Targeting the Lung: Preclinical and Comparative Evaluation of Anticancer Aerosols in Dogs with Naturally Occurring Cancers

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Abstract: Pet dogs with naturally occurring cancers offer a novel opportunity for the study of both cancer biology and therapy. The following review will provide the rationale for the use of these spontaneous cancer models in translational research, particularly in the development of anticancer aerosols. A summary of work involving pet dogs with primary and metastatic cancers to the lung and the investigation of therapeutic chemotherapy and cytokine immunotherapy aerosols will be presented.

INTRODUCTION

The significant anatomic and physiologic similarities that exist between dogs and humans have been the basis for the use of dogs in biomedical research for over 70 years. Reports of the use of dogs in the field of cancer drug development extend to the first form of systemic chemotherapy, nitrogen mustard. [1] Dogs continue to be used to define the safety profiles for novel cancer agents destined for use in human phase I clinical studies. Similarly the preclinical evaluation of aerosol-based therapies has a long history in dogs. Dogs have been used to define particle distribution to the lung following inhalation of therapeutic aerosols. [2] These studies provided in vivo validation of particle distribution and deposition hypotheses that are still used to characterize inhaled aerosols based on particle size. More recently, dogs have been used to assess the effects of environmental exposure to inhaled radon gas [3]; acute and chronic studies of inhaled allergens [4]; safety and activity of bronchodilator therapy [5]; the direct carcinogenic effects of inhaled cigarette smoke [6]; and the potential health risks associated with second hand cigarette smoke.

To date the use of dogs in biomedical research has largely focused on research animals, i.e. Beagle dogs. A largely un-used model are pet dogs with naturally occurring disease. The spontaneous development of disease in the pet animal population provides significant opportunities for translational research and human drug development. Studies that include pet dogs included the evaluation of novel treatments for infectious diseases the optimization of techniques for wound healing, gene therapy to modulate hereditary immune disorders, and the study of novel cancer therapies [7-11].

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NATURALLY OCCURRING CANCERS IN PET DOGS

Inbred rodent models and laboratory derived canine populations have been the primary population of experimental animals used for the preclinical development of cancer therapeutics. Working with inbred populations in controlled, artificial laboratory environments raises some concern regarding the applicability of data as it relates to the treatment of cancers in people. Many of these concerns may be allayed through the study of naturally occurring tumors in our companion animal population, (i.e. dog and cat pet population). Companion animals with naturally occurring tumors may provide an excellent opportunity to investigate many aspects of malignancy from etiology to treatment.

Several aspects of companion animal disease make for attractive comparative models. Companion animals share a common environment with people and represent a more natural outbred population. Exposure to environmental carcinogens should, therefore, be similar to that of people. Malignancies in companion animals develop spontaneously, whereas experimental laboratory models utilize induced tumors either through exposure to known carcinogens or transplantation, often in the context on an immunocompromized animal. The relative abundance of cancer in companion animals provides a potentially large population of cases for investigation. Over half of all households in the United States include a companion animal. This represents approximately 55 million dogs and 60 million cats at risk for developing cancers in the U.S. [11] Being resistant to atherosclerosis-associated cardiovascular disease, cancer is the number one cause of death overall in dogs. In a necropsy series of 2000 dogs, 23% of all dogs, regardless of age, and 45% of dogs 10 years of age or older died of cancer. Estimates of age-adjusted overall cancer incidence rates per 100,000 individuals/years at risk range from 243 to 381 for dogs and 156 to 264 for cats. [12] These rates are comparable to those reported by the National Cancer Institute SEER program for human beings (approximately 300 per 100,000). Additionally, incidence rates for certain malignancies in companion animals (e.g., canine osteosarcoma, non-

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Hodgkin's lymphoma [NHL]) are higher than those observed in people, which may provide a large population for study. Conversely, certain neoplasms, such as hemangiosarcoma and mast cell neoplasia, are extremely rare in humans but abundant in companion animals, and allow meaningful clinical data to be generated for tumor types with "orphan" status in humans. [13-15] Table I presents a list of cancers seen in cats and dogs that may be relevant models for the study of the same human cancers. Tumors in companion animals generally progress at a more rapid rate than their human counterparts. This time-course is both long enough to allow comparison of response times, but short enough to ensure rapid collection of data. Companion animal cancers more closely resemble human cancers than rodent models in terms of size, cell kinetics, and biologic variables such as hypoxia and clonal variation. [16-18] By virtue of their body size, sample collection (i.e., serum, urine, cerebrospinal fluid, multiple tissue samples), surgical interventions, and imaging are more feasible than in rodent models.

Table I. Naturally Occurring Cancers Seen in Pet Animals that may be Relevant Models of Human Cancer Conditions

Cancer/Model Histology	Species
Osteosarcoma	Canine
Non-Hodgkin's Lymphoma	Canine
Prostate Carcinoma	Canine
Mammary Carcinoma	Canine, Feline
Melanoma	Canine
Lung Carcninoma	Canine, Feline
Head and Neck Carcinoma	Canine, Feline
Soft Tissue Sarcoma	Canine, Feline
Bladder Carcinoma	Canine
Renal Cystadenocarcinoma	Canine
C-kit driven cancers (canine mast cell tumor – Human GIST)[1]	Canine
Brain Tumors	Canine
Lymphoma (Gut associated)	Feline

[1] Similar juxtamembrane mutation in c-kit is seen in canine mast cell tumors and human gastrointestinal stromal tumors. Canine mast cell tumors may represent an important molecular model of a c-kit driven tumor.

In addition, an expanding animal rights movement is making investigations with laboratory animals more difficult. Provided that well-designed humane guidelines are adhered to, clinical trials involving companion animals may be more acceptable. Because the "standard of care" is not established for many tumors encountered in the veterinary profession, more latitude in prospective clinical trials is allowable, and it is easier and morally acceptable to attempt new and innovative treatment strategies. Importantly, such latitude should not be abused, and present-day veterinary institutions consistently use informed client consent and institutional review boards to ensure study design and ethical standards are maintained.

In general, clinical trials using veterinary patients can be completed at significantly lower expense than similar human clinical trials. Professional services, clinical pathology, and diagnostic imaging, while of the highest quality, are of lower cost in comparison to human clinical trials. Most companion animal caregivers are highly committed, and are actively seeking innovative and promising new therapies for their companion's cancer. As a whole, either through personal experience with family members and friends, or media coverage of available and leading-edge therapy, the pet-owning public now demands the highest quality of care, often only available through clinical trials. Compliance with treatment and recheck visits is exceptional, and autopsy compliance approaches 85%, significantly better than most human clinical trials.

Finally, companion animal and human tumors share a great deal in terms of etiopathogenesis and biology. Mutations in many oncogenes and tumor suppressor genes commonly seen in human cancer, such as p53. [19-28] Rb,[21] Ras, [29-32] Myc , [19, 32] and bcl-2 [33, 34] have been detected in a variety of canine and feline tumors. Likewise, overexpression of telomerase [35-38] and matrix metalloproteinases [39-42] have been detected in various canine tumors. A variety of tyrosine kinase growth factors and receptors have been detected in canine and feline tumors, and thus they may serve as excellent models for the preclinical development of small molecule inhibitors of these growth factor pathways. Additionally, analogs of most major angiogenic growth factors and their receptors exist in dogs and cats, and elevations in serum or urine VEGF and/or bFGF have been detected in several canine tumors. [43-45]

EVALUATION OF ANTICANCER AEROSOLS IN DOGS

Many of the advantages for the study of cancer in dogs are particularly relevant to the evaluation of aerosol approaches for the treatment of cancer. We have successfully used pet dog cancer models to evaluate the delivery of cytotoxic chemotherapy and cytokines by aerosol for the treatment of primary cancers of the lung and cancers metastatic to the lung. In both cases the use of the dog models was essential for the development of each anticancer aerosol therapy. A summary of preclinical studies undertaken in dogs in the development of inhalation therapies are presented below. The utility of dogs with naturally occurring cancers in the translation and optimization of this therapeutic modality to human cancer patients is emphasized.

DELIVERING CHEMOTHERAPEUTICS BY INHALATION

The concept of delivering chemotherapeutics by inhalation for the treatment of lung cancer, either primary or metastatic, has received little attention. The outcome for treatment of primary and metastatic lung cancer in both companion animals and people has not changed dramatically in spite of the availability of new chemotherapeutic agents. The reasons for treatment failure are diverse, but one possibility may be the inability to deliver adequate drug

concentrations to the tumor site using systemic administration. Pulmonary delivery of antineoplastic drugs offers the theoretical advantage of achieving high local pulmonary concentrations of drug while minimizing systemic exposure. Thus the possibility exists to optimize local action of chemotherapeutics with significantly lower overall dose and fewer systemic side effects. Efficacy of locoregional application of chemotherapy has been demonstrated in a variety of cancers including liver, bladder, and ovarian cancer. Isolated lung perfusion has been used with limited success in a small number of patients, but is unlikely to be widely used because it requires surgery. [46]

Few studies exist documenting the feasibility of delivering antineoplastic agents by inhalation. Tatsumura *et al.* evaluated inhaled administration of 5-fluoruracil (5-FU) in dogs and achieved high concentrations of drug in trachea, hilar bronchi, and regional lymph nodes within 2 hours of treatment. [47] He also reported responses in 6 of 10 patients with non-small cell lung cancer given aerosolized 5-FU.

Preclinical studies, performed in normal rodents and dogs, have demonstrated the feasibility of delivering chemotherapeutic agents by the inhalation route without serious toxicity. [48] As part of an effort to determine the safety and efficacy of pulmonary delivered antineoplastic drugs, a study using pet dogs with primary and metastatic tumors to lung was recently completed by our group. [48] In this proof of principle trial twenty-eight privately-owned dogs with spontaneously occurring primary or metastatic lung tumors received newly developed proprietary formulations of either paclitaxol (PTX) or doxorubicin (DOX) by inhalation once every 2 weeks using an aerosol device specially designed to capture all exhaled and fugitive drug. The targeted dose delivery to the pulmonary tree was 3 mg of DOX and 40 mg of PTX per inhalation therapy event and the amount of each drug delivered was controlled by the duration (in minutes) of inhalation exposure based on the dog's stabilized minute volume of respiration under light anesthesia. A total of 6 biweekly therapies were scheduled per patient unless additional therapy was warranted based on continued response.

Tumor regression was achieved in 25% of dogs with measurable lung tumors and the delivery of efficacious doses of PTX and DOX did not result in side effects normally associated with systemic administration of these drugs. Five of 25 dogs had partial responses and 1 dog had a complete response to therapy (Fig. **1**). Five of 6 responding animals had metastatic sarcoma (4 were treated with DOX and 1 with PTX) and 1 animal had metastatic mammary carcinoma (treated with inhalational PTX). Three of the six responders were dogs with pulmonary metastatic osteosarcoma (OSA) and one dog demonstrated partial regression of metastatic hilar lymphadenopathy, suggesting uptake of inhaled drug into regional lymph nodes. Similar observations have been made by Tatsumura *et al.*, who demonstrated high concentrations of 5-FU in hilar lymph nodes in dogs undergoing inhaled administration of the drug. [47] Similarly, exposure of the lung to DOX by isolated perfusion was capable of achieving significantly higher drug levels in lymph nodes and lung. [46, 49] These responses in dogs with OSA are particularly noteworthy for two reasons. First, systemic chemotherapy is virtually ineffective for

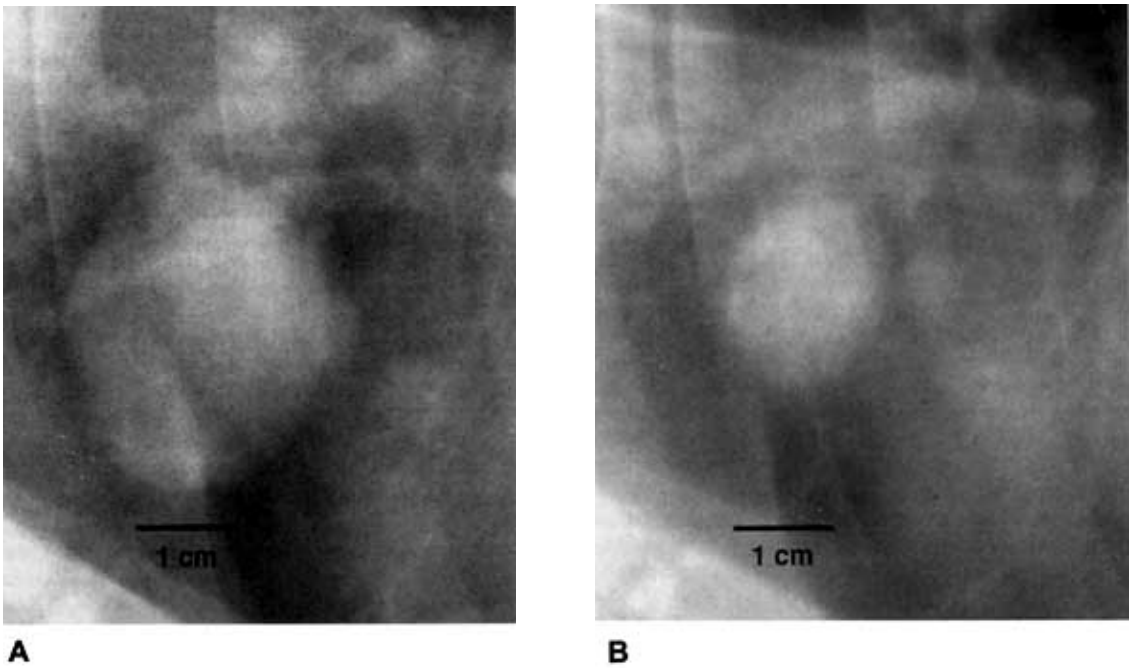


Fig. (1). Inhalation of paclitaxol in dogs every 2 weeks (x 6) resulted in greater than 50% volume regression of a mammary gland carcinoma pulmonary metastasis in a dog. (A) A lateral thoracic radiograph taken just prior to initiation of inhalation therapy. (B) Lateral thoracic radiograph 14 days following a single treatment with paclitaxel by the inhaled route. Reprinted with permission from Hershey, *et al.*, *Inhalation chemotherapy for macroscopic primary or metastatic lung tumors: proof of principle using dogs with spontaneously occurring tumors as a model.* Clin Cancer Res, 1999. **5**(9): p. 2653-2659.

canine OSA once gross pulmonary metastases develop. [50] Secondly, all 3 dogs with OSA exhibiting responses to inhaled drug had received prior systemic chemotherapy; two of whom demonstrated responses to inhaled DOX after having received systemic DOX prior to development of their pulmonary metastases.

Administered doses of drugs were sufficient to result in regression of tumors, without associated toxicity. Acute side effects of myelosuppression, nausea, and vomiting frequently associated with intravenous administration of PTX and DOX were not observed in any dog. Cardiotoxicity, a cumulative dose-limiting effect of intravenous administration of DOX, was not observed in any dog receiving DOX inhalation alone. These findings are consistent with the results of distribution of ^{14}C-DOX following intravenous or inhalation administration in normal beagle dogs[A]. Levels of ^{14}C in lung were demonstrated to be 15-20 times higher following inhalation during the first 24 hours after dosing. By contrast, uptake of ^{14}C in systemic circulation and heart was significantly lower following inhalation administration.

Acute local pulmonary effects of DOX were observed in nearly 50% of the dogs and consisted of an intermittent, nonproductive cough. These side effects were not dose limiting in any animal, usually self-limiting and only rarely required antitussive therapy. This side effect is likely related to the direct effect of DOX on pulmonary tissues, although the primary cancer and irritation from the endotracheal tube may be contributing factors. Histologic examination of dogs at necropsy revealed mild or moderate pneumonitis, multifocal interstitial fibrosis or alveolar histiocytosis that was not clinically evident. In contrast, doses up to 90 mg/m^2 of paclitaxel could be safely delivered through this route with no local toxicity. Mild systemic leukopenia was observed only at the highest paclitaxel dose levels.

The alternate-week dosing schedule used in this trial was chosen based on the cyclic dosing of cytotoxic drugs given intravenously, in which myelosuppression is generally dose-limiting. However, for inhalational chemotherapy, where loco-regional responses and toxicity may be dose-limiting, a schedule based on non-pulmonary drug effects may not be appropriate. Further evaluation of dose and schedule are warranted.

As the toxicities of inhalational chemotherapy are for the most part locoregional, it can be theorized that concomitant use of chemotherapeutics by both the inhaled route and the intravenous route would not add to overall toxicity and may enhance efficacy. To test this hypothesis 17 additional pet dogs with splenic hemangiosarcoma (HSA) were treated with concurrent inhalational and systemic chemotherapy. [45] Splenic hemangiosarcoma (HSA) in the dog is an aggressive malignancy with a high potential for widespread metastasis, including abdominal viscera and the lungs. Two weeks following splenectomy for their primary disease, dogs received systemic DOX and cyclophosphamide at their known maximally tolerated doses every 3 weeks for a total of 4 treatments. Concurrently, DOX was administered via the inhalation route to a targeted dose of 3 mg as described above on the same day as systemic chemotherapy. No increase in systemic toxicity grades (hemataologic, gastrointestinal or cardiac) were observed following combination therapy. While median survivals were superior to a similar group of historical control dogs treated with an identical systemic chemotherapy protocol without inhaled chemotherapy [51], a randomized prospective trial would be necessary to further evaluate the efficacy of combined therapy. These results do suggest, however, that inhalation chemotherapy combined with systemic chemotherapy may offer some improvement in efficacy over systemic chemotherapy alone without adding significant treatment related toxicity. These studies, undertaken in dogs with naturally occurring cancer have established the potential activity and safety of this therapeutic approach for human cancer patients.

INHALED CYTOKINE IMMUNOTHERAPY IN DOGS

Cytokines including, interleukin-2, interleukin-12, GM-CSF and others have demonstrated profound antitumor activity in human and animal cancers [52-56]. A limitation to the use of many cytokines, including interleukin-2 in patients has been the development of dose dependent toxicity [55, 57, 58]. Toxicities associated with intravenous IL-2 have included pulmonary vascular leakage (most common dose limiting toxicity), fever, weight gain and anasarca, malaise, rigors, azotemia, anemia, and thrombocytopenia [59, 60]. Adverse effects of IL-2 are dependent on the dose, route of delivery, and formulation of IL-2 [57, 61]. Novel approaches to reduce the toxicity of IL-2 may increase the therapeutic index of this central cytokine of the cell-mediated immune system and allow greater application to the treatment of cancer. Given that a primary problem in the management of cancer patients is the development of pulmonary metastases the evaluation of the aerosol route of delivery of cytokine based immunotherapy held the promise of increased local delivery of interleukin-2 to limit toxicity and potentially improve local biodistrtibution of IL-2 to the lung following inhalation. Huland et al have demonstrated the clinical activity inhaled IL-2 for patients with metastatic renal cancer [62, 63]. The optimization of this therapeutic approach from the standpoint of dose, regimen (qd, bid, tid) and aerosol formulation (free IL-2 or liposomal IL-2) was necessary. To answer these questions we initiated a series of preclinical studies that included the study of pet dogs with naturally occurring cancers.

In unpublished data we used a mildly immunogenic murine pulmonary metastases model, the MCA 106 pulmonary sarcoma model, to evaluate the potential anti-tumor activity of inhaled IL-2 and IL-2 liposomes. Groups of 10 mice were then injected with 5 x 10^5 tumor cells to the tail vein. On the day of tail vein injection, mice were treated in groups of 10 in a plastic inhalation chamber with aerosols of 1.0 x 10^6 IU free IL-2 (n=10), 1.0 x 10^6 IU IL-2 liposomes (n=10), and saline alone (n=10) twice daily. Survival of mice treated with aerosols of IL-2 (free or

[A] Zutshi, A., Townsend, R.W., Moutvic, R., Trigg, N., Watts, J.K., and Imondi, A.R. *Disposition of inhaled (IH) 14C-Doxorubicin (14C-DOX) in dogs*. Proc Amer Assoc Canc Res, 1999. **40**: p. 416-421

liposomal) was significantly prolonged compared to the saline treated mice.

The murine model confirmed the potential antitumor activity of inhaled IL-2; however, it was not possible to reliably predict delivered dose and inhaled dose, investigate intermediate longitudinal endpoints of immunologic activation, and define relevant toxicities associated with inhalation. The dog was thus considered to be an appropriate animal (model) for further investigation of the safety and biological activity of inhaled IL-2 liposomes. The potential to investigate antitumor activities associated with inhaled IL-2 cytokine immunotherapy in dogs with intact immune responses and relevant tumor-host microenvironment was a particularly attractive component of the use of the dog.

Initial studies in normal research dogs defined the feasibility, biological activity and pulmonary biodistribution of aerosols of IL-2 delivered to dogs [64]. A major difference between the evaluation of inhalation chemotherapy (as discussed above) and inhalation cytokine therapy is the regimen of therapy. Based on work with inhaled IL-2 in human patients, multiple daily treatments with cytokine were considered to be necessary over long periods of time (days to months). This repeated delivery regimen required the training of dogs to accept inhalation while awake rather than though closed capture anesthesia. Using a polyurethane re-breathing bag that covered the dog's muzzle, normal research dogs were trained to receive repeated inhalation treatments (Fig. **2**). Within 7 days all dogs were easily trained to receive inhalation treatments that lasted 15-30 minutes. We later found this same delivery mechanism to be effective in the training of pet owners to deliver cytokines to their cancer bearing pet dogs. Most of the delivered aerosol entered the lung following oral inhalation (i.e. during mouth breathing); however, the re-breathing bag was associated with aerosol dose loss that resulted from particle trapping in the rhinarium of the dog. These losses are thought to be slightly greater than upper airway losses associated with nebulization in human subjects. Alternative strategies have been employed for the delivery of aerosols to non-anesthetized spontaneously respiring (awake) dogs. Most techniques have included tracheostomy implants that could be used to deliver aerosols by nebulizer or metered dose inhaler [65-67]. Such techniques are best suited for experimental dogs and may not be as useful for aerosol delivery to pet dogs whose owners may not be comfortable with such surgical procedures.

A significant advantage of the use of dogs to assess the biological activity of inhaled cytokine immunotherapy was the fact that longitudinal collection and analysis of bronchoalveolar leukocyte effectors before and after exposure to aerosols was possible. The collection of such biological samples would be difficult in other animal models of inhalation and not easy to justify in human clinical trials. These biological endpoints allowed the optimization of inhalation regimen, IL-2 dose, and selection of the most active IL-2 formulation for delivery. Biological studies in

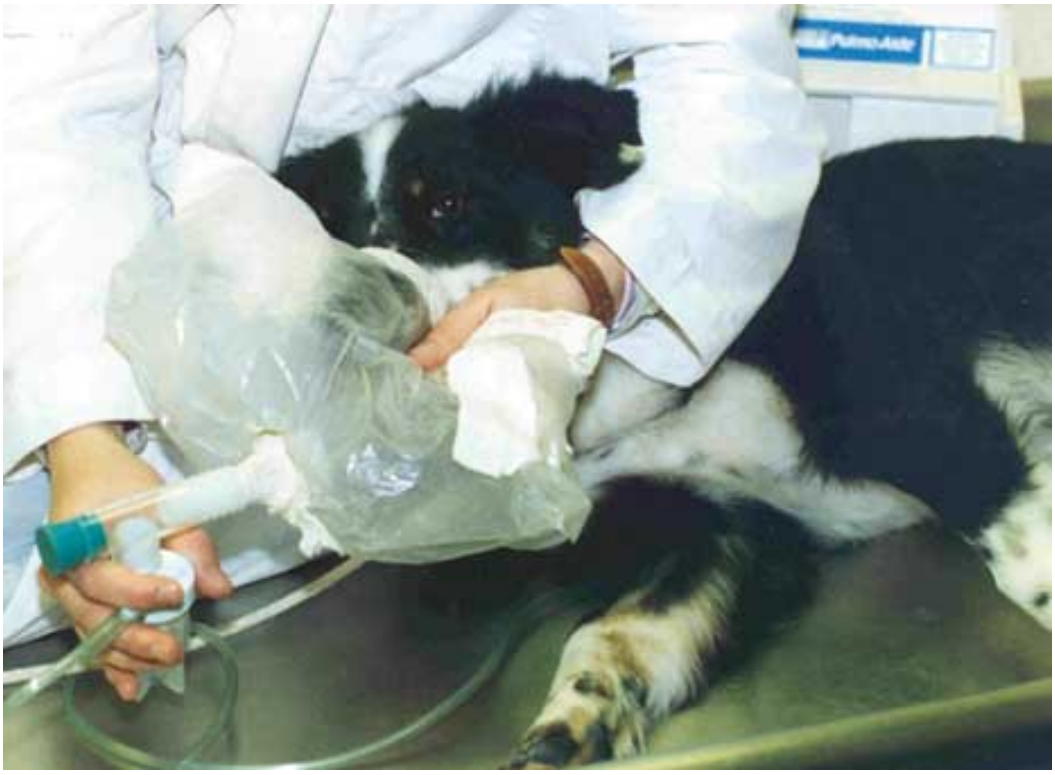


Fig. (2). Pet dogs were easily trained to receive nebulization therapy. Nebulization was accomplished using a standard compressor and a Puritan Bennet Twin Jet nebulizer connected to a polyurethane rebreathing bag. Nebulized interleukin-2 formulations filled the rebreathing bag and allowed inhalation therapy of awake dogs. Treatments were performed twice daily for 20 minutes. Treatment was administered under active ventilation to limit secondary inhalation by the human handler.

Table II. Summary of Clinical Outcomes for Dogs with Primary Lung Cancers and Pulmonary Metastases Treated with Inhaled Interleukin-2 Liposomes

Tumor Histology	Clinical Stage	Response[1]
Osteosarcoma metastases	(TIIb-resectedM1-lymph nodeM1-lung)[2]	CR > 700 days
Osteosarcoma metastases	(TIIb-resectedM1-lung)	PD
Mammary carcinoma metastases	(T2-resectedN0M1-lung)	PD
Digital melanoma metastases	(TresectedN0M1-lung)	PD
Fibrosarcoma metastases	(T3-resectedN0M1-lung)	PD
Osteosarcoma metastasis	(TIIbM1-lung)	CR – 420 days
Primary lung	($T_2N_0M_0$)	SD > 250 days
Primary lung	($T_1N_0M_0$)	PD
Osteosarcoma metastasis	(TIIbM1-lung)	PD
Oral Melanoma	(T3-resectedN2b-resectedM1-lung)[3]	SD >180 days
Osteosarcoma metastasis	(TIIbM1-lung)	PD

1 Tumor responses: CR-complete response; PR-partial response; SD-stable disease; PD-progressive disease; all dogs treated with inhaled interleukin-2 liposomes (1.0×10^6 IU; BID x 30 days).

2 World Health Organization stage assigned to dogs at the time of entry to phase I/II trial of inhaled IL-2 liposomes.

3 Primary oral tumor and regional lymph nodes were minimally resected with tumor present at surgical margins.

dogs demonstrated that optimal pulmonary immune activation was associated with an IL-2 liposome aerosol formulation (compared to free IL-2) delivered at least twice daily (compared to once daily; no difference when compared to three times daily inhalation), at a dose of 1.0×10^6 IU. Within the same experimental model the inhalation of technetium labeled aerosols allowed the evaluation of aerosol retention, pulmonary biodistribution, and systemic uptake of aerosols of IL-2 as a function of dose and IL-2 formulation [68]. Results suggested prolonged retention of aerosols within the lung following the delivery of both free and liposomal formulations. Clearance of IL-2 liposome formulations from the lung was slower and appeared to accumulate in hilar lymphoid structures, whereas free IL-2 clearance into the systemic circulation was evident.

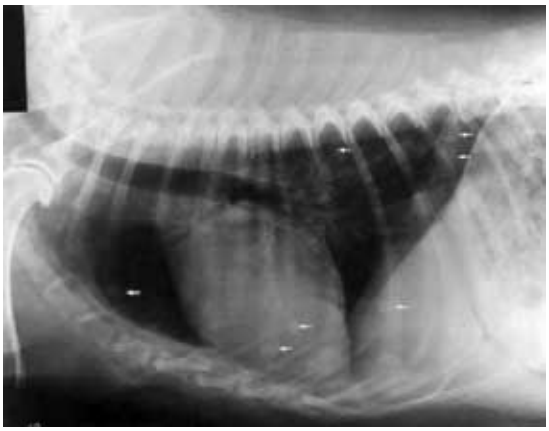
Collectively these studies in normal dogs provided significant information on the biological activity and biodistribution of inhaled IL-2 formulations that would have been difficult to perform in other animal models or in human subjects. The broader benefit of dogs in the study of aerosol therapy for cancers came from the application of information generated in experimental dogs to studies of pet dogs with naturally occurring cancers of the lung (primary lung cancer or pulmonary metastases) [69]. Pilot studies in tumor bearing dogs allowed the evaluation of the feasibility, biological activity, antitumor activity and safety of inhaled IL-2 liposome inhalation therapy (1.0×10^6 IU; BID) in cancer bearing dogs. These endpoints were then correlated with endpoints of pulmonary immune activation.

Results of the first nine dogs treated with inhalation therapy have been previously reported [69]. Table II includes an updated summary of the treatment of these cases and two additional dogs treated since the initial publication. Eligibility for therapy included measurable pulmonary metastasis or lung cancer, favorable performance status, and no evidence of significant cardiac, renal or hepatic insufficiency. Response to therapy was monitored with serial chest radiographs and collection of pulmonary effector populations, by broncho-alveolar lavage (BAL) and from heparinized whole blood. Effector populations were assessed for cell type, immunophenotype, and tumor cytolytic activity. BAL cell numbers increased greater than 4 fold ($p=0.01$) and included significantly greater proportions and total numbers of eosinophils ($p=0.006$) and lymphocytes ($p=0.008$). Mean BAL effector lytic activity was significantly greater after 15 days of IL-2 liposome inhalation compared to pretreatment activity ($p=0.01$); however mean BAL lytic activity decreased after 30 days and was no longer significantly greater than pretreatment BAL lytic activity. Toxicity was minimal and restricted to mild coughing immediately following inhalation. No allergic reactions or changes in pulmonary function were associated with inhaled IL-2 liposome therapy. Two dogs had primary lung cancers and the remaining 9 had pulmonary metastases. (Fig. **3**).

The interleukin-2 used in these studies was the human recombinant protein. Human IL-2 has been shown to interact with the canine IL-2 receptor and to activate canine lymphocytes. In tumor bearing dogs we detected the presence of neutralizing canine antibodies against human IL-2 as early as 30 days after therapy. For future cytokine inhalation studies the use of canine cytokines, which are increasingly available, will be necessary.

This preclinical work in dogs including dogs with naturally occurring pulmonary metastases and primary lung cancers has demonstrated the safety, and clinical and biological activity of inhaled cytokine immunotherapy. This work was rapidly translated to the management of human patients with pulmonary metastases in a feasibility trial of inhaled IL-2 liposomes [70] and has provided proof of

(A)



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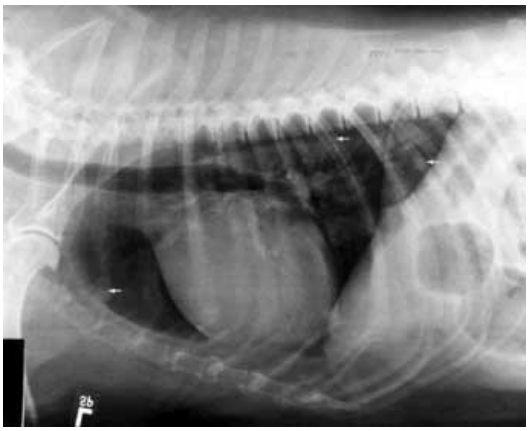

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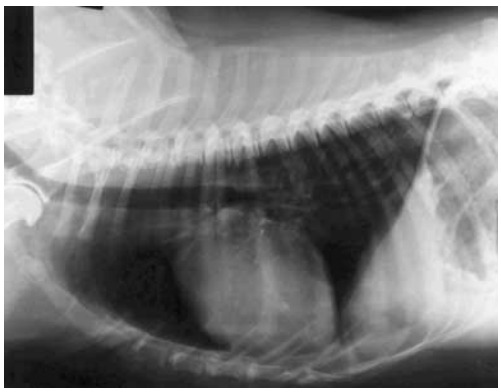

Fig. (3). Complete regression of pulmonary metastases from a skeletal osteosarcoma in a dog treated for 30 days with inhaled IL-2 liposomes. (A) A lateral thoracic radiograph taken at day 0 demonstrates 6 pulmonary metastases. (B) Lateral thoracic radiograph after 15 days of inhaled IL-2 liposome immunotherapy demonstrates a reduction in the number of metastases (from 6 to 3 metastases. (C) Lateral thoracic radiograph after 30 days of inhaled IL-2 liposome immunotherapy demonstrates a complete regression of pulmonary metastases. This complete regression of pulmonary metastases has been stable for over 420 days. Metastatic lesions are labeled with arrows. Reprinted with permission from Khanna, *et al.*, Inhaled interleukin-2 liposome immunotherapy in dogs with spontaneous primary lung cancers and cancer metastatic to the lung. *Cancer*, **1997**, *79*(7), 1409-1421.

principle support for inhaled free GM-CSF for pediatric patients with pulmonary metastases [71]. As clinical studies progress in the field of both cytokine and chemotherapy inhalation new questions will arise. Dogs with naturally occurring cancer should be considered to assist with these questions and the further development of this therapeutic approach.

REFERENCES

[1] Kidwai, M.; Venkataramanan R.; Mohan R.; Sapra P. Cancer Chemotherapy and Heterocyclic Compounds. *Curr. Med. Chem.* **2002**, *9*, 1209-1228.

[2] Knight, V.; Waldrep, J. C. New Approaches in Aerosol Drug Delivery for the Treatment of Asthma, In *Allergy and Allergic Disease*, B. Kay, Editor. **1995**, Blackwell Scientific: London, Chapter 41.

[3] Fisher, H. L.; Jr. A Model for Estimating the Inhalation Exposure To Radon-222 and Daughter Products from the Accumulated Lead-210 Body Burden. *Health Phys.* **1969**, *16*, 597-616.

[4] Redman, T. K.; Rudolph, K.; Barr, E. B.; Bowen, L. E.; Muggenburg, B. A.; Bice, D. E. Pulmonary Immunity to Ragweed in A Beagle Dog Model of Allergic Asthma. *Exp. Lung Res.* **2001**, *27*, 433-451.

[5] Auclair, B.; Wainer, I. W.; Fried, K.; Koch, P.; Jerussi, T. P.; Ducharme, M. P. A Population Analysis of Nebulized (R)-Albuterol in Dogs Using a Novel Mixed Gut-Lung Absorption PK-PD Model. *Pharm. Res.* **2000**, *17*, 1228-1235.

[6] Auerbach, O.; Hammond, E. C.; Kirman, D.; Garfinkel, L. Effects of Cigarette Smoking on Dogs. II. Pulmonary Neoplasms. *Arch. Environ. Health*. **1970**, *21*, 754-768.

[7] Legendre, A. M.; Rohrbach, B. W.; Toal, R. L.; Rinaldi, M. G.; Grace, L. L.; Jones, J. B. Treatment of Blastomycosis with Itraconazole in 112 Dogs. *J. Ve.t Intern. Med*. **1996**, *10*, 365-731.

[8] Carmagnola, D.; Berglundh, T.; Lindhe, J. The Effect of a Fibrin Glue on the Integration of Bio-Oss with Bone Tissue. An Experimental Study in Labrador Dogs. *J. Clin. Periodontol*. **2002**, *29*, 377-383.

[9] Foureman, P.; Whiteley, M.; Giger, U. Canine Leukocyte Adhesion Deficiency: Presence of the Cys36Ser Beta-2 Integrin Mutation in an Affected US Irish Setter Cross-Breed Dog and in US Irish Red and White Setters. *J. Vet. Intern. Med*. **2002**, *16*, 518-523.

[10] Khanna, C.; Prehn. J.; Hayden, D.; Cassaday, R. D.; Caylor, J.; Jacob, S.; Bose, S. M.; Hong, S. H.; Hewitt, S. M.; Helman, L. J. A Randomized Controlled Trial of Octreotide Pamoate Long-Acting Release and Carboplatin Versus Carboplatin Alone in Dogs with Naturally Occurring Osteosarcoma: Evaluation of Insulin-Like Growth Factor Suppression and Chemotherapy. *Clin. Cancer Res*. **2002**, *8*, 2406-2412.

[11] Vail, D. M.; Macewen, E. G. Spontaneously Occurring Tumors of Companion Animals as Models for Human Cancer. *Cancer Invest*. **2000**, *18*, 781-792.

[12] Bronson, R. T. Variation in Age at Death of Dogs of Different Sexes and Breeds. *Am. J. Vet. Res.* **1982**, *43*, 2057-2059.

13] Dorn, C. R.; Taylor, D. O.; Schneider, R.; Hibbard, H. H.; Klauber, M. R. Survey of Animal Neoplasms in Alameda and Contra Costa Counties, California. II. Cancer Morbidity in Dogs and Cats from Alameda County. *J. Natl. Cancer. Inst*. **1968**, *40*, 307-318.

[14] Dorn, C. R. Epidemiology of Canine and Feline Tumors. *Comp. Cont. Ed. Pract. Vet*. **1976**, *12*, 307-312.

[15] Priester, W. A.; F. W. Mckay. The Occurrence of Tumors in Domestic Animals. *Natl. Cancer Inst. Monogr.* **1980,** *54*, 1-210.

[16] Larue, S. M.; Fox, M. H. Withrow, S. J.; Powers, B. E.; Straw, R. C.; Cote, I. M.; Gillette, E. L. Impact of Heterogeneity in the Predictive Value of Kinetic Parameters in Canine Osteosarcoma. *Cancer Res.* **1994**, *54*, 3916-3921.

[17] Vail, D. M.; Kisseberth, W. C.; Obradovich, J. E.; Moore, F. M.; London, C. A.; Macewen, E. G.; Ritter, M. A. Assessment of Potential Doubling Time (TPOT), Argyrophilic Nucleolar Organizer Regions (AGNOR), and Proliferating Cell Nuclear Antigen (PCNA) as Predictors of therapy Response in Canine Non-Hodgkin's Lymphoma. *Exp. Hematol*. **1996**, *24*, 807-815.

[18] Zeman, E. M.; Calkins, D. P.; Cline, J. M.; Thrall, D. E.; Raleigh, J. A. the Relationship Between Proliferative and Oxygenation Status in Spontaneous Canine Tumors. *Int. J. Radiat. Oncol. Biol. Phys*. **1993**, *27*, 891-898.

[19] Korkolopoulou, P.; Oates, J.; Kittas, C.; Crocker, J. p53, C-Myc p62 and Proliferating Cell Nuclear Antigen (PCNA) Expression in Non-Hodgkin's Lymphomas. *J. Clin. Pathol*. **1994**, *47*, 9-14.

[20] Levine, R. A.; Fleischli, M. A. Inactivation of p53 and Retinoblastoma Family Pathways in Canine Osteosarcoma Cell Lines. *Vet. Pathol.* **2000**, *37*, 54-61.

[21] Mendoza, S.; Konishi, T.; Dernell, W. S.; Withrow S. J.; Miller, C. W. Status of the P53, Rb and MDM2 Genes in Canine Osteosarcoma. *Anticancer Res.* **1998**, *18*, 4449-4453.

[22] Nambiar, P. R.; Jackson, M. L.; Ellis, J. A.; Chelack, B. J.; Kidney, B. A.; Haines, D. M. Immunohistochemical Detection of Tumor Suppressor Gene P53 Protein in Feline injection Site-Associated Sarcomas. *Vet. Pathol,* **2001**, *38*, 236-238.

[23] Nasir, L.; Rutteman, G. R.; Reid, S. W.; Schulze, C.; Argyle, D. J. Analysis of p53 Mutational Events and MDM2 Amplification in Canine Soft- Tissue Sarcomas. *Cancer Lett*. **2001**, *174*, 83-89.

[24] Roels, S.; Tilmant, K.; Ducatelle, R. p53 Expression and Apoptosis in Melanomas of Dogs and Cats. *Res. Vet. Sci.* **2001**, *70*, 19-25.

[25] Rungsipipat, A.; Tateyama, S.; Yamaguchi, R.; Uchida, K.; Miyoshi, N.; Hayashi, T. Immunohistochemical Analysis of C-Yes and C-Erbb-2 Oncogene Products and p53 Tumor Suppressor Protein in Canine Mammary Tumors. *J. Vet. Med. Sci.* **1999**, *61*, 27-32.

[26] Veldhoen, N.; Stewart, J.; Brown, R.; Milner, J. Mutations of the p53 Gene in Canine Lymphoma and Evidence for Germ Line p53 Mutations in the Dog. *Oncogene* **1998**, *16*, 249-255.

[27] Veldhoen, N.; Watterson, J.; Brash, M.; Milner, J. Identification of Tumour-Associated and Germ Line p53 Mutations in Canine Mammary Cancer. *Br. J. Cancer* **1999**, *81*, 409-415.

[28] Wakui, S.; Muto, T.; Yokoo, K.; Yokoo, R.; Takahashi, H.; Masaoka, T.; Hano, H.; Furusato, M. Prognostic Status of p53 Gene Mutation in Canine Mammary Carcinoma. *Anticancer Res.* **2001,** *21*, 611-616.

[29] Ahern, T. E.; Bird, R. C.; Bird, A. E.; Wolfe, L. G. Overexpression of C-Erbb-2 and C-Myc but not C-Ras, in Canine Melanoma Cell Lines, is Associated with Metastatic Potential in Nude Mice. *Anticancer Res*. **1993**, *13*, 1365-1371.

[30] Griffey, S. M.; Kraegel, S. A.; Madewell, B. R. Rapid Detection of K-Ras Gene Mutations in Canine Lung Cancer Using Single-Strand Conformational Polymorphism Analysis. *Carcinogenesis* **1998**, 19, 959-963.

[31] Merryman, J. I.; Buckles, E. L, Bowers. G.; Neilsen, N. R. Overexpression of C-Ras in Hyperplasia and Adenomas of the Feline Thyroid Gland, an Immunohistochemical Analysis of 34 Cases. *Vet. Pathol*. **1999,** *36*, 117-124.

[32] Watzinger, F.; Mayr, B.; Gamerith, R.; Vetter, C.; Lion, T. Comparative Analysis of Ras Proto-Oncogene Mutations in Selected Mammalian Tumors. *Mol. Carcinog*. **2001,** *30*, 190-198.

[33] Madewell, B. R.; Gandour-Edwards, R.; Edwards, B. F.; Walls, J. E.; Griffey, S. M. Topographic Distribution of Bcl-2 Protein in Feline Tissues in Health and Neoplasia. *Vet. Pathol.* **1999**, *36*, 565-573.

[34] Madewell, B. R.; Gandour-Edwards, R.; Edwards, B. F.; Matthews, K. R.; Griffey, S. M. Bax/Bcl-2, Cellular Modulator of Apoptosis in Feline Skin and Basal Cell Tumours. *J Comp. Pathol*. **2001**,*124*, 115-121.

[35] Funakoshi, Y.; Nakayama, H.; Uetsuka, K.; Nishimura, R.; Sasaki, N.; Doi. K. Cellular Proliferative and Telomerase Activity in Canine Mammary Gland Tumors. *Vet. Pathol.* **2000**, *37*, 177-183.

[36] Nasir, L.; Devlin P, Mckevitt T, Rutteman G, Argyle DJ. Telomere Lengths and Telomerase Activity in Dog Tissues, A Potential Model System to Study Human Telomere and Telomerase Biology. *Neoplasia* **2001**. 3(4), 351-359.

[37] Yazawa, M.; Okuda, M.; Setoguchi, A.; Nishimura, R.; Sasaki, N.; Hasegawa, A.; Watari, T.; Tsujimoto, H. Measurement of Telomerase Activity in Dog Tumors. *J. Vet. Med. Sci.* **1999**, *61*, 1125-1129.

[38] Yazawa, M.; Okuda, M.; Setoguchi, A.; Iwabuchi, S.; Nishimura, R.; Sasaki, N.; Masuda, K.; Ohno, K.; Tsujimoto, H. Telomere Length and Telomerase Activity in Canine Mammary Gland Tumors. *Am. J. Vet. Res*. **2001**, *62*, 1539-1543.

[39] Cakir, Y.; Hahn, K. A. Direct Action by Doxycycline Against Canine Osteosarcoma Cell Proliferation and Collagenase (MMP-1) Activity *in Vitro*. *In Vivo* **1999,** *13*, 327-331.

[40] Lana, S. E.; Ogilvie, G. K.; Hansen, R. A.; Powers, B. E.; Dernell, W. S.; Withrow, S. J. Identification of Matrix Metalloproteinases in Canine Neoplastic Tissue. *Am. J. Vet. Res*. **2000**, *61*, 111-114.

[41] Leibman, N. F.; Lana, S. E.; Hansen, R. A.; Powers, B. E.; Fettman, M. J.; Withrow, S. J.; Ogilvie, G. K. Identification of Matrix Metalloproteinases in Canine Cutaneous Mast Cell Tumors. *J. Vet. intern. Med*. **2000**, *14*, 583-586.

[42] Yokota, H.; Kumata, T.; Taketaba, S.; Kobayashi, T.; Moue, H.; Taniyama, H.; Hirayama, K.; Kagawa, Y.; Itoh, N.; Fujita, O.; Nakade, T.; Yuasa, A. High Expression of 92 Kda Type IV Collagenase (Matrix Metalloproteinase- 9) in Canine Mammary Adenocarcinoma. *Biochim. Biophys. Acta* **2001**,*1568*, 7-12.

[43] Allen, D. K.; Waters, D. J.; Knapp, D. W.; Kuczek, T. High Urine Concentrations of Basic Fibroblast Growth Factor in Dogs with Bladder Cancer. *J. Vet. Intern. Med.* **1996**, *10*, 231-234.

[44] Clifford, C. A.; Hughes, D.; Beal, M. W.; Mackin, A. J.; Henry, C. J.; Shofer, F. S.; Sorenmo KU. Plasma Vascular Endothelial Growth Factor Concentrations in Healthy Dogs and Dogs with Hemangiosarcoma. *J. Vet. Intern. Med.* **2001**. 15(2), 131-135.

[45] Mohammed, S. I.; Craig, B. A.; Mutsaers, A. J.; Glickman, N. W.; Snyder, P. W.; Degortari, A. E.; Schlittler, D. L.; Coffman, K. T.; Bonney, P. L.; Knapp, D. W. Effects of the Cyclooxygenase inhibitor, Piroxicam, On Tumor Response, Apoptosis, and Angiogenesis in a Canine Model of Human Invasive Urinary Bladder Cancer. *Cancer Res.* **2002**, *62*, 356-358.

[46] Johnston, M. R.; Minchen, R. F.; Dawson, C. A. Lung Perfusion with Chemotherapy in Patients with Unresectable Metastatic Sarcoma to the Lung or Diffuse Bronchioloalveolar Carcinoma. *J. Thorac. Cardiovasc. Surg*. **1995**, *110*, 368-373.

[47] Tatsumura, T.; Koyama, S.; Tsujimoto, M.; Kitagawa, M.; Kagamimori, S. Further Study of Nebulisation Chemotherapy, A New Chemotherapeutic Method in the Treatment of Lung Carcinomas, Fundamental and Clinical. *Br. J. Cancer* **1993**, *68*, 1146-1149.

[48] Hershey, A. E.; Kurzman, I. D.; Forrest, L. J.; Bohling, C. A.; Stonerook, M.; Placke, M. E.; Imondi, A. R.; Vail, D. M. Inhalation Chemotherapy for Macroscopic Primary or Metastatic Lung Tumors, Proof of Principle Using Dogs with Spontaneously Occurring Tumors as a Model. *Clin. Cancer Res*. **1999**, *5*, 2653-2659.

[49] Johnston, M. R. Lung Perfusion and Other Methods of Targeting Therapy to Lung Tumors. *Chest Surg. Clin. N. Am*. **1995**, *5*, 139-156.

[50] Ogilvie, G. K.; Straw, R. C.; Jameson, V. J.; Walters, L. M.; Lafferty, M. H.; Powers, B. E.; Withrow, S. J. Evaluation of Single-Agent Chemotherapy for Treatment of Clinically Evident

Osteosarcoma Metastases in Dogs, 45 Cases (1987-1991). *J. Am. Vet. Med. Assoc*. **1993**, *202*, 304-306.

[51] Vail, D. M.; Macewen, E. G.; Kurzman, I. D.; Dubielzig, R. R.; Helfand, S. C.; Kisseberth, W. C.; London, C. A.; Obradovich, J. E.; Madewell, B. R.; Rodriguez, C. O. Jr. Liposome-Encapsulated Muramyl Tripeptide Phosphatidylethanolamine Adjuvant Immunotherapy for Splenic Hemangiosarcoma in the Dog, a Randomized Multi-institutional Clinical Trial. *Clin. Cancer Res*. **1995,** *1*, 1165-1170.

[52] Glaspy, J.A. therapeutic Options in the Management of Renal Cell Carcinoma. *Semin Oncol,* **2002**, *29*, 41-46.

[53] Atkins, M. B. Interleukin-2, Clinical Applications. *Semin. Oncol*. **2002**, *29*, 12-17.

[54] Rubin, J. T. interleukin-2, Its Biological and Clinical Applications in Patients with Cancer. *Cancer Invest*. **1993**, *11*, 460-472.

[55] Menzel, T.; Schomburg, A.; Korfer, A.; Hadam, M.; Meffert, M.; Dallmann, I.; Casper, S.; Kirchner, H.; Poliwoda, H.; Atzpodien, J. Clinical and Preclinical Evaluation of Recombinant PEG-IL-2 in Human. *Cancer Biotherapy* **1993**, *8*, 199-212.

[56] Wigginton, J. M.; Komschlies, K. L.; Back, T. C.; Franco, J. L.; Brunda, M. J.; Wiltrout, R. H. Administration of interleukin-12 with Pulse interleukin-2 and the Rapid and Complete Eradication of Murine Renal Carcinoma. *J. Natl. Cancer Inst.* **1996**, *88*, 38-43.

[57] Anderson, P. M.; Sorenson, M. A. Effects of Route and formulation On Clinical Pharmacokinetics of Interleukin-2. *Drug Disposition* **1994,** *27*, 19-31.

[58] Guida, M.; Latorre, A.; Mastria, A.; De Lena, M. Long-Term Subcutaneous Recombinant interleukin-2 As Maintenance Therapy, Biological Effects and Clinical Implications. *Cancer Biotherapy* **1995**, *10*, 195-203.

[59] Siegal, J.; Puri, R. Interleukin-2 toxicity. *J. Clin. Oncol.* **1991**, *9*, 694-704.

[60] Smith, K. A. Interleukin-2, Inception, Impact, and Implications. *Science* **1988**, *240*, 1169-1176.

[61] Maas, R. A.; Dullens, H. F.; Den Otter, W. Interleukin-2 in Cancer Treatment, Disappointing or (Still) Promising? A Review. *Cancer Immunol. Immunother.* **1993.** 36(3), 141-148.

[62] Huland, E.; H. Huland. Local Continuous High Dose interleukin-2, A New therapeutic Model for the Treatment of Advanced Bladder Cancer. *Cancer Res.* **1989,** *49*, 5469**.**

[63] Huland, E.; H. Huland, and H. Heinzer. interleukin-2 By inhalation, Local therapy for Metastatic Renal Carcinoma. *J. Urology* **1992**, *147*, 344-348.

[64] Khanna, C.; Hasz, D. E.; Klausner, J. S.; Anderson, P. M. Aerosols of interleukin-2 Liposomes Are Non-toxic and Biologically Active, Canine Studies. *Clin. Cancer Res*. **1996**, *4*, 719-736.

[65] Coggins, C. R. A Review of Chronic Inhalation Studies with Mainstream Cigarette Smoke, in Hamsters, Dogs, and Nonhuman Primates. *Toxicol. Pathol.* **2001**. *29*, 550-755.

[66] Lee, L. Y.; Widdicombe, J. G. Modulation of Airway Sensitivity to Inhaled Irritants, Role of Inflammatory Mediators. *Environ. Health Perspect*. **2001**, *109*, 585-589.

[67] Boykin, A. B.; Anderson, D.; Mcnamee, G. M.; Mcnamee, J. E. Effect of Entrainment Time on Pulmonary Deposition of Cigarette Smoke in Dogs. *J. Appl. Physiol*. **1993**, *74*, 1862-1868.

[68] Khanna, C.; Waldrep, J. C.; Anderson, P. M.; Weischelbaum, R. W.; Hasz, D. E.; Katsanis, E.; Klausner, J. S. Nebulized interleukin-2 Liposomes: Aerosol Characteristics and Biodistribution. *J. Pharm. Pharmaceut*. **1997**, *49*, 960-971.

[69] Khanna, C.; Anderson PM, Hasz, D. E.; Katsanis, E.; Neville, M.; Klausner, J. S. Inhaled interleukin-2 Liposome Immunotherapy in Dogs with Spontaneous Primary Lung Cancers and Cancers Metastatic to the Lung. *Cancer* **1997**. *79*, 1409-1421.

[70] Skubitz, K. M.; Anderson, P. M. Inhalational interleukin-2 Liposomes for Pulmonary Metastases, A Phase I Clinical Trial. *Anticancer Drugs* **2000,** *11*, 555-563.

[71] Anderson, P. M.; Markovic, S. N.; Sloan, J. A.; Clawson, M. L.; Wylam, M.; Arndt, C. A.; Smithson, W. A.; Burch, P.; Gornet, M.; Rahman, E. Aerosol Granulocyte Macrophage-Colony Stimulating Factor, A Low toxicity, Lung-Specific Biological therapy in Patients with Lung Metastases. *Clin. Cancer Res*. **1999,** *5*, 2316-2323.