of treatment ($p = 0.01$). B) Representative IHC images of TAMs (brown staining) from dog tumor biopsies obtained on day 0 and day 21 after LC administration (40X).

Figure 2.

Effects of LC treatment on tumor MVD. Tumor tissue biopsies were analyzed for MVD before and after LC treatment, using CD146 IHC. A) Quantitation of CD146+ cells per 100X high-powered field from 3 independent fields. The density of CD146+ cells (MVD) was significantly reduced on day 21 of treatment ($p = 0.05$). B) Representative IHC images of tumor microvessel endothelial cells (brown staining) from tumor tissue obtained on day 0 and day 21 of LC treatment (100X).

Figure 3.

Liposomal clodronate induces a rapid decrease in serum IL-8 concentrations. Serum samples collected prior to LC treatment and 24 hours after treatment were evaluated for IL-8 concentrations using a Luminex-based canine multiplex cytokine assay. Serum samples collected 24 hours after LC administration contained significantly ($p = 0.02$) lower concentrations of canine IL-8 than pre-treatment serum samples.

Table 1

Patient characteristics

Median age, years (range)	10.6 (7–13)
Sex	
Spayed female	7
Castrated male	6
Tumor Grade	
I	4
II	5
III	2
ND	2
Tumor location	
appendage	5
facial	4
perianal	2
thoracic	1
multiple	1
Tumor Histology	
Fibrosarcomas	3
Peripheral Nerve Sheath Tumor	3
Undifferentiated	1
ND	6
Breeds	
Golden Retriever	2
Mixed Breed	2
Other (one each)	9

ND: not determined

Table 2

Hematological changes following LC administration

	Pre-Tx × 10³/μl (±SD)	24-hr post × 10³/μl (±SD)	p-value
Neutrophil number	6386 (2546)	9982 (3343)	0.003 *
Neutrophil percentage	71.9 (8.4)	82.8 (6.2)	0.002 *
Monocyte number	342 (175)	448 (226)	0.24
Monocyte percentage	3.9 (1.5)	3.9 (2.1)	0.978
Platelet number	380 (70)	238 (72)	0.0002 *

SD: standard deviation

*
p-value < 0.05