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PEQUEÑOS ANIMALES

Nº 4

- Immunotherapeutic Strategies for Canine Lymphoma: Changing the Odds Against Non-Hodgkin Lymphoma

  Estrategias inmunoterapéuticas para el linfoma canino: cambiar las probabilidades contra el linfoma no Hodgkin

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Immunotherapeutic Strategies for Canine Lymphoma: Changing the Odds Against Non-Hodgkin Lymphoma

Estrategias inmunoterapéuticas para el linfoma canino: cambiar las probabilidades contra el linfoma no Hodgkin

Palabras clave:
cáncer, oncología comparativa, linfoma no Hodgkin, linfoma canino, inmunoterapia contra el cáncer

Keywords:
cancer, comparative oncology, non-Hodgkin lymphoma, canine lymphoma, cancer immunotherapy

https://www.frontiersin.org/articles/10.3389/fvets.2021.621758/full

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La nueva era de la inmuno-oncología ha traído complejidades y desafíos que enfatizan la necesidad de identificar nuevas estrategias y modelos para desarrollar terapias exitosas y rentables. La inclusión de un modelo canino en el desarrollo farmacológico de inmunoterapias contra el cáncer está siendo ampliamente reconocida como una solución válida para superar varios obstáculos asociados con los modelos preclínicos convencionales. Impulsado por el éxito de las inmunoterapias en el tratamiento del linfoma no Hodgkin humano (LNH) y por las notables similitudes del LNH canino con su contraparte humana, el LNH canino ha sido uno de los principales focos de la investigación comparativa. En la presente revisión, resumimos una visión general de los desafíos y perspectivas de las inmunoterapias actuales contra el cáncer y el papel que la medicina comparativa podría desempeñar en la solución de las limitaciones que plantea este campo en rápida expansión.

The new era of immune-oncology has brought complexities and challenges that emphasize the need to identify new strategies and models to develop successful and cost-effective therapies. The inclusion of a canine model in the drug development of cancer immunotherapies is being widely recognized as a valid solution to overcome several hurdles associated with conventional preclinical models. Driven by the success of immunotherapies in the treatment of human non-Hodgkin lymphoma (NHL) and by the remarkable similarities of canine NHL to its human counterpart, canine NHL has been one of the main focus of comparative research. Under the present review, we summarize a general overview of the challenges and prospects of today's cancer immunotherapies and the role that comparative medicine might play in solving the limitations brought by this rapidly expanding field. The state of art of both human and canine NHL and the rationale behind the use of the canine model to bridge the translational gap between murine preclinical studies and human clinical trials are addressed. Finally, a review of currently available immunotherapies for canine NHL is described, highlighting the potential of these therapeutic options.

Introduction

In 2018 alone, cancer was responsible for an estimated 9.6 million deaths worldwide in countries of all income levels, ranking second place in the leading causes of death, behind cardiovascular diseases (1). Owing to population growth, aging, and adoption of lifestyle behaviors associated with cancer risk, this number is expected to rise by about 70% over the next 20 years (2, 3). Still, even though these impressive numbers demonstrate that cancer burden remains a major challenge worldwide, recent developments in personalized medicine and novel treatment

Abbreviations: *ADCC, antibody-dependent cellular cytotoxicity; CAR, chimeric antigen receptor; CAR-T, chimeric antigen receptor T-lymphocytes; CDC, complement dependent cytotoxicity; CDV, canine distemper virus; CHOP, cyclophosphamide, doxorubicin, vincristine and prednisolone; cNHL, canine lymphoma; CTLA-4, cytotoxic T-lymphocyte associated protein 4; DLBCL, diffuse large B-cell lymphoma; EGFR, epidermal growth factor receptor; FDA, US Food and Drug Administration; GM-CSF, Granulocyte-macrophage colony- stimulating factor; HER2, human epidermal growth factor receptor 2; hNHL, human non-Hodgkin lymphoma; HSP, heat shock proteins; HSPPC, immunogenic tumor specific peptides; LMI, large multivalent immunogen; mAbs, monoclonal antibodies; NHL, non-Hodgkin lymphoma; PBMC, peripheral blood mononuclear cells; PD-1, programmed-death 1; PD-L1, PD ligand 1; scFv, single chain variable fragment; TERT, telomerase reverse transcriptase; USDA, United States Department of Agriculture.*

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approaches, such as immunotherapy, have raised hope of significantly improving cancer survival (2).

The concept of harnessing the host's immune system to treat cancer can be traced back decades, however only in recent years immunotherapies have emerged as a clinically validated and effective treatment strategy (4). Nowadays, cancer immunotherapy has emerged as a fast-growing field and rapidly became the fourth pillar of cancer care, along with surgery, cytotoxic therapy and radiotherapy (5). More recently the successes of clinical breakthroughs, such as checkpoint inhibitors and engineered T cells, revitalized the field and highlighted the opportunities that immunotherapeutic approaches can offer, which culminated in the nomination of "cancer immunotherapy" as 2013's Breakthrough of the Year by Science (6, 7). In 2018, the Nobel Prize in Physiology or Medicine was jointly awarded to James Allison (University of Texas MD Anderson Cancer Center) and Tasuku Honjo (Kyoto University School of Medicine) for their discoveries leading to new approaches in harnessing the immune system to fight cancer (8–12).

However, by transforming the cancer therapeutic landscape, this complex modality brought unique challenges to the drug discovery community. In fact, as more cancer patients have received immunotherapies, some of the major drawbacks of these treatments have become clear. One of the major issues is to determine the sub-populations of patients who will respond and who will experience significant toxicities (13). In fact, the challenge now is to extend the range of patients that benefit from immunotherapy while minimizing treatment-related adverse events. To address this, it is crucial to identify factors predictive of response that may help to properly select patients for treatment, identify rational combination therapies, and define progression and resistance (14). This is particularly critical when developing cancer immunotherapies, considering that the patient's immune system is expected to be as significant as tumor-related aspects when determining response and toxicity (15).

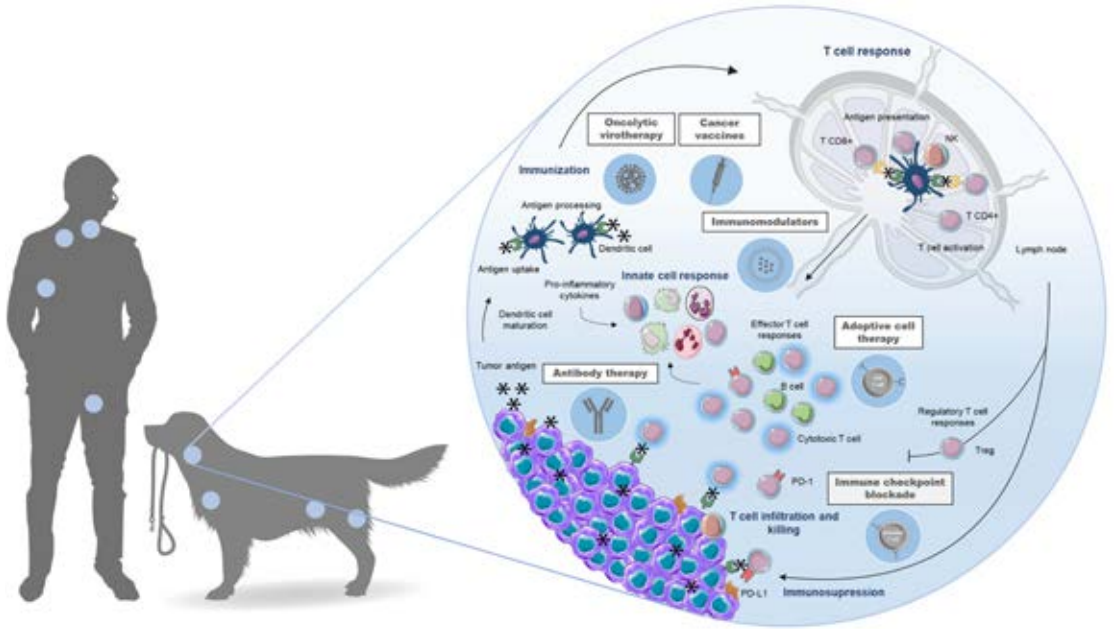
Clinical translation of cancer immunotherapy depends on preclinical investigation and rodent models have been the foundation of preliminary basic investigation and safety assays (16). However, these models underrepresent the heterogeneity and complex interaction between the human immune cells and cancers. Indeed, laboratory mice rarely develop spontaneous tumors, are housed under specific-pathogen free conditions that greatly impact immune development, and incompletely model main characteristics of the tumor/immune microenvironment, creating challenges for clinical translation. As a result, these murine models have failed to correlate with clinical success rates, demonstrating an urgent need for innovative pre-clinical models (17–19). Thereby, the use of alternative animal models is pivotal to bridge the translational gap between murine models and human clinical studies. In particular, preclinical models displaying intact immune systems that closely resemble the human immune response, present comparable, spontaneous oncogenesis and immune interactions similar to humans, and can model key clinical outcomes such as efficacy, dose response, and toxicity, will be critical for translational cancer immunotherapy research (15).

Thus, comparative medicine offers an important platform with innovative complex cross-species models that allow the research of novel therapeutic strategies and agents for diseases that are common to animals and humans (20, 21). Notably, the canine model represents a powerful resource of models for cancer immunotherapy research. Dogs are an appealing outbred combination of companion animals that experience spontaneous cancer development in the setting of an intact immune system (15). Besides, naturally occurring tumors in dogs present many clinical, pathological, immunologic, molecular, diagnostic and therapeutic sim-

ilarities to those observed in humans, that are difficult to reproduce in other models (22–25). This allows studying the complex immune interactions during the course of treatment while also addressing long-term efficacy and toxicity of cancer immunotherapies (15).

Nevertheless, the integration of the canine model in immunotherapeutic approaches research requires diagnosis, staging and treatment response assessment, optimization and standardization, to perform large and organized clinical trials and to achieve conformity when analyzing data (26).

Driven by the great success accomplished with the application of immunotherapies in the treatment of human non-Hodgkin (hNHL) and by the remarkable similarities of canine non-Hodgkin lymphoma (cNHL) to its human counterpart, cNHL has been one of the main focus of comparative research regarding the development of immunotherapeutic approaches for dogs (Graphical abstract).



GRAPHICAL ABSTRACT. *Graphical Abstract. The application of canine lymphoma as an animal model for immunotherapeutic approaches in comparative medicine provides an integrated drug discovery platform that maximize interdisciplinary cooperation and leverage commonalities across humans and dogs for the development of novel immunotherapies against non-Hodgkin lymphoma, benefiting both species.*

Rationale for a Canine Model of Lymphoma

For a long time, research in lymphoma has benefited from traditional mouse models, however the paucity of truly representative models has hindered complete understanding of disease biology and drug development. With the introduction of genomics technology, non-traditional animal models have been more accessible and the leverage of these opportunities may represent a novel strategy to accelerate disease research and new drug discovery (27). Furthermore, there is an increasing number of studies demonstrating that spontaneously arising lymphoma in dogs could be an invaluable resource to study the biology and treatment of this disease (28). As such, the cNHL model may help to bypass many of the limitations associated with the use of murine models while presenting other additional advantages (29, 30).

The cNHL shares many remarkable similarities with its human counterpart (29, 31–34). The incidence of cNHL of 15–30/100 000 is similar to human incidence (35, 36), though additional studies indicate that the incidence of cNHL may be higher (37). Classification and grading schemes of cNHL were designed to reflect the equivalent in peo-

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ple and facilitate comparison. In fact, the 2008 revised World Health Organization classification based on the Revised European American Lymphoma classification system, which attempts to group lymphomas by cell type, phenotypic, genetic and molecular aspects, is the current standard for the diagnosis and classification of human lymphoma, also serves as the basis for the current canine recommendations (38, 39). The use of these current World Health Organization guidelines as a template, allowed describing 20 cNHL entities, among nearly 50 discrete subtypes of hNHL. Moreover, B-cell lymphoma is more prevalent than T-cell lymphoma in both species and diffuse large B-cell lymphoma is the most common type of non-Hodgkin lymphoma (NHL) in both humans and dogs (38). Finally, treatment modalities for cNHL are similar to those used for human lymphoma (radiation, corticosteroids, chemotherapy) and CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisolone)-based chemotherapy agents are typically used to treat it. Response to treatment and resistance also present clinical patterns similar to hNHL (27).

From a drug development perspective, the canine model represents a large and long-lived animal model, evolutionarily more closely related to humans than rodents, that provides a more accurate assessment of the pharmacokinetic/pharmacodynamic parameters, while determining safety and efficacy of new therapeutic agents and approaches (27, 40). Moreover, the relatively fast disease progression rate allows obtaining early conclusions from clinical trials. In fact, a randomized clinical trial in pet dogs requires ~1–3 years, whereas a human clinical trial takes about 15 years to be completed. This short timeline allows to integrate the findings of pet trials on human trials, including toxicity, response, pharmacodynamics, dose, regimen, schedule, biomarkers and responding histology assessment (28).

Another main advantage of the canine model is that cNHL is a spontaneously occurring tumor in an immune-competent host, in contrast to murine xenograft or genetically engineered mouse models. This natural occurring cancer setting offers genetic diversity similar to human lymphoma and allows studying biological mechanisms, such as tumor initiation and promotion. Moreover, the pet dog model harnessed by the evolutionary conservation allows to identify similarities between canine and human lymphomagenesis, for example in identifying key "driver" gene mutations common to both species (27).

The benefits of the cNHL model extend beyond the biological advantages of a spontaneously occurring tumor in a large animal. Pet dogs share the same living environment as their caregivers, allowing to study environmental risk factors of developing lymphoma (27, 28). For example, an epidemiological study in France demonstrated a correlation between the incidence of cNHL and hNHL and reported a strong association between cNHL and the distribution of waste incinerators, radioactive waste or other polluted sites (41). Moreover, there is an increased prevalence of lymphoma within specific dog breeds (42) and a breed-specific distribution of B-cell and T-cell lymphomas (43). This in association with the well-organized multi-generational pedigrees kept by many breeders, represents a unique genetic advantage that allows mapping of lymphoma predisposition genes with strategies that are not possible in humans (28).

The final rationale for using dogs with lymphoma as an animal model relies on the dual benefit concept of this comparative research approach. Improved current health care have promoted the increase of dogs lifespan, allowing the diagnosis of late-in-life diseases such as cancer (44). Lymphoma particularly is one of the most common malignancies in dogs (28). In addition, the social status of dogs as companion animals allows them to benefit from high quality health care and the ethical exploration of translational approaches. Moreover, these initiatives are also motivated by the increasing healthcare standards

demanded by pet owners, creating a need for novel cancer therapies in veterinary settings (20, 21, 45). Altogether, the use of the cNHL model represents a unique opportunity to strengthen the collaboration between human and veterinary medicine in lymphoma research, that ultimately will lead to advances in the care of people and dogs affected by NHL, a critical medical unmet need of today's society (22, 27).

A Critical Unmet Need for Novel Treatment Options for Non-Hodgkin's Lymphoma in Comparative Oncology

NHL, an heterogeneous group of cancers characterized by a diverse class of lymphocyte proliferations, represents one of the most common neoplasias in both humans and pet dogs (38, 46). hNHL constitutes the most commonly reported hematological malignancy worldwide, comprising nearly 3% of all cancer diagnoses. The highest incidence rates are found in Australia/New Zealand, Northern America, and Europe. In the United States, NHL is the seventh most common and sixth most common cause of cancer-related death, in Europe is the eleventh most common and the fourteenth most deadly malignancy and its incidence has nearly doubled since the early 70s (47, 48). NHL represents 90% of all lymphomas and encompasses an heterogeneous group of cancers characterized by the proliferation of malignant lymphocytes, 85–90% of which arise from B lymphocytes, whereas the remaining derive from T cells or natural killer cells. This diverse group of malignancies usually develops in the lymph nodes, but can occur in almost any tissue, ranging from the more indolent follicular lymphoma to the more aggressive diffuse large B-cell (DLBCL) and Burkitt's lymphoma (49). NHL patients typically present with persistent painless lymphadenopathy, but some patients may present with constitutional symptoms or with involvement of organs other than those from the lymphoid and hematopoietic system (50).

The basis of treatment selection requires an accurate diagnosis, a careful staging of the disease, and the identification of adverse prognostic factors. Regardless, NHL patients most commonly receive chemoimmunotherapy as initial treatment. Radiation therapy may be performed if patients have early-stage disease (50). Response rates to conventional chemotherapy are generally >50%; however, most patients eventually relapse. Moreover, the toxicity of conventional chemotherapy often limits its efficacy (47).

In the last decades, the scientific community has been reporting cases of therapeutic success using monoclonal antibodies (mAbs) in the treatment of NHL in humans. One of the most successful examples has been the application of mAbs targeting the surface antigen of CD20 (Rituximab®) in combination with chemotherapy regimen CHOP, which has revolutionized the treatment of B-cell lymphoma by significantly improving disease-free interval and overall survival, with minimal toxicity (51, 52). Even though current therapy strategies have significantly improved prognosis of patients diagnosed with NHL, a substantial fraction of patients relapse or are refractory to these treatments. Several treatment shortcomings have been identified as research priorities, however rituximab resistance and refractory/relapsed disease represent major current and emerging challenges (53–55).

Thus, a plethora of new immunotherapeutic approaches to treat lymphoma have been ensued. The most exciting classes of immunotherapies comprise chimeric antigen receptor T-cells, bispecific antibodies, immune checkpoint inhibitors, and vaccines. The advent of such innovative therapies brought unique challenges that need to be considered, including the assessment of the appropriate timing of treatment, optimal patient population, duration of therapy, toxicity, and cost. Hence, future studies need to focus on the de-

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velopment of new strategies, models and paths in order to optimize the drug development of novel immunotherapies for hNHL (56).

Owing to shared molecular, incidence, genetic, histopathologic and clinical features, cNHL has been proposed as a comparative animal model for the research of novel therapeutic agents and approaches for hNHL (22–24, 30). cNHL displays several histological subtypes and patients can manifest a wide range of symptoms. However, most suffer from generalized lymphadenopathy (multicentric form) and are diagnosed with intermediate to high-grade lymphoma, more commonly of B-cell origin. Without treatment, the disease has high mortality (28), requiring prompt chemotherapy to achieve temporary remission and prolonged survival. Chemotherapy still remains the mainstay for the treatment of cNHL and regardless of the numerous published chemotherapeutic protocols, it seems we have reached a stalemate concerning what this treatment modality has to offer in standard settings (57). Yet, cure is rarely achieved and the majority of dogs relapse with lethal, drug-resistant lymphoma. The 12 month median survival barrier and the 20 to 25% 2 years survival rates demonstrate an urgent and unmet need in veterinary medicine to develop new treatment strategies for refractory disease (58–61).

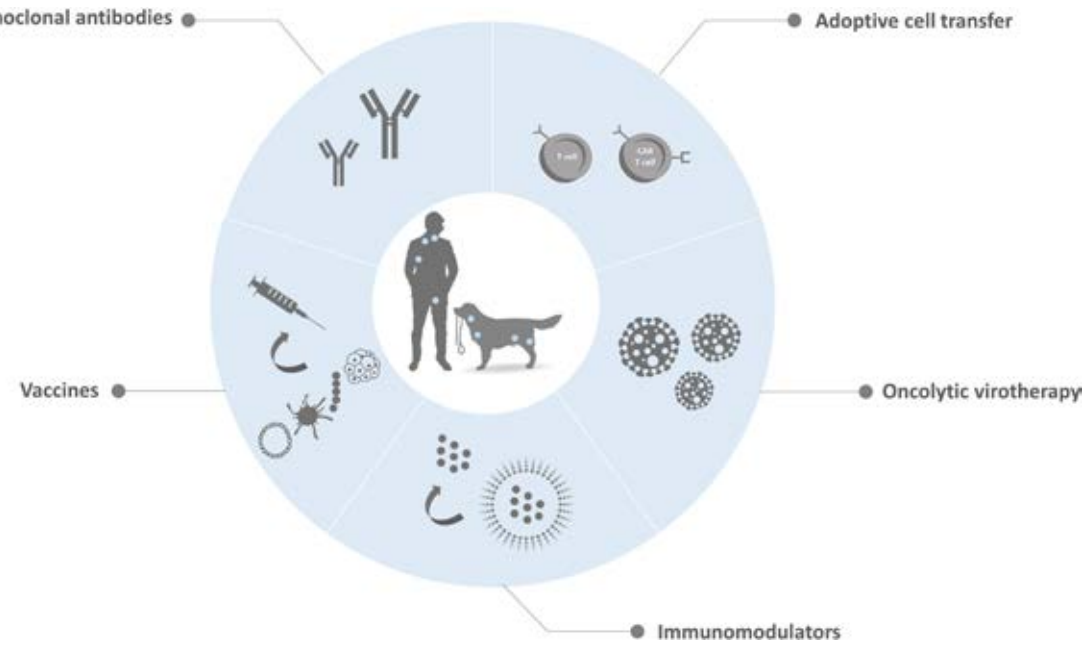

Figure 1. *Schematic representation of available and under development immunotherapy strategies for cNHL. Currently, several research groups are actively investigating new immunotherapies that mobilize the patient's own immune system to treat NHL in both pets and pet owners. These treatment modalities include therapeutic mAbs that promote the direct or indirect death of cancer cells, adoptative cell transfer that uses a patient's own cells to induce antitumor activity, oncolytic virotherapy that involves the replication-competent virus in the elimination of cancer, immunomodulators that aim to enhance immune responses and tumor control and vaccines that stimulate a patient's own immune system against cancer cells.*

Thus, immunotherapies for cNHL are a promising approach for the development of a new class of anti-cancer therapeutics, which will in many cases benefit humans and man's best friends. To demonstrate the potential of these strategies, available and under development immunotherapies for cNHL will be summarized below (**Figure 1**).

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Monoclonal antibodies therapy	Study	References
mAb 231	Preclinical and clinical	(64–66)
Anti-HLA-DR (L243)	Preclinical and clinical	(67)
Anti-HLA-DR (IMMU-114)	Preclinical and clinical	(67)
Anti-CD20 (6C8)	Preclinical	(51)
Anti-CD20 (1E4-cIgGB)	Preclinical and clinical	(68)
Anti-CD20 (NCD1.2)	Preclinical	(69)
Anti-CD20 (AT-004)	Preclinical and clinical	Aratana Therapeutics®
Anti-CD52 (AT-005)	Preclinical and clinical	Aratana Therapeutics®
Anti-CD20 (1E4-cIgGB) plus CD47 blockade	Preclinical	(45)
Anti-CD20 (4E1-7-B_f)	Preclinical and clinical	(70)
Adoptive cell transfer therapy	**Study**	**References**
Autologous T cells	Preclinical and clinical	(71, 72)
Autologous T-cells		Aurelius BioTherapeutics®
Autologous T-cells plus tumor vaccination		Elias Animal Health®
CD20 CAR-T cells	Preclinical and clinical	(73)
Oncolytic virotherapy	**Study**	**References**
Canine distemper virus (pCDVeGFPN)	Preclinical and clinical	(74, 75)
Newcastle disease virus	Preclinical and clinical	(76, 77)
Reovirus (dearing strain)	Preclinical and clinical	(78, 79)
Immunomodulator therapy	**Study**	**References**
Autologous tumor antigen-coated microbeads with IL-2 and GM-CSF	Preclinical and clinical	(80)
Vaccine therapy	**Study**	**References**
Intralymphatic autologous tumor vaccine	Preclinical and clinical	(81–83)
Autologous CD-40-activated B-cells loaded with total RNA from autologous lymphoma cells	Preclinical and clinical	(84)
DNA-vaccine targeting canine telomerase reverse transcriptase	Preclinical and clinical	(85–87)
Autologous tumor heat shock proteins (APAVAC)	Preclinical and clinical	(88–90)

Table 1. *Immunotherapy approaches developed and under development for cNHL.*

Current Immunotherapies for Canine Non-Hodgkin's Lymphoma

After decades of weakening or even eliminating the patient's immune system with chemotherapy, now the trend is to harness the ability of the immune system to eradicate cancer (62). Over the past decades immunotherapy has moved into the forefront of cancer care due to unprecedented clinical success in a wide range of malignancies, sometimes even in late stages of disease (63). The field of veterinary immunotherapy holds similar promise for companion animals with cancer, and several efforts have been made in order to develop veterinary specific immunotherapies (**Table 1**). In the nearby future, it is hoped that tumor immunotherapy will become a valid therapeutic tool in veterinary oncology, along with chemotherapy, radiotherapy and surgery.

Monoclonal Antibodies

In cancer therapy, the main purpose of antibody treatment is to promote the direct or indirect death of cancer cells and a number of strategies have been successfully employed. MAbs can bind to target cancer cells and directly promote signaling-induced death or can mediate an anti-tumor immune response by promoting antibody-dependent cellular cytotoxicity (ADCC) and inducing complement-dependent cyto-

toxicity (CDC) (91). In the case of ADCC responses, mAbs bind to target tumor cells while the mAb Fc region engage with the FcγRs on the surface of effector cells, including natural killer cells and macrophages. These immune cells cause phagocytosis, apoptosis or lysis of the target cells. In CDC responses, mAbs promote directly target cell death through the development of a complement cascade membrane attack complex. Furthermore, mAb-based therapies can also block growth-promoting pathways, such as angiogenesis or can directly regulate the anti-tumoral activity of adaptive immune cells by blocking inhibitory signals responsible for limiting T cell activation (92). Most marketed mAbs consist of a full-length IgG molecule. By providing a long half-life and effector functions, these molecules have been presenting a quite successful application in therapeutics. However, this conventional antibody format present some drawbacks that limit their clinical use and there is a range of therapeutic applications in which other antibody formats may be more appropriate. To address these major issues, smaller antibody scaffolds such as the Fab or the single chain variable fragment (scFv) or single domain antibody are emerging as alternative therapeutic agents (93) (**Figure 2**).

MAbs are the most commonly used and approved cancer immunotherapy method in clinical practice (94). The use of an antibody targeting the human surface antigen CD20 (Rituximab®), expressed on B-lymphocytes has revolutionized the treatment of B-cell lymphoma (51, 52). Rituximab is a chimeric antibody and was the first US Food and Drug Administration (FDA) approved mAb for the treatment of human cancer, being used for the treatment of most B-cell NHL and subtypes of acute lymphocytic leukemia (95–97). This immunotherapy provided significant enhancements in the efficacy of treatment vs. existing non-mAb therapies, increasing the rate of durable remissions from 30 to 60% (51).

Even though immunotherapy has a crucial role in the treatment of B-cell malignancies in humans, its role in canine

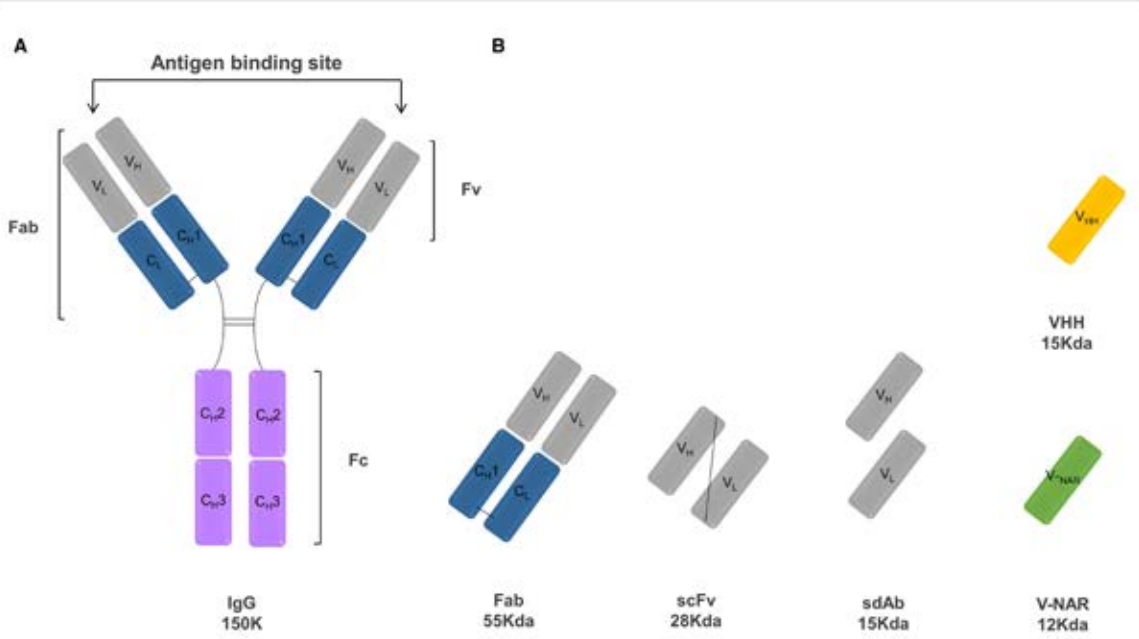

Figure 2. *Schematic representation of various antibody formats including a conventional IgG antibody (**A**) and antibody fragments (**B**) of interest. (**A**) The basic unit of a conventional IgG antibody is a polypeptide consisting of a pair of identical heavy and light chains held together by disulfide bonds. Light chains are comprised of one constant domain (CL) and one variable domain (VL), whereas heavy chains are comprised of three constant domains (CH1, CH2, and CH3) and one variable domain (VH). The antigen-binding site is composed by the variable domains of both the heavy and light chains. In turn, the Fc constant region is responsible for the recruitment of the immune system effector functions. (**B**) Antibody fragments that can be engineered from a conventional IgG include: antigen-binding fragment (Fab), single-chain Fv fragment (scFv), heavy and light single domains antibodies (sdAbs) and natural camelid variable domain (VHH) and shark variable domains (V-NAR).*

lymphoma remains limited. Immunohistochemistry using mAbs that recognize the CD20 intracellular domains demonstrated the presence of CD20 in canine lymphoma tissue samples (98, 99). However, Rituximab® and other anti-human and anti-mouse antibodies that recognize the CD20 extracellular domains, failed to bind to canine CD20, even though the reported epitopes are conserved between human and canine CD20 (100). For that reason, it is evident that technology to speciate antibodies is essential when developing similar passive immunotherapy strategies for canine cancer patients.

Interestingly, in 1992, prior to FDA approval of Rituximab, the United States Department of Agriculture (USDA) approved the licensing of mAb 231 for use in cNHL. mAb 231 consists of a murine-derived mAb that showed both in vitro (64) and in vivo activity and served as adjuvant therapy following remission induction with chemotherapy (65, 66, 81). Unfortunately, subsequent clinical trials failed to confirm the initial study results and the antibody epitope was never identified, which culminated in its commercial suspension (65).

Since then, driven by the great potential of the canine lymphoma model for immunotherapeutic approaches, academic research groups and industry began exploiting the dual benefit approach of comparative medicine.

One of the first examples was a pilot study that aimed to assess the suitability of the canine lymphoma model to evaluate endpoints with clinical relevance of anti-HLA-DR mAb treatment before proceeding to an extensive trial in pet dogs, and eventually human research. In vitro studies revealed that L243, a murine IgG1 anti-HLA-DR, binds to canine healthy lymphocytes and lymphoma cells, inducing apoptosis in cNHL cells. In turn, in vivo studies confirmed the L243 treatment safety in healthy dogs and dogs with lymphoma and its binding activity to lymphoma affected lymph node samples. Preliminary data also showed that a subset of patients with advanced lymphoma achieved transient disease stabilization after L243 treatment (67). Furthermore, this work also reported that hL243γ4P (IMMU-114), a humanized IgG4 anti-HLA-DR, under preclinical evaluation for human trials, also bound to cNHL cells. Finally, the assessment of IMMU-114 treatment in healthy canine patients indicated a safety and pharmacokinetic profile similar to L243. Overall, these findings supported the use of cNHL in safety and efficacy studies of anti-HLA-DR mAbs for both veterinary and human medicine (67).

Advances in speciation technology has also led to several clinical trials in pet dogs since "caninization" of antibodies is crucial when approaching canine patients with cancer. With this in mind, research groups focused on the technique to generate caninized antibodies, which resulted in the development of a canine anti-EGFR (epidermal growth factor receptor) mAbs (101) and nowadays is also being offered as a service by companies (Creative Biolabs).

Considering the success achieved with Rituximab in human medicine, several studies also focused on developing canine anti-CD20 antibodies. An anti-canine CD20 mAb (6C8) that recognized the extracellular domain of canine CD20 and showed high-affinity binding to canine CD20 in solution and its native conformation on canine B-cells was developed. This mAb promoted phagocytosis of B-cell lymphoma cells by macrophages, but in its current framework did not induce direct cytotoxicity or CDC (51). In the same year, Rue et al. reported the development of an anti-canine CD20 antibody (1E4) and the generation of a canine chimeric molecule for therapeutic use. This clone bound a similar extracellular domain as rituximab, and flow cytometry analysis confirmed that 1E4-based chimeric versions were able to stain canine B cells and canine CD16a, a receptor that mediates ADCC responses. Moreover, the best chimeric mAb candidate depleted the number of circulating B cells in healthy beagles in an in vivo study. Though, the clinical efficacy in dogs with canine B cell lym-

phoma remains unknown (68). Likewise, a new anti-CD20 mAb (NCD1.2) that bound both human and canine CD20 has been developed, in order to strengthen human-canine comparative model. NCD1.2 bound to clinically derived canine cells including B-cells in peripheral blood and in different histologic types of B-cell lymphoma. Heavy chain and light chain genes from the NCD1.2 hybridomas were cloned and packaged as scFv into a phage-display library. Recombinant anti-CD20 scFv were identified and selected as a possible useful tool for evaluation in bioconjugate-directed anti-CD20 immunotherapies in comparative medicine (69). Although these works established several canine anti-CD20 mAbs candidates with high potential for therapeutic use, their clinical efficacy in dogs bearing B-cell lymphoma remains unknown.

A canine anti-CD20 mAb (AT-004) has been fully approved by USDA for clinical usage in dogs with B-cell Lymphoma and is currently being commercialized in the United States and Canada. Treatment with AT-004 (Aratana Therapeutics), an anti-canine CD20 was subject to a prospective randomized clinical trial and preliminary results suggested an improved median progression-free survival of dogs with B-cell lymphoma (102). Yet, these results were published in a conference abstract and peer-reviewed results are still lacking. Another work evaluated the combination of CD47-blockade with 1E4-cIgGB, a canine-specific antibody to CD20. Although 1E4-cIgGB could elicit an in vivo therapeutic response against canine lymphoma as a single agent, superior responses were observed when combined with agents targeting CD47, an immune checkpoint that enables the evasion of tumor cells to phagocytosis promoted by therapeutic antibodies, such as anti-CD20 mAbs. The combination of CD47-blocking therapies with 1E4-cIgGB resulted in synergic antitumoral effects in vitro and in vivo, eliciting cures in 100% of mice bearing canine lymphoma (45). However, there is no anti-CD20 antibody treatment for cNHL currently available. More recently a novel approach of developing an anti-canine CD20 monoclonal antibody using rats as a host species renewed hopes of finally obtaining an antibody-based therapy for cNHL. This work culminated in the generation of a mAb capable of inducing cell death of B cell lymphoma cell lines, however this mAb was incapable of eliciting CDC and ADCC responses. To tackle these limitations, this antibody was modified into a canine/rat anti-CD20 chimeric, which resulted in the alterations of its characteristics into a potent CDC and ADCC inducer. Furthermore, its defucosylation resulted in a 10-fold higher ADCC activity. The in vivo antitumor activity of this improved mAb version was assessed, revealing a tumoral growth inhibition in a cNHL xenograft mouse model and a peripheral B cell depletion in healthy beagles (70). Finally, AT-005 (Aratana Therapeutics), a caninized mAb targeting CD52 on T cells, has obtained conditional USDA approval for the treatment of T-cell lymphoma and is currently being evaluated in clinical trials (62).

The success of mAbs in human medicine strongly encourages veterinary medicine to develop similar therapeutics for our pets. Regardless of their potential, little speciated mAbs have been established for veterinary application and fewer were investigated in clinical trials enrolling companion animals. Nonetheless, the approval of the first mAb by the European Union Agency for the treatment of atopic dermatitis in dogs—Lokivetmat, a caninized, anti-canine IL-31 mAb (103), highlighted the impact that biological therapies may have in veterinary practice. In the oncology setting, mAbs have the capacity to treat a diversity of hematological and solid malignancies, do not need to be a personalized product and manufacturing methods are well-established, minimizing the cost associated limitation. Hence, mAb-based therapy is one of the most promising immunotherapy strategies in veterinary settings (63).

Adoptive T-Cell Transfer

Adoptive cell therapy is a term that was first used to describe the infusion of lymphocytes to mediate rejection of organ allografts and to treat tumors (104). This immunotherapeutic option represents the most effective treatment for patients with metastatic melanoma inducing visible cancer regression in ~50% of patients. Adoptive cell therapy is also associated with clinical improvement in selected patients with post-transplant lymphoproliferative diseases caused by Epstein–Barr virus infection (105). More recently, gene transfer techniques developed in the 1990s allowed to convert normal lymphocytes into lymphocytes with anti-cancer activity by redirecting the specificity of T cells with the use of T-cell receptors or chimeric antigen receptor (CARs). CARs are engineered receptors that graft a defined specificity onto an immune effector cell, typically a T cell, resulting in the augment of T-cell function (104). This innovation represented a possibility of extending adoptive cell immunotherapy to patients with a large diversity of cancer types (105). In humans, treatment of advanced B-cell leukemia or lymphoma using CAR T-cells has demonstrated promising clinical responses, resulting in the approval of two autologous CAR T-cell therapies (Kymriah™ and Yescarta™) by the FDA (106, 107). These therapies are both genetically modified autologous T cells expressing a CD19-specific CAR, lysing CD19-positive targets (107).

By displaying an intact immune response and genetic similarities to humans, dogs may potentially inform the development of the later-stages of human clinical trials, while studying the use of adoptive cell therapy in veterinary malignancies, including hematologic neoplasias (71, 72). In fact, there is evidence that canine cancer, and specifically cNHL, respond to cell-based immunotherapy. Half a century ago, the Fred Hutchinson Cancer Center established hematopoietic cell transplantation for canine lymphoma (108). At first, the therapeutic value of this practice was solely associated with the administration of high-dose chemotherapy and radiation prior to the transplant. Yet, a larger retrospective study confirmed that, despite the use of the same chemotherapy and radiation protocols, dogs that received an allogeneic transplant from a littermate exhibited a significantly lower relapse rate, in contrast to dogs that received their own (autologous) bone marrow stem cells. This effect was later known as the "graft vs. leukemia/tumor effect" and is mainly promoted by activated allogeneic T cells that recognize and react to antigen differences, and therefore also attack residual tumor cells (109).

Since then, few studies have focused on the scientific and clinical investigation of cell-based immunotherapies for canine patients. O'Connor et al. conducted a clinical trial to test non-specific autologous T cells isolated from dogs with NHL and expanded ex vivo using a novel artificial antigen presenting cell protocol (71, 72). Infused cells were detected in the blood for longer than 49 days and trafficked to secondary lymphoid organs, confirming the safety of adoptive transfer of autologous T cells in dogs. Furthermore, this adoptive immunotherapy demonstrated to be viable and effective in improving first remission and overall survival periods in dogs with multicentric lymphoma (71, 72).

Notably, a few biotech companies have emerged in the area of autologous T-cell based therapy for veterinary medicine. One example is Aurelius BioTherapeutics that provides a service that expands for 2–3 weeks autologous lymphocytes collected from dogs with canine lymphoma, in order to increase T cell numbers exponentially and to activate them to be responsive to antigens presented by the tumor cells before reinfusion. However, the methods used for the activation and expansion of dog's immune cells and the clinical benefit of this therapy are not disclosed. In turn, Elias Animal Health included a vaccination procedure prior to cell collection, aim-

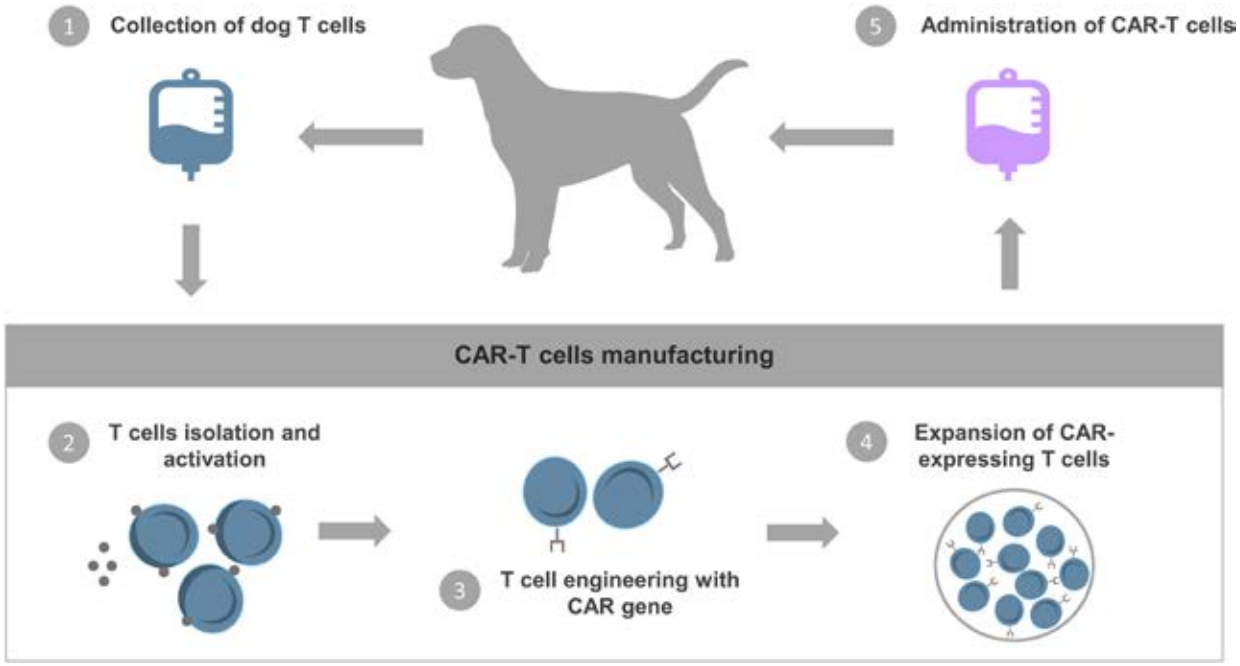

Figure 3. *CAR-T cells therapy. The basic procedures for CAR-T cell therapy start with the collection and extraction of T cells from the pet's peripheral blood. The T cells are then genetically engineered in vitro to express chimeric antigen receptors (CARs) that can recognize specific tumor-associated antigens and activate self-proliferation and cytotoxicity. Finally, CAR-T cells are expanded and reinfused into the patient.*

ing to improve cancer-cell specificity of their autologous T-cell therapy. The vaccine is obtained from the excised tumor material and is given through an intradermal route. Additionally, a brief cycle of chemotherapy may be administered prior to the infusion, which has shown to result in better acceptance of the lymphocyte therapy in humans. The preliminary results revealed that overall survival may be prolonged with this adoptive cell-based therapy, indicating that this immunotherapy prompts an antitumor vaccine-like effect that extends canine patients' lives, even when the disease is not fully eradicated. The holding company is pursuing regulatory approval, which would qualify it as the first approved and commercialized cell therapy for dogs (106).

More recently, researchers have started to explore chimeric antigen receptor T-lymphocytes (CAR-T) cell therapy for dogs (**Figure 3**). CARs engineering consists of modifying T-cells to express artificial receptors formed by a tumor-antigen specific scFv linked to an intracellular signaling domain and co-stimulatory molecules. Because CARs work in a MHC independent manner, antigen presentation do not rely on patient antigen presenting cells. Moreover, CARs do not have to be syngeneic to the patient immune system (63). Canine T cells expressing a HER2 (human epidermal growth factor receptor 2)-specific CAR have been produced and showed anti-tumoral activity in vitro against canine osteosarcoma cells expressing HER2 (110). This work proved that a successful ex vivo expansion of HER2-CAR specific T-lymphocytes is possible. Yet, no canine patients have been treated. Ongoing studies aim to develop a canine CAR-T cells for the treatment of B-cell lymphomas and other malignancies (63). Importantly, protocols for the propagation of CD20 CAR-T cells have been reported (73, 111). Researchers transfected the CD20 CAR into the expanded T-cells us-

ing electroporation of CAR mRNA. Unfortunately, even though this strategy allows to avert using retro or lentivirus, mRNA transfection results in variable efficiency and transient transcriptional activity that ceases following 24 to 48 h. It was reported the treatment of one dog diagnosed with lymphoma with these transfected T-cells, however it only presented a short-term partial response (73, 106). This limited clinical response can be due to the inability of these transfected cells to expand in vivo, considering that human studies demonstrated that in vivo expansion is a requirement for durable responses. Furthermore, this treatment protocol did not include chemotherapy sessions prior to the CAR-T cells infusion, a common practice used in the human treatment to deplete inhibitory immune cells that has shown to potentiate clinical efficacy. In the case of dogs, the addition of this procedure could also minimize the risk of triggering a canine anti-mouse antibody immune response, considering that most scFvs derived from murine mAbs, thereby increasing the risk for an anti-CAR T cell immune response. To conclude, reported data proved the feasibility of generating canine CAR-T cells, however the necessary logistics and expenses are expected to be considerable.

Oncolytic Virotherapy

Oncolytic virotherapy is a new concept of immunotherapy recently introduced that involves the replication-competent virus in the elimination of cancer. By infecting tumor cells, oncolytic virotherapy can stimulate de novo or enhance pre-existing native immune response. The majority of developed oncolytic virus are genetically altered to promote tumor tropism while reduce virulence against healthy host cells. Thereby, oncolytic virotherapy have the ability to promote a proinflammatory environment by improving antigen release/recognition and promoting immune activation, while reverting immunosuppression of tumor cells and improving the efficacy of other forms of immunotherapy (112, 113). Although several oncolytic virotherapies are being developed in preclinical and clinical settings, currently the only oncolytic viral therapy approved by FDA is talimogene laherparepvec (T-Vec or Imlygic) for advanced melanoma (114). In veterinary medicine, several studies evaluated natural and genetically modified oncolytic viruses for dogs diagnosed with cancer, showing some encouraging results. However, the majority of the developed research work focused on in vitro results, with a few reporting in vivo studies, of which most were isolated clinical case reports (115).

Regarding cNHL, a study reported that a recombinant strain of the canine distemper virus (CDV)—pCDVeGFPΔN—was capable of infecting cNHL cell lines in vitro, inducing significant apoptotic cell death. The pCDVeGFPΔN strain also efficiently infected primary canine B and T-cell lymphoma cells, though its oncolytic efficacy was not proved (74). Another work evaluated the anti-tumoral effect of CDV infection using an attenuated strain in seven dogs with naturally occurring lymphoma. For this purpose, single or multiple doses of the virus were injected intratumorally. This study reported low toxicity with a severe fibrotic reaction in the injection site. Immunohistochemistry analysis revealed a variable positive detection of CDV antigen in treated lymph nodes, while co-culturing enabled virus isolation from treated lymph nodes, but not from distant nodes or from peripheral blood mononuclear cells (PBMCs). Furthermore, this treatment promoted a strong anti-CDV antibody response (75). However, one of the major drawbacks of this immunotherapy is that CDV belongs to the regular vaccination schedule in dogs and pre-existing antibodies can limit its efficacy (116). Another group explored the oncolytic properties of a vaccine strain of Newcastle disease virus, an attenuated lentogenic strain presenting low virulence, on a human large B-cell lymphoma cell

line and on primary canine B-cell lymphoma cells. The group used as controls healthy PBMCs from humans and dogs. Newcastle disease virus infection decreased cell viability in both human and dog lymphoma cells when compared to untreated controls, with minimal tropism toward healthy PBMCs. In the same work the authors reported the viral biodistribution in a canine patient diagnosed with T-cell lymphoma, 24 h following the virus intravenous injection. Immunohistochemistry and endpoint PCR demonstrated viral dissemination in the salivary gland, kidney, stomach and lung, but not in tumor samples, with no abnormal findings on the histopathological evaluation (76). Curiously, a complete and long-term clinical response was reported in a dog diagnosed with lymphoma resistant to chemotherapy (76, 77). Although these preliminary data revealed that Newcastle disease virus could represent a promising oncolytic virotherapy, future studies are required to determinate the best therapeutic regimen and define the proper safety protocol (117).

One of the oncolytic virotherapies that has gathered most interest amongst the scientific community, due to the promising results obtained in multiple phase I and II clinical trials, is the dearing strain of Reovirus (Reolysin®, from OncolyticsTM Biotech Inc., Calgary, AB, Canada) (118). In dogs, Reolysin® showed promising in vitro results for the treatment of a variety of malignancies, such as mastocytoma, lymphoma, mammary gland tumors and melanoma. In fact, in vitro studies showed apoptosis induction and a significant cell viability reduction in both T and B-cell lymphoma. Furthermore, a mouse xenograft model of canine T-cell lymphoma treated via intratumoral injection revealed significant tumor growth inhibition, compared to the control group treated with reovirus inactivated by ultraviolet (78). Notably, the safety profile of Reolysin® was proven in a clinical trial enrolling dogs with advanced cancer, including mastocytoma, lymphoma, oral melanoma and soft tissue sarcoma. In this work, dogs received virotherapy by intratumoral injection or intravenous injection daily for 5 days, during one or several treatment cycles. Live virus was only detected in the serum of one dog in the first chemotherapy cycle, but not in the subsequent treatment cycles. While all dogs exhibited an increase in the titer of anti-reovirus neutralizing antibodies, tumor volume reduction was observed in five dogs and six dogs presented alleviation of clinical manifestations. Furthermore, a subset of dogs revealed a good safety profile, as well as clinical response. Taking into account the experience gathered in human medicine, the combination of this immunotherapy with conventional therapies such as chemotherapy, radiotherapy, or other could be investigated in dogs (79).

Overall, these studies provide preliminary results that support the development of oncolytic virotherapy as canine cancer therapy to benefit pets and pet-owners (115).

Immunomodulators

Cytokine therapy aims to enhance immune responses and tumor control in a variety of spontaneous oncologic diseases. In human medicine, modest success has been obtained with a low-dose IL-2 therapy delivered subcutaneously, with few side effects (119–124). Additionally, subcutaneous GM-CSF (Granulocyte-macrophage colony-stimulating factor) therapy boosts cell-mediated immune responses and improves anti-idiotype vaccines efficacy in human lymphoma (125). In canine patients, IL-2 delivered subcutaneously, intralesionally, by inhalation and via liposome-DNA complexes encoding IL-2 gene, as a monotherapy or in combination with other modalities, promoted regression in dogs with oral melanoma, soft tissue sarcoma, squamous cell carcinoma and pulmonary metastases from osteosarcoma (126–131). Likewise, in dogs with oral melanoma, combination therapy including GM-CSF delivered intralesionally, either via liposome–DNA complexes or via GM-CSF secreting transgenic xenogeneic

cells, resulted in regression (126, 132). Through the Comparative Oncology Trials Consortium, a Phase I safety/dose escalation study of human IL12 administered subcutaneously to dogs with melanoma was conducted. Data gathered from this study and other preclinical data allowed to inform the design of a Phase I clinical trial of IL12 in human cancer patients (133).

A phase I study enrolling 15 dogs with B-cell lymphoma tested a therapy with a combination of autologous tumor antigen-coated microbeads (large multivalent immunogen—LMI) with cytokine therapy including IL-2 and GM-CSF, following induction of remission with conventional chemotherapy. Results demonstrated no significant toxicity, no adverse effects in disease-free interval and half of the animals presented quantifiable delayed-type hypersensitivity reactions to intradermal LMI, suggestive of a specific cell-mediated immune response (80).

Although these studies show that human cytokines can be effectively used in dogs, the often-needed higher doses and the immunogenicity that they generate, limits their use. Nonetheless, the development of canine IL-15 has led to a renewed interest in cytokine therapy as an immunotherapy strategy for veterinary settings (134).

Vaccines

Therapeutic vaccines represent a viable and attractive cancer immunotherapy strategy that aim to treat late stage disease by stimulating a patient's own immune system against cancer cells (135).

Several attempts to use vaccines as a treatment for cNHL have been made. In the initial studies, Freund's adjuvant was added to lymphoma cell extracts lysates and used as a cancer vaccine strategy. Despite de fact that these early studies reported some treatment benefit (136), this was later attributed to the use of the Freund's adjuvant (137).

Later, Jeglum et al. described the use of an autologous tumor vaccine administrated via intralymphatic injection following remission induction with chemotherapy. However, results using this strategy have been conflicting (81–83).

In a clinical trial, autologous CD40-activated B-cells loaded with total RNA from autologous lymphoma cells were administered to 19 dogs with NHL as an adjuvant, following induction of a complete response with chemotherapy. Vaccination promoted an anti-tumor response and increased a lasting second remission rate, however median time to disease progression and overall survival did not show differences between groups (84).

Moreover, a new approach targeting canine telomerase reverse transcriptase using a genetic vaccine, Tel-eVax, is reported. As telomerase confers immortality to cells, telomerase reverse transcriptase is overexpressed in cancer cell lines and in several tumors and undetectable in the majority of normal tissues, establishing a possible target for translational cancer immunotherapy. A DNA-vaccine targeting canine telomerase reverse transcriptase was able to prompt an immune response against telomerase in dogs diagnosed with multicentric lymphoma, and conventional chemotherapy seems not to alter the immunotherapy effects (85). The combination of this vaccine with chemotherapy using the cyclophosphamide, vincristine and prednisolone protocol resulted in a durable immune response, as well as prolonged survival in dogs with B-cell lymphoma (86). On other clinical study including 17 pet dogs, Tel-eVax in association with CHOP chemotherapy demonstrated to be safe and immunogenic and presented a significant impact on DLBCL canine patients' survival. Antibody response induced by Tel-eVax against telomerase reverse transcriptase (TERT) protein was also evaluated considering the potential that these anti-TERT antibodies may possess as surrogate biomarkers of the immune response in vaccinated dogs. Curiously, most dogs developed

a low but detectable seroconversion against the N-terminal of TERT protein (87).

More recently, an autologous vaccine APAVAC®, comprised of hydroxylapatite ceramic powder with autologous heat shock proteins (HSP) purified from affected lymph node biopsy is currently available (88). HSPs resultant from tumor cells, including gp96, hsp90, hsp70, calreticulin, hsp110, and hsp170, present strong immunogenicity. Furthermore, the chaperone function of HSPs allows their combination with immunogenic tumor specific peptides (HSPPC), exposing the host to a large repertoire of tumor associated antigens for immunization. In addition, hydroxylapatite vehicles and HSPPCs functions as an adjuvant. In order to reproduce the tumor heterogeneity, each vaccine is produced for each dog. Vaccination protocol consists of four administrations within 4 weeks followed by one injection a month for 4 months in combination with dose-intense chemotherapy. In an initial phase, preliminary results showed that the administration of this autologous vaccine is effective in prolonging overall survival and the time to progression in dogs with DLBCL and multicentric indolent B-cell neoplasia, without increasing treatment toxicity (88, 89). Following, to better characterize the safety and efficacy of APAVAC®, and to find the best candidates for immunotherapy, a larger retrospective study was conducted, which included all dogs treated with chemo-immunotherapy to date. Overall, compared to dogs treated with chemotherapy only, dogs receiving the chemo-immunotherapy protocol survived significantly longer, regardless of histotype and evaluated prognostic factors. The study also confirmed the excellent tolerability of the vaccine in dogs diagnosed with B-cell lymphomas (90). Unfortunately, until now there is no information regarding the chemo-immunotherapy treatment response in T-cell lymphoma dogs.

Altogether these works clearly demonstrate the potential of the cNHL model to advance cancer vaccine strategies research to treat lymphoma both in humans and dogs.

Immune Checkpoint Blockade

Immune checkpoint inhibitors, such as those targeting CTLA-4 and the PD-1 (programmed-death 1)/PD-L1 (PD ligand 1) axis, have shown unprecedented and durable clinical effect in a wide range of malignancies and are rapidly transforming the practice of medical oncology in humans (138).

Tumor cells can successfully evade immunosurveillance and progress through different mechanisms, including activation of immune checkpoint pathways that hinder antitumor immune responses. By interrupting co-inhibitory signaling pathways, immune checkpoint inhibitors reestablish antitumor immune responses and promote immune-mediated elimination of malignant cells (139). Hematologic malignancies such as lymphoma are likely targets for this type of treatment. Several clinical trials of checkpoint blockade have been performed in hematological malignancies, with promising preliminary results, suggesting the therapeutic benefit of this approach. These results were specially promising regarding PD-1 blockade in Hodgkin lymphoma (140). To date, there are currently seven approved immune checkpoint inhibitors for the treatment of various cancers in human medicine.

Clinical trials using checkpoint inhibitors for the treatment of cNHL have yet to be conducted. Nevertheless, expression of canine PD-L1 has been reported on a variety of canine tumor types, including mastocytoma, melanoma and renal cell carcinoma (141). A preliminary study suggests that anti-PD-L1 might play a significant role in the treatment of dogs with tumors expressing PD-L1, by demonstrating that treatment of canine tumor infiltrating lymphocytes with this molecule improved interferon-γ production (141). It was recently reported that PD-L1 is elevated in canine B cell lymphomas compared to normal B cells. Tumor cells from

T-cell cNHL and healthy canine patients both showed low to negative expression of PD-1 and PD-L1. In addition, tumor infiltrating lymphocytes from both B-cell and T-cell lymphoma cells presented an increased expression of both PD-1 and PD-L1 expression compared to B and T cells from lymph nodes of healthy animals. In vitro, chemotherapy-resistant canine B-cell and T-cell lymphoma cell lines exhibited increases in both PD-1 and PD-L1 expression, compared to non-chemotherapy selected tumor cells (142). In line with this, a panel of 5 canine PD-1/PD-L1 mAbs were generated and are being studied for in vitro activity in T cell assays (143). Moreover, the immunomodulatory effects of c4G12, a canine-chimerised anti-PD-L1 mAb, were evaluated in vitro, demonstrating significantly enhanced cytokine production and proliferation of dog PBMCs. Then, a pilot clinical study was performed on seven dogs with oral malignant melanoma and two with undifferentiated sarcoma, revealing that this antibody can be a safe and effective treatment option for canine cancers (144).

Importantly, canine CTLA-4 (cytotoxic T-lymphocyte associated protein 4) has also been described and cloned (145). An agonistic recombinant canine CTLA has been efficiently used to promote tolerance in a transplant model (146), suggesting that the mechanism of action of CTLA-4 in dogs is similar to humans and that CTLA-4 checkpoint blockade could represent a novel immunotherapy for canine cancer. Importantly, Tagawa et al. (147) demonstrated an up-regulation expression of CTLA-4 on CD4+ T cells from peripheral blood obtained from dogs with B cell high grade lymphoma. CTLA-4 expression on T cells was also associated with a poor prognosis.

With the development of new checkpoint molecule targeted drugs for dogs, multiple opportunities emerge in which the dog model may provide relevant clinical information, especially regarding the rational combination of immunotherapies, including checkpoint inhibitors.

Discussion

The current landscape of cancer research is facing a profound transformation with the introduction of immune-oncology as the fourth pillar for cancer therapy. Not only have immunotherapies resulted in unprecedented clinical responses, rapid drug development and several first-in-class approvals from the FDA in the past few years, but the advent of such innovative therapies is also revolutionizing treatment paradigms and algorithms in current oncology and hemato-oncology practice (148). As a result, clinical and translational research need to adapt to a rapidly changing scenario to effectively translate novel concepts into sustainable and accessible therapeutic options for cancer patients (149). The complexities and challenges of the new era of immune-oncology strongly emphasize the need to identify new strategies, models and paths to develop fast, successful, and cost-effective therapies (13, 149). The inclusion of a canine model in the drug development path of cancer immunotherapies is being widely recognized as a valid solution to overcome several hurdles associated with conventional preclinical models (150). Dogs with naturally occurring tumors are highly translational models that represent an opportunity to investigate the clinical potential of novel immunotherapies in a comprehensive manner. By complementing murine studies and human clinical trials, dogs allow monitoring the "scaling up" effects of a therapeutic approach that depends on complex interactions between tumor and immune cells, while assessing long-term efficacy and toxicity (15). Taken together, these features may allow the establishment of solid foundations to rapidly translate the results obtained from canine patients to human patient management, with benefits for both species (151).

Importantly, the benefits of these collaborative studies can more easi-

ly translate into clinical success in emerging technologies, such as immune checkpoint inhibitors and CAR T cells therapy, where the rapid pace of its clinical applicability is proving critical challenges. In fact, a lot remains to be understood about patient selection, delivery, and off-target effects of emerging immunotherapy used alone or in combination. While clinicians have learned during the last decades to deal with clinical responses and toxicities related to the use of antibodies in cancer therapy, emerging therapies, such as those mentioned, are much less familiar to oncologists. Therefore, cancer research needs to develop better predictive clinical models to make these emerging immunotherapies universally available to those patients with cancer who need immune intervention in addition to other therapies (152).

However, the implementation of such canine clinical trials is far from being an easy quest. It requires multiple organized efforts to validate the canine model, which still lacks a thorough characterization of the canine immune system and its effector cells and molecules, the evaluation of common tumor epitopes, the development of canine-specific/cross-reactive agents and the establishment of preclinical models for veterinary oncological settings (62, 153, 154). Furthermore, this also requires veterinary scientific community to join forces to implement diagnosis, staging and treatment response assessment optimization and standardization, to perform large and organized clinical trials and to achieve conformity when analyzing data (26).

Regardless of the challenges that implementing immunotherapies for cNHL lymphoma may pose, cNHL treatment is facing a paradigm shift. With several new immunotherapies emerging, it is expected that in the nearby future, immunotherapy will become a valid therapeutic tool, along with chemotherapy, radiotherapy and surgery. Furthermore, these advances also provide an integrated drug discovery platform that maximize interdisciplinary cooperation and leverage commonalities across humans and dogs, for the development of novel immunotherapies against NHL, benefiting both species.

Author Contributions

JD: writing—original draft preparation and visualization. AA: visualization and writing—review and editing. SA, SG, LT, and FA-d-S: writing—reviewing and editing. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by the Portuguese Funding Agency, Fundação para a Ciência e Tecnologia, FCT IP, [SAICT/2017/32085 and Ph.D. fellowship SFRH/BD/131468/2017 to AA]. CIISA has provided support through Project UIDB/CVT/00276/2020, funded by FCT. Gilead Génese has provided support through Project PGG/050/2019.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Acknowledgments

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Abbreviations

ADCC, antibody-dependent cellular cytotoxicity; CAR, chimeric antigen receptor; CAR-T, chimeric antigen receptor T-lymphocytes; CDC, complement dependent cytotoxicity; CDV, canine distemper virus; CHOP, cyclophosphamide, doxorubicin, vincristine and prednisolone; cNHL, canine lymphoma; CTLA-4, cytotoxic T-lymphocyte associated protein 4; DLBCL, diffuse large B-cell lymphoma; EGFR, epidermal growth factor receptor; FDA, US Food and Drug Administration; GM-CSF, Granulocyte-macrophage colony-stimulating factor; HER2, human epidermal growth factor receptor 2; hNHL, human non-Hodgkin lymphoma; HSP, heat shock proteins; HSPPC, immunogenic tumor specific peptides; LMI, large multivalent immunogen; mAbs, monoclonal antibodies; NHL, non-Hodgkin lymphoma; PBMC, peripheral blood mononuclear cells; PD-1, programmed-death 1; PD-L1, PD ligand 1; scFv, single chain variable fragment; TERT, telomerase reverse transcriptase; USDA, United States Department of Agriculture.

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Citation: Dias JNR, André AS, Aguiar SI, Gil S, Tavares L and Aires-da-Silva F (2021) Immunotherapeutic Strategies for Canine Lymphoma: Changing the Odds Against Non-Hodgkin Lymphoma. Front. Vet. Sci. 8:621758. doi: 10.3389/fvets.2021.621758

Received: 26 October 2020; Accepted: 27 July 2021; Published: 26 August 2021.

Edited by: Steven E. Suter, North Carolina State University, United States

Reviewed by: Maria Gärtner, University of Porto, Portugal Jillian M. Richmond, University of Massachusetts Medical School, United States

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Animal models of cancer metastasis to the bone

Modelos animales de metástasis del cáncer al hueso

Palabras clave: metástasis óseas, modelos animales, cáncer de mama, cáncer de próstata, líneas celulares

Keywords: *bone metastases, animal models, breast cancer, prostate cancer, cell lines*

https://www.frontiersin.org/journals/oncology/articles/10.3389/fonc.2023.1165380/full

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La metástasis del cáncer es una causa importante de mortalidad por varios tumores, incluidos los de mama, próstata y glándula tiroides. Dado que el tejido óseo es uno de los sitios más comunes de metástasis, el tratamiento de las metástasis óseas es crucial para la curación del cáncer. Por lo tanto, se deben desarrollar modelos de enfermedad para comprender el proceso de metástasis ósea con el fin de diseñar terapias para ello. Se han desarrollado varios modelos traslacionales de diferentes tumores metastásicos óseos, incluidos modelos animales, modelos de inyección de líneas celulares, modelos de implantes óseos y modelos de xenoinjertos derivados de pacientes. Sin embargo, actualmente no se dispone de un compendio sobre diferentes cánceres metastásicos óseos. Aquí, hemos recopilado varios modelos animales derivados de experimentos actuales sobre metástasis óseas, principalmente con cáncer de mama y próstata, para mejorar el desarrollo de modelos preclínicos y promover el tratamiento de la metástasis ósea.

Cancer metastasis is a major cause of mortality from several tumors, including those of the breast, prostate, and the thyroid gland. Since bone tissue is one of the most common sites of metastasis, the treatment of bone metastases is crucial for the cure of cancer. Hence, disease models must be developed to understand the process of bone metastasis in order to devise therapies for it. Several translational models of different bone metastatic tumors have been developed, including animal models, cell line injection models, bone implant models, and patient-derived xenograft models. However, a compendium on different bone metastatic cancers is currently not available. Here, we have compiled several animal models derived from current experiments on bone metastasis, mostly involving breast and prostate cancer, to improve the development of preclinical models and promote the treatment of bone metastasis.

Introduction

Metastasis is a frequent malignant manifestation of cancer in the mid to late stages of tumor progression. Metastasis to the bone, one of the most common sites, occurs when cancer cells migrate from the original site and invade bone tissue. It indicates adverse prognosis, and can cause severe pain, fractures, impaired mobility, and death. The invasion of cancer cells into target sites involves several stages. Initially, they invade the surroundings of the original site, breaching the vasculature and entering the circulation. Then, depending on molecular signals on cell membranes or in their microenvironment, they invade a particular target organ along their path of circulation (1, 2). Although the precise process has not been elucidated yet, the invasion appears to last many months if not years (3). Once a bulk of invasive cancer cells agglomerate into a mass, metastasis begins. Cancer cells modify the surrounding tissues and vasculature to favor their growth. Cancer treatment often involves a combination of radiation, chemotherapy, and medications to reduce the pain and inflammation.

Breast cancer, one of the most prevalent malignant tumors, exhibits a 40% likelihood to eventually develop bone metastases (4, 5). Bone tissue is the most common target site of breast cancer. Bone metastasis reflects potential skeletal-related events and poor clinical results. To improve the current therapies for bone-metastasized breast cancer, animal models that mimic the human tumor microenvironment have been used in preclinical experiments (6). Prostate cancer is the second most frequently occurring cancer in men.

It preferentially metastasizes to the bone, and presents a worse prognosis at the metastatic stage. Rarely lethal when restricted to its primary site, the 5-year-survival rate of prostate cancer decreases by 29.8% when it metastasizes to the bone, explaining its rank as the fifth leading cause of tumor-related mortality in males (7). Antimetastatic agents need to be urgently developed and the prognosis following bone metastasis must be improved.

Multiple animal models have been used in clinical research to explore the mechanisms and prognosis of tumor metastasis. Translational models have been used to study the advanced stages of tumor metastases, reveal potential protein targets, and develop metastasis-related treatments. However, fully reproducing human bone metastases in animal models is difficult. Nevertheless, by selecting different cell lines, animal strains, and tumor transplantation methods, animal models can be constructed to answer various questions.

In this review, we have discussed the animal models of bone metastasis most commonly used in preclinical experiments and their underlying mechanisms. No single model can represent all the genetic mechanisms of bone metastasis, which requires whole-body organisms. Here, we have compiled a

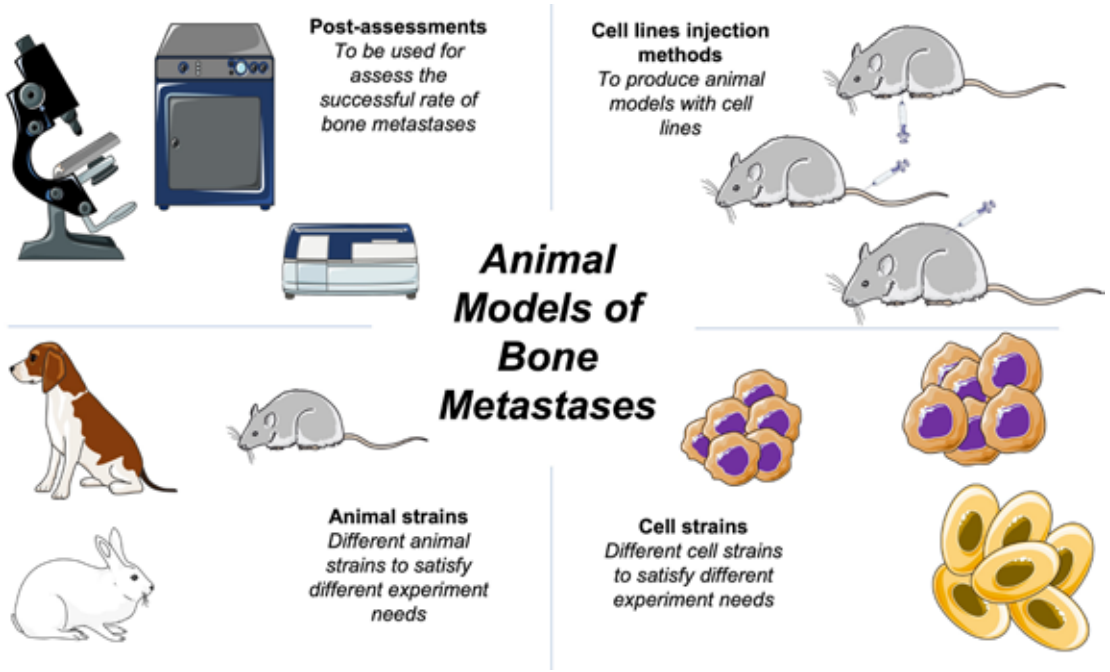

Figure 1 *Schematic of basic bone metastases animal models methods.*

selection of animal models to assist in future studies (**Figure 1**).

Commonly used animals in building animal models

Basing animal models of bone metastasis on general disease models is unreliable. Because the etiology of bone metastasis of human and animal cancers is different, different cancers have different metastatic targets. For example, mouse breast cancer may preferentially metastasize to the lung, while human breast cancer mainly metastasizes to the bone (2). Lung tumors may specifically metastasize to the vertebral column (8, 9). Hence, researchers are required to modify the animal models based on their experiments. The mouse is the most common animal of choice to construct bone metastasis models.

Breast cancer

Animal models based on human breast cancer cells are commonly constructed using rodents, such as mice or rats, and used in preclinical experiments (10). Both immunodeficient and immunocompetent animals are used. Nude mice of the Balb/c background are frequently used because they are

susceptible to both human and rodent breast cancer cell lines (2). Due to the lack of a thymus, immune responses are hardly generated in most of these mice following the injection of cancer cells, which significantly improves the success rate of model construction. Non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice are immunodeficient mice commonly used in xenograft experiments. Disabilities in the immune system of NOD/SCID mice affect the growth of lymph cells as well as immune signaling. Yin's team used NOD/SCID mice paired with the MDA-MB-231 cell line to investigate how runt-related transcription factor 2, an osteogenesis-related factor, promotes breast cancer and bone metastasis (11).

The demand for crossbred or genetically engineered mice has also increased to better meet experimental needs (12–16). Mice that have been crossed and repeatedly backcrossed can offer an in vivo environment better suited to investigate the mechanism of breast cancer bone metastasis (13). In Laura's experiment, Col1a-Krm2 mice were backcrossed with NOD/SCID/IL-2rγnull (NSG) mice for 10 generations to introduce an immunocompromised background (13). They found that cancer metastasis to other organs like the spine may be prevented in rather young animals. By modifying the animal model into adult mice and backcrossing over 10 generations, they could focus on the early stages of human breast cancer metastasis. Devignes' team also backcrossed Floxed mice bred in previous experiments with FVB/n wild-type mice for 10 generations to achieve genetic reconstitution consistent with their experimental requirements. Based on whether the HIF gene was expressed, mice were divided into two groups to verify whether the HIF signaling pathway in osteoblasts could promote breast cancer cell invasion and bone metastasis (14).

Unlike these experiments, Mercatali's team used zebrafish as a special model to study bone metastasis (17). Visualizing zebrafish embryos and easy genetic manipulation provide researchers with a new method of studying cancer progression.

Prostate cancer

The first model of prostate cancer – the Dunning rat – exhibits a spontaneous development of the disease (7). However, this model did not show a tendency for bone metastasis, and R-3327 cells derived from the Dunning rat can only metastasize to the lymph nodes. Dogs are also listed as candidate animal models, but they rarely develop prostate cancer due to the lack of androgen receptors on their cell membranes (7). The internal organization of mice femur includes a high-woven bone structure that is less fibrolamellar in nature, providing conditions amenable for bone metastasis (10, 18).

Transgenic mouse models have the advantage of lacking immune responses to injected cells or xenografts (19). Transgenic adenocarcinoma of the mouse prostate (TRAMP) is one of the most famous transgenic models, exhibiting metastases to the lung and lymph nodes rather than the bone (19, 20). The promoters expressed in neuroendocrine cells, such as the probasin promoter in TRAMP, drive transgenic oncogene expression. NOD/SCID mouse is one of the most used immunodeficient animal models in prostate cancer bone metastasis experiments (21–25). Landgraf created a new model for studying prostate cancer bone metastasis by modifying NSG mice with a humanized tissue-engineered bone construct (hTEBC), which facilitates cancer cell growth (23). Ganguly's team injected PC3 cells into the tibia of 6-week-old NSG mice to explore whether NOTCH3 induces tumor-specific elevation and secretion via MMP-3 (21).

However, the existing models are still limited to some of the detectable cancer-related factors, and cannot provide a comprehensive or linear picture of bone metastasis.

Cancer cell lines

Both patient-derived cancer tissues and immortalized cancer cell lines are used for transplantation. Patient-derived cancer tissues show genetic concordance between the clinic and the animal models, and help to establish consistent animal models specific to particular cancer cell lines. However, these models may face obstacles in the form of ethics and tissue availability. Cell lines, after several passages, can generate stable primary or secondary cancer sites. Moreover, researchers can genetically edit cell lines by using luciferase genes or knocking out certain genes (26–28).

Breast cancer

Immortalized human breast cancer cell lines, such as MDA-MB-231, 4T1, and MCF-7, are more easily available than patient-derived tissues. They possess obvious breast cancer target characteristics, and can also exhibit a tendency for bone metastasis after multiple passages (**Table 1**) (2, 5, 11, 51). They can help restore human bone metastasis in animal models. The bone-homing capabilities of MDA-MB-231 sub-lines can be enhanced via generation injections, and up to 90% of MDA-MB-231-bone cells can form neoplasms (52–54). Using 5–8-week-old mice is vital to achieve bone metastasis via intracardiac, intra-arterial, or intravenous injections. Farhoodi injected 4T1 cells into the mammary fat pad of Balb/c mice, and then examined their legs for bone metastases. Once its incidence was confirmed, the mice were sacrificed to collect the metastatic tumor cells from the leg bones. These cells were cultivated to purify tumor cells with bone-metastatic tendencies (51). They purified their experimental cells to improve the success rate.

Different pairs of cell lines can also be combined to test certain concepts. Yin's team compared MCF-7 and HCC1954 to validate whether KRT13, a protein from the keratin family, promotes stemness, metastasis, and cellular invasiveness (55). Han's group estimated the metastatic rate of different cell lines (56). They found that the proliferation of

Table 1 *Common cancer cell lines in bone metastases.*

Cancer	Cell Lines	Origin	Model System	Metastases Preference
BCa	MDA-MB-231	Human mammary adenocarcinoma from a 51-year-old Caucasian female	Balb/c nude, MF1 nude, NSG	Mouse long bones, spine and jaw (29–34)
	MCF-7	Human mammary adenocarcinoma from a 69-year-old Caucasian female	Balb/c nude, NOD/SCID	Mouse long bones (32–34)
	T47D	Human mammary ductal carcinoma isolated from a pleural effusion	Balb/c nude, NOD/SCID	Mouse long bones (35, 36)
	4T1	Stage IV mammary tumor from a female Balb/c cfC3H mouse	Balb/c cfC3H	Mouse long bones, Spine, jaw, lungs, and spleen (37–40)
PCa	PC3	Bone metastases from a 62-year-old white man	Balb/c nude, NOD/SCID, NSG	Mouse long bones, spine (33, 41–45)
	LNCaP	Supraclavicular lymph node from a 50-year-old white man	Balb/c nude, SCID	Mouse long bones, spine (29, 46–48)
	DU145	Brain metastases from a 69-year-old white man	Balb/c nude, Ncr nu/nu, NOD	Mouse long bones (25, 45, 47, 49, 50)

BCa, breast cancer; PCa, prostate cancer.

MDA-MB-453, UACC-893, and HCC-202 cells increased in the eighth week, while MDA-MB-361, UACC-812, BT-474, and ZR-75-1 cells exhibited moderate proliferation but obvious migration. Using HCC-2218 and HCC1419 cells, tumors did not form, suggesting that both lack the ability to metastasize to the bone. The tumors formed by HCC-202 and MDA-MB-361 cells decreased in size after the sixth week, indicating that these two cell lines may not survive long-term metastasis (56). Eckhardt et al. also tested several cell lines, and NSG mice were used in xenograft studies involving MDA-MB-231 and SUM159 cells (37).

Prostate cancer

Like other cancer cell lines, those of prostate cancer also originate from both humans and animals (**Table 1**). R-3327, derived from the Dunning rat, has been used to investigate human prostate cancer due to its spontaneous neoplasm development (57). Other animal-derived cell lines, such as PA-III or AT6-1, naturally form osteolytic and osteoblastic lesions similar to human bone metastases in animal models (57–59). RM1, derived from the mouse prostate, is a highly metastatic cell line, but does not metastasize to the bone (60). Although it can induce consistent bone lesions in mouse models, it is a transformed cell line, not a natural one. PC3, DU145, and LNCaP are patient-derived cell lines commonly used in prostate cancer animal models. They are easily available and possess the basic prostate cancer cell targets. PC3, derived from the bone metastases of a 62-year-old white man, was selected by isolating highly invasive cells from bone metastatic lesions. Landgraf implanted an hTEBC structure based on the bone-homing properties of PC3 cells, followed by an intracardiac injection of Luc-transfected cancer cells, facilitating the construction of models for transferring the human osteoblast line PC3 to hTEBC and the murine femur (23). Studies on LNCaP, PC3, and DU145 cells, all of which differ in their sensitivity to androgens, showed that prostate cancer-secreted growth differentiation factor 15 modulates the potential for bone remodeling in metastatic bone lesions (49, 61). Lang's team grouped five common prostate cancer cell lines to verify whether PCAT7, a bone metastasis-related long non-coding RNA, activates the transforming growth factor-β/suppressor of mothers against decapentaplegic signaling pathway by upregulating transforming growth factor-β receptor 1. Its negative correlation with miR-324-5p was also investigated (62). Sohn's team tried to intracardiacally inject LNCaP cell lines grouped with CD133+. The overexpression of CD133+ in LNCaP cells enhanced their cancer stem cell-like characteristics in terms of colony formation, migration, etc. The CD133+ group exhibited a bone metastasis rate of 80%, compared with 20% in the Vec group. Moreover, the CD133+ group showed a significant violation of the diffuse osteolytic characteristics of the spinal cord and the vertebral bodies (29).

Preparation of cell lines for transplantation

Orthotopic inoculation of cells

In situ injection of cancer cells best reproduces the process of cancer metastasis in the human body. Injected into mouse mammary fat pads, tumor cells can be seeded through the vasculature towards the target organs – a method that achieves 40–60% of bone metastases in breast cancer animal models (63). To study the function of TIE2, a tyrosine kinase receptor, in osteolytic bone metastasis, Drescher's team administered both bilateral mammary fat pad injections and left ventricular injections to the grouped mice. The correlation between carcinoma in situ and bone metastasis was evaluated to determine whether TIE2 inhibition stimulates the dormant breast cancer cells and promotes bone metastasis (34). Likewise, Spadazzi's team injected MCF-7 cells into the left ventricle and

mammary fat pads of NSG mice to investigate whether trefoil factor-1 could exert estrogen-induced effects (64).

However, this method suffers from a considerable variation in metastatic tumor growth, besides the comorbidity caused by development of the tumor (Table 2) (73). In addition, it poses the problem of small bone metastases while the primary tumor has grown beyond an ethically reasonable size (5), which seriously compromises the detection of stimulated bone metastases.

Some scientists have also suggested subcutaneous allografts to model bone metastasis. Peiffer's team provided a detailed protocol of resecting subcutaneous prostate cancer allografts from immunocompetent mice (65). Bone metastases, abdominal cavity metastases, and local invasion all occurred in eight mice. This study demonstrated that resection of subcutaneous allografts from mice can lead to the development of metastasis; however, the duration of the experiment was extended by the removal of the prostate gland and precise operations.

Intravascular injection

Intravascular injection is a way of inoculating cells into the blood circulation. Unlike in orthotopic or ectopic inoculation, tumor cells injected via this method can localize to the target site through the intravascular circulation (**Table 2**) (66). Intra-arterial injections are usually administered to the left ventricle, limiting the clearance of cells that occurs when they pass through the lung capillaries (10, 53, 67). Tail vein injection, which is the more common intravenous injection today, effectively increases the rate of bone metastasis while also increasing the rate of mortality in mice (51).

Animal models currently rely on intracardiac injections to realize the process of bone metastasis. Tumor cells are injected into the circulation through the left ventricle of mice, after which they go through the processes of adhesion, degradation, and migration to finally cause metastases in different organs, thereby simulating the process of bloodway metastasis of tumors. Using intracardiac injections to probe the role of cancer-associated factors in the regulation of tumor bone metastasis has become the preferred modeling approach (44–46). Zheng et al. used this method to prove that osteoblastic Niche-derived Jagged1 sensitizes bone metastases (15). Wang's team showed that the bone sialoprotein–αvβ3 integrin axis functioned significantly more efficiently in cancer cell bone metastasis when integrin was overexpressed. For comparison, stained specimens of the brain, lung, tibia, and femur were collected after left ventricular injection in nude mice (52). Although the postop-

Cell Injection Methods	Module of metastases studied	Advantages	Disadvantages
Orthotopic Inoculation	Primary tumor and invasively distant metastases	Study of tumor growth *in situ* and distant metastases	Unstable bone metastasis success rate (65–67)
Intracardiac	Circulation and metastases	Easily producing metastases	Requiring sophisticated skills (68–70)
Caudal Vessels	Circulation and metastases	More visualization of circulation inoculation	Potential lung metastases (7, 24, 51)
Intraosseous	Bone metastases	Most convenient and successful method for bone metastases models	Not reflecting the complete course of tumor metastasis (71)
Allografts/Xenografts	Depend on location	Reflecting natural heritability and cellular heterogeneity	Usually requiring immunodeficient mice and high maintenance (23, 72)

Table 2 *Implantation methods for bone metastases models.*

erative mortality is relatively high, the survival rate can still exceed 90% with practice.

Caudal vessel injection can produce a higher rate of metastasis to the leg bone than to other vital organs. This method offers better accuracy than intracardiac injection because the visibility of tail vessels enables researchers to observe the flow of cancer cell fluids within (74). Caudal vascular injections can either be intravenous or arterial. Injecting through the tail artery will reduce the elimination of tumor cells in pulmonary capillaries and improve the success rate of colonization to the bone, while tail vein injection will promote tumor metastasis to the lung (2, 51, 74). In Farhoodi's experiments, the 4T1 cell model tail artery injection mice showed a significant number of tumor cells localized to the subinguinal fat pad and the leg bone (51). Tumor cells were found in the leg bones of all 32 mice injected through the tail artery, and the rate of bone metastasis following complete tail veil injection was greater than 90% as well. Metastases were also detected in 70% of other target locations 2 weeks post-injection. Hamaidi et al. determined the effect of Lim1 on the adhesion, epithelial–mesenchymal transition, invasion, and metastatic progression of cancer cell surface targets after injection of the renal carcinoma cell line Caki2/786 through the lateral caudal vein of nude mice (75). However, caudal vein injection also resulted in metastatic foci in the lungs of mice.

Multiple factors affect the success of experiments involving vascular injection. Operator skill gaps, standard cell operation procedures, and pressure within the caudal vessels can all influence the growth rate and success of tumor bone metastasis (51). Dilation of the caudal vessels prior to injection or the use of fluorescein to reveal vessel flow can improve the effectiveness of the injection. Non-directed intracardiac injection is still associated with a risk of thrombosis due to the procoagulant activity of tumor cells after accurate completion. The mortality of post-inoculation animal models may be reduced by injecting low-molecular weight heparin into the tail vein 10 minutes before inoculation (76).

Intraosseous injection

Metastatic tumors can bypass the pre-metastatic process if they are directly ectopically implanted into the bone. The growth of tumor cells inside the bone depends on their interaction with bone cells and the bone microenvironment (Table 2) (77, 78). Therefore, while intraosseous injection can help examine local tumor behavior within the bone microenvironment, it cannot be used to study the early stages of bone metastasis (79). Researchers typically inject 50,000–100,000 cancer cells directly into the tibia or femurs of mice, avoiding the possible comorbidity of the animals' primary tumor (80, 81). Chen et al. observed that Brachyury, one gene affects tail length in mice, was expressed at a low level in the highly metastatic MDA-MB-231 cell line while it was highly expressed in the poorly metastatic T47D cell line when breast cancer cells were injected into the top anterior condylar region of the right tibia of mice. Nude mice showed significant swelling at the injection site 4 weeks post-injection, and X-ray revealed tumor-induced osteolytic lesions (35). After injecting prostate cancer cells into the left tibia of Balb/c nude mice, Thulin's team performed bone tumor development status assays using peripheral quantitative computed tomography (CT) and microCT to investigate the effect of signal transducer and activator of transcription 3 (STAT3) inhibitors on STAT3-regulated prostate cancer bone metastasis. The STAT3 inhibitor treatment resulted in an intact tibial bone microenvironment with no tumor formation or sclerotic response in mice, whereas the VCaP group showed sclerotic bone tumor response up to 85% (48).

Allograft and xenograft models

Transplanting allogeneic or xenogeneic tissues into animal models is a com-

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mon way of modeling bone metastasis (Table 2). Since animals with different genetic backgrounds respond to allogeneic tissues differently, selecting the appropriate tissue source is especially important. In the case of xenografts, patient-derived tumor tissues can better reflect the biological characteristics of tumor bone metastasis in humans (82). Patient-derived xenografts aim to directly transplant human tumor tissue into immunodeficient mice, which represents natural heritability and cellular heterogeneity in human cancer better than simple cell-transplantation models (83). Among animal models, xenografts can only be performed in immunocompromised or immunodeficient animals. Aoki et al. first grew tumor tissue from bone metastases by intraperitoneally injecting it into male thymus-free nu/nu nude mice (42). The tumors were surgically processed to 1-mm3 fragments to be implanted into the proximal left tibia of the nude mice when they reached 10 mm in diameter. They observed tumor growth in all eight mice. Landgraf's hTEBC model is likewise based on the low immune response of NSG mice to xenografts, while adding humanized components to mimic human tumor bone metastasis as satisfyingly as possible in mice (23).

Assessment of animal models of bone metastasis

After injecting cancer cells into mice, bone lesions develop quickly, necessitating researchers to detect physiological conditions, bone changes, and tumor lesions in a timely manner.

Establishing bone metastasis models using luciferase or fluorescent protein-labeled cell lines allows researchers to monitor tumor development in the bones of living animals (15, 39–41). Oliemuller et al. studied the effects of SOX11 on cell invasion and bone metastasis using DCIS-Luc cells, generated by transducing the cells with luciferase 2 lentiviral particles (84). Arriaga's team bred NPKEYFP mice by crossing NPK mice with the Rosa-CAG-LSL-EYFP-WPRE reporter allele, facilitating in vivo fluorescence visualization and quantification of YFP-positive prostate tumors and metastases (85).

In turn, instrumentation such as the IVIS system can provide more accurate quantitative indicators through fluorescent or bioluminescent readings obtained from tumors (76–78). Typically, tumor growth in the bone is measured once or twice a week. The area of osteolytic lesions and abnormal bone remodeling can be assessed visually by X-ray or in vivo microCT (45–47, 85). Hinz's team then used the IVIS system. After injecting MDA-MB-231 cells into the left ventricle of NSG mice, they performed IVIS bioluminescence assays weekly to assess osteolytic lesions caused by bone metastasis from triple-negative breast cancer. The inoculation of AKT3-knockout 231-BO cells into NSG mice resulted in enhanced bone metastases (86). Another team validated the effect of intracardiacally injecting MDA-MB-231-derived osteotropic cells into nude mice by examining osteolytic lesions in their hind tibia and femurs by microCT. MicroCT images showed that NKX2-8-silenced cell lines were more likely to produce earlier bone metastases, while its overexpression delayed the appearance of metastases, inhibited osteoclast activity, and reduced bone metastatic lesions (87).

At the end of the animal test, the mice should be examined simultaneously for extraosseous metastases. All relevant organs and metastases are fixed in 10% formalin for analysis. For histological studies, samples are fixed in paraformaldehyde for 24–48 hours and then decalcified in paraformaldehyde/ethylenediaminetetraacetic acid solution for 2 weeks. The decalcified paraffin-embedded bone should be sectioned for hematoxylin and eosin staining and evaluated using image analysis software. Bone conversion-related growth factors in the serum can also be assayed (88, 89). Metastases from the lung, liver, and brain tissue

can likewise be analyzed and studies investigating the correlation between the area and the number of bone metastases can be performed (90).

Conclusion

Bone metastasis is a common manifestation of cancer deterioration in the mid and late stages of the disease. Much research has been done on the invasion of cancer cells, from migration to the bone tissue and beyond; however, much needs to be understood yet. Animal models are vital tools in preclinical metastatic experiments that can help identify the key steps in bone metastasis. Here, we have summarized the experimental animals, cell lines, cell implantation techniques, and evaluation methods used while studying common breast and prostate cancer bone metastases. For preclinical animal testing, immunodeficient animals are used to achieve xenograft growth without eliciting a host immune response. In preclinical studies, many investigators have successfully improved the success of tumor cell colonization to the bone by backcrossing cell lines and transgenic mice. More importantly, most animal tests related to cancer bone metastasis have been performed using cancer cell line injection models. Although the early stages of bone metastasis cannot be studied, these models are effective for studying the interaction between cancer cells and the bone microenvironment.

However, using mice to study human tumor immunity has its limitations. The differences in bone metastasis pathways between humans and animal models can explain why the success of preclinical treatments is not perfectly reproduced in humans. The inability to present a complete and comprehensive picture of the whole process of bone metastasis is also a problem that needs to be addressed while engineering animal models today.

Author contributions

All authors contributed equally to this work. All authors contributed to the article and approved the submitted version.

Funding

National Natural Science Foundation of China.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Keywords: bone metastases, animal models, breast cancer, prostate cancer, cell lines

Citation: Yu Y, Li K, Peng Y, Wu W, Chen F, Shao Z and Zhang Z (2023) Animal models of cancer metastasis to the bone. Front. Oncol. 13:1165380. doi: 10.3389/fonc.2023.1165380

Received: 14 February 2023; Accepted: 10 March 2023;Published: 05 April 2023.

Edited by:Wenwen Zhang, Nanjing Medical University, China

Reviewed by:Qiuyu Liu, Henan Provincial People's Hospital, ChinaYanping Yang, Shanghai University of Traditional Chinese Medicine, China

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Tabla de contenido:

ROYAL CANIN® Urinary es una gama diseñada para el manejo nutricional de los problemas del tracto urinario inferior

Incluye dietas con enfoques nutricionales diferentes en función del tipo de cálculo.

La presentación húmeda es especialmente interesante, ya que su alto contenido de humedad favorece la dilución urinaria y es la primera elección en caso de cistitis idiopática felina.

Gemma Baciero

Veterinaria, Acre. GTNC AVEPA.
Comunicación Científica Royal Canin.

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¿Cómo definirías la gama Urinary de Royal Canin?

ROYAL CANIN® Urinary es una gama formulada específicamente para el manejo dietético de las patologías de tracto urinario inferior en el gato y el perro, como urolitiasis y cistitis.

Incluye dietas con enfoques nutricionales diferentes en función del tipo de cálculo. Por un lado, para estruvita y oxalato, las urolitiasis más comunes tanto para gatos como perros, están las dietas Urinary S/O que provocan una baja sobresaturación relativa (SSR) para ambos, y que se caracterizan por su capacidad de disolver la estruvita, su efecto acidificante de la orina y su aporte controlado de los precursores como el magnesio. Al mismo tiempo, favorece la dilución urinaria y es indicado para ayudar a reducir la reaparición de los cálculos de oxalato, que no se pueden disolver.

Por otro lado, está la dieta Urinary UC, con un enfoque totalmente diferente, alcalinizante, con un contenido controlado de proteína y seleccionada por dejar menos residuos purínicos, indicada en el caso de los llamados cálculos metabólicos: urato, cistina y xantina.

¿Hay mucha diferencia entre los productos para perro y los productos para gato?

La estrategia nutricional de las dietas Urinary S/O es la misma para gatos y perros: baja SSR, dilución urinaria, efecto acidificante y control de precursores. La diferencia está en que en cada caso están ajustadas a las necesidades de cada especie.

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¿En qué formatos se presenta? ¿Qué opciones hay disponibles?

La gama Urinary se presenta en formato seco y húmedo, disponible este último en diferentes texturas: bocaditos en salsa para gatos, en finas láminas en salsa para perros y paté para ambos, con el fin de satisfacer las distintas preferencias.

En el caso de las patologías de tracto urinario inferior, la presentación húmeda es especialmente interesante, ya que su alto contenido de humedad favorece la dilución urinaria y es la primera elección en caso de cistitis idiopática felina.

Además, es importante destacar que dentro de las dietas Urinary S/O disponemos de distintas opciones para dar una respuesta más precisa según el caso: si se trata de perros pequeños (de menos de 10kg), Urinary S/O Small Dogs; para perros mayores Urinary S/O Ageing 7+, especialmente adaptado a los cambios de las necesidades asociados a la edad y para aquellos gatos o perros con tendencia a engordar o un ligero sobrepeso, Urinary S/O Moderate Calorie.

¿Qué tipo de patologías podemos abordar con el uso de la gama Urinary?

La gama Urinary nos permite ofrecer un manejo nutricional específico en el caso de cálculos de estruvita y oxalato con las dietas Urinary S/O, también indicada como apoyo en casos de cistitis bacterianas en el perro y cistitis idiopática felina. La dieta Urinary UC es la opción para los cálculos de urato, cistina y xantina.

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¿Cuáles son los tipos de cálculos más frecuentes en el perro y en el gato?

En ambas especies, las urolitiasis más comunes son las de estruvita y oxalato, seguidas de lejos por las de urato.

Aunque los datos pueden variar según el estudio que se consulte, los cálculos de estruvita en el perro suponen en torno al 35% en perros y el 40% en gatos y los de oxalato, aproximadamente un 45% y un 50% respectivamente. Los de urato son los siguientes, pero mucho menos frecuentes, con cerca de un 3% y un 4%.

¿Existe algún tipo de variabilidad dependiendo de la edad del animal? ¿Y de la raza? Nos referimos a si hay razas más predispuestas que otras.

Existen factores de riesgo de aparición de cálculos urinarios como la edad, la raza y el sexo, que dependen de cada tipo de urolito. En general, sabemos que los perros de razas pequeñas son más predispuestos a la formación de cálculos, que en las perras es más frecuente la estruvita porque presentan más infecciones urinarias y que en cuanto a los cálculos de urato, los más propensos son los Dálmata.

¿Qué otro tipo de cuidados serían complementarios para nuestro animal mientras usamos la gama Urinary? ¿Recomendáis una visita periódica al veterinario? ¿Cada cuánto tiempo?

Cuando se inicia el manejo nutricional con las dietas Urinary S/O es muy importante que la dieta sea el único alimento que reciba el animal, ya que es la manera en que la dieta puede ser eficaz. Si se mezcla con otros productos o se le añaden alimentos como comida de la mesa, trocitos de carne u otros complementos, la dieta perderá efectividad.

Si se quiere dar algo más que el alimento seco, siempre se puede combinar con la versión equivalente de la dieta en presentación húmeda, que además de mantener el mismo enfoque nutricional, aporta gran cantidad de agua que favorece la dilución urinaria.

Por supuesto, siempre tiene que haber un seguimiento por parte del veterinario, mientras se está intentando la disolución de los cálculos y también después para controlar que no reaparezcan de nuevo, detectando los primeros signos lo antes posible. Según el caso el veterinario indicará la frecuencia de las visitas

Por último, ¿dónde podemos encontrar esta línea de productos?

ROYAL CANIN® Urinary S/O solo debe utilizarse bajo prescripción del veterinario, por lo que podrán encontrarse en clínicas veterinarias.

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TODOS LOS PROBLEMAS URINARIOS MERECEN SER SOLUCIONADOS

La enfermedad del tracto urinario inferior abarca una gran variedad de afecciones, se manifiesta a través de diversos signos y puede estar causada por múltiples problemas o la comorbilidad de diversas patologías.

Gracias a más de 50 años de ciencia, a una observación meticulosa y a la colaboración con veterinarios, sabemos que una nutrición especializada puede tener un papel fundamental en la recuperación de pacientes con problemas urinarios, así como en la salud general de los animales.

Por eso, disponemos de una amplia gama de soluciones nutricionales a medida para los problemas específicos del sistema urinario, ahora con innovaciones de última generación.

URINARY

Animal models in osteosarcoma

Modelos animales en osteosarcoma

Palabras clave: osteosarcoma, modelos condicionales de ratón, modelos de ratón de línea germinal, modelos animales, p53, RB

Keywords: *osteosarcoma, conditional mouse models, germ-line mouse models, animal models, p53, RB*

https://www.frontiersin.org/journals/oncology/articles/10.3389/fonc.2014.00189/full

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El osteosarcoma (SG) es el tumor óseo primario no hematológico más frecuente en niños y adultos. La quimioterapia citotóxica en dosis altas y la resección quirúrgica han mejorado el pronóstico, con una supervivencia a largo plazo para la enfermedad no metastásica cercana al 70%. Sin embargo, la mayoría de los tumores de SG son de alto grado y tienden a desarrollar rápidamente metástasis pulmonares. A pesar de los avances clínicos, los pacientes con enfermedad metastásica o recaída tienen un mal pronóstico. Para una mejor comprensión de la patogénesis molecular de la SG humana, se han desarrollado varios modelos de ratón con SG modificados genéticamente y se revisarán aquí.

Osteosarcoma (OS) is the most common non-hematologic primary tumor of bone in children and adults. High-dose cytotoxic chemotherapy and surgical resection have improved prognosis, with long-term survival for non-metastatic disease approaching 70%. However, most OS tumors are high grade and tend to rapidly develop pulmonary metastases. Despite clinical advances, patients with metastatic disease or relapse have a poor prognosis. Toward a better understanding of the molecular pathogenesis of human OS, several genetically modified OS mouse models have been developed and will be reviewed here. However, better animal models that more accurately recapitulate the natural progression of the disease are needed for the development of improved prognostic and diagnostic markers as well as targeted therapies for both primary and metastatic OS.

Introduction

Osteosarcoma (OS) is a highly malignant form of bone cancer characterized by osteoid production. Although OS comprises <1% of cancers diagnosed in the United States, it is the most common primary malignancy of the bone (1, 2). It occurs predominantly after the first decade of life during periods of skeletal growth, with a second peak incidence in the geriatric patient population (1, 3). The vast majority of OS in children, adolescents, and young adults is high grade and begins in the intramedullary space of metaphyseal locations in long bones of the lower extremity. This suggests a relationship with active growth plates. After a low incidence in individuals between 25 and 59 years of age, the incidence of OS rises again in individuals over 60 years of age, and is most often associated with Paget's disease or radiation exposure (1, 2). This may suggest that the underlying pathogenesis is not identical in young and older patients. Conventional OS presents in three major subtypes based on histological classification: osteoblastic, fibroblastic, and chondroblastic. Osteoblastic is the most common (around 60%) with fibroblastic and chondroblastic being equally represented (4).

Osteosarcoma is characterized by a local invasion of bone and soft tissue, loss of the function of the affected extremity, and distant metastasis, most often to the lung (90%). Metastases are also found in bone (8–10%) and rarely in lymph nodes (5). Treatment involves aggressive removal of the primary tumor to afford local control via limb sparing surgery or amputation. Systemic chemotherapy (both prior to and after tumor removal) is used to suppress development of metastasis and effect cure. The most common chemotherapy

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regimens comprise the drugs, cisplatin, doxorubicin, and high-dose methotrexate in combination (6–8). Although chemotherapy slows tumor growth, it can induce cardiomyopathy, hearing loss, and risk of secondary malignancy (8, 9). In patients without metastases at the time of diagnosis (80–90%), surgical treatment in combination with chemotherapy has resulted in long-term survival rates that approach 70%. In contrast, for patients with established metastases there is currently no reliable therapeutic option to provide long-term tumor control. Despite intensive efforts to improve both chemotherapeutics and surgical management, 40% of all OS patients succumb to the disease. Specifically, the clinical outcome for metastatic OS remains poor; fewer than 30% of patients who present metastases survive 5 years after initial diagnosis. Therefore, there is an urgent need for the development of novel therapeutics for OS agents with increased capacity to eliminate systemic tumor burden as well as reduced toxicity in healthy tissues.

Etiology of OS

Osteosarcoma is characterized by a complex karyotype and a lack of recurrent translocations. Genetic approaches have identified several genes of potential importance in the development and progression of the disease (10–12). However, the widespread chromosomal alterations of the OS genome have limited the interpretation of these findings. Genetic alterations of OS are usually sporadic though genetic predisposition has been documented in patients with Li-Fraumeni and retinoblastoma syndrome. Somatic deletions and point mutations in P53 occur in approximately 50% of human OS (13–16) and half of those mutations are associated with loss of the remaining allele (14). Additionally, almost 70% of OS have at least one RB allele alteration (17, 18). Homozygous deletions of RB are seen in 23% of tumors, while point mutations appear in 6% (18, 19). In addition, numerous alterations that disrupt the RB pathway have also been reported; for example, the loss of function at the INK4a/ARF locus and the amplification of CDK4 have been found to occur (one or the other) in 22% of OS (20–22). The prevalence of these alterations would suggest that the deregulation of both G1/S and G2/M checkpoint in the cell cycle are a common event in OS.

For this, a tumor of unknown origin, chaotic genetics, early onset, and aggressive behavior, there is a need for more representative models to learn more about the biology of OS.

Animal Models in OS

Animal models hold significant promise in increasing our understanding of the genetic basis of OS and more importantly, in advancing preclinical studies aimed to the rational development of new therapeutic approaches as well as their validation prior to clinical trials.

In order for any animal model of human disease to be useful and informative, it is preferable to accurately recapitulate the natural course of the disease. Unfortunately, the etiology and pathogenesis of OS are not completely understood; therefore, the establishment and induction of representative experimental models are challenging and incomplete. Currently, there is not a robust animal model of OS that fully represents its biological and clinical features. The ideal would be one in which there was a naturally occurring primary bone lesion and spontaneous pulmonary metastases. To date, the major species used to generate OS models are mouse and rat; however, OS arising in dogs is also of note as a validated model of spontaneous OS.

Many aspects of the biology of the disease have been determined from a variety of animal model approaches. Genetically modified mouse models of OS have given the field much insight. However, spontaneous OS, secondary OS as a consequence of animals receiving

radiation, human and murine OS cell lines, and xenotransplantation studies are also important to understand the biology of this malignancy.

Canine Models

Spontaneous OS is much more common in large dogs than in humans, making the dog an attractive candidate model to study human disease (23). Canine OS is indistinguishable from human tumors at the histological and gene expression levels (24–27). The primary differences between the two are the age of development and the prevalence of the disease. In dogs, OS is a disease of older, large breed dogs (6–12 years of age), and it is estimated that over 10,000 cases occur annually in the United States. The median disease-free interval following surgery alone is 4 months, and after surgery with chemotherapy, 13 months. This high prevalence and the relatively rapid rate of disease progression provide the opportunity to model metastasis development and progression and evaluate novel treatment options in a relatively short period of time (28–32). Many of the genes involved in human OS pathogenesis appear to participate in canine OS, including P53, RB, and PTEN (33–36).

Although canine OS serves as an excellent comparative tumor model for human OS, there are some limitations to be considered. First, OS affects skeletally mature, geriatric dogs, which is different from humans where the peak of incidence occurs during adolescence. Second, some breeds have specific heritable germ-line mutations in certain genes that may influence OS biology, progression, and response to treatment without driving the initiation of the disease (37).

Secondary OS after Radiation

The development of rodent OS models began with the exposure of rats and mice to chemical and radioactive carcinogens (38–40). Of note, among those was the development of OS in rats treated with P32-orthophosphate, which resulted in a high incidence (41). These models yielded tumors that histologically resembled the human cancer and produced cell lines that complement human OS studies (42). Despite the high penetrance of the models, their relevance remains unclear since the majority of OS in humans is sporadic, while the carcinogen-induced murine model is more representative of a therapy induced disease.

Xenotransplantation Studies

There is a significant amount of literature related to the development and use of xenograft and allograft models of human and murine OS cells injected into immunocompromised mice. The injected cells form a solid tumor locally grown within days or weeks after implantation (42, 43). The use of these systems has become a prominent tool in current oncological research due to the quick onset, its affordable cost, and ease of handling and maintenance. In addition, OS donor-derived cells may metastasize to the lungs, providing an opportunity to investigate primary and secondary tumor growth. The principal limitation is that the approach uses fully developed OS cells and therefore does not provide information about the initiation of the tumor and its etiology. Furthermore, since the tumor microenvironment can contribute significantly to the tumor behavior, such interactions may be lost when establishing the disease by direct introduction into a recipient animal (44–46). In certain circumstances, the injected cell line may not be metastatic in the rodent context, making it impossible to study the dissemination of the disease. Despite these limitations, many groups have successfully used this model to identify factors involved in OS migration (47, 48) and more importantly for screening drugs with tumoricidal potential (49). Distinct advantages of the

subcutaneous cell suspension injection model are high rate of incidence and reproducibility that allows for accurate titration of cell numbers in the inoculum to quantify tumorigenic potential of the injected cells.

A variation of injecting cell suspensions into recipient animals is to transplant pieces of tumor directly harvested from the patient. The advantage is that the human malignant cells can grow in its native environment maintaining the heterogeneity that may be required for their proliferation, which in some reports has been shown to enhance tumor growth and metastasis. With the use of cell suspension and transplants, murine host cells can infiltrate the tumor, possibly influencing the activities of the tumor cells, and in some cases, cells of the rodent host can overgrow the human cell population (50). Alternatively orthotopic, intratibial implantation of OS cells has been shown to induce OS at local and metastatic sites (proximal tibia and lung) (43, 51–53). This approach allows the study of primary tumor formation within a more native context as well as the early stages of metastatic progression of OS, thereby reconstituting the entire metastatic process. Its use, however, is limited by a lack of reproducibility due in part to the technical skill required to perform the implantation and the associated lack of quantifiable inoculum.

Genetically Engineered Mouse Models

Of the sarcomas with complex karyotypes, OS is one of the most well-studied as exemplified by the development of numerous mouse models available for this disease. The ability to alter specifically the expression of individual genes (by loss or gain of function) became available in the mouse with the evolution of gene targeting technologies (54, 55).

Many murine OS models have been developed to recapitulate the P53 and RB mutations in hereditary and sporadic human OS. Germ-line deletion of P53 resulted in an OS incidence of 4% in homozygous P53 null mice (56) and 25% in heterozygous P53 mice (57), underlying the importance of altered P53 in driving OS. This unexpected ratio of tumor formation, though, is likely due to the early lethality seen in the homozygous null population. Further, the rapid development, the higher incidence of other tumors (mostly lymphomas), and the long latency of OS (58) necessitate the sacrifice of the mice before OS onset, hampering in many cases the utility of these models. The role of P53 was further highlighted by tumor analysis of P53 knock-in mice containing a mutant copy of P53R172H (corresponding to the R175H hot-spot mutation in humans) that not only develop primary tumors but also metastasize to the lungs as well as other organs (59, 60). Conversely, mice with germ-line deletions of Rb did not develop OS: homologous deletion of Rb is embryonic lethal and the heterozygotes are not predisposed to OS (61, 62).

The application of conditional gene regulation and the availability of tissue specific Cre expressing mouse lines (63) have greatly enhanced our ability to generate specific models of mesenchymal osteogenic lineage that more faithfully resemble human OS (55, 64). The majority of these models have used the loss of P53 with or without the disruption of the Rb pathway to generate penetrant OS models (54). They use conditional gene deletion approaches restricted to multipotent mesenchymal progenitors, early committed osteoblasts (pre-osteoblasts) and the osteoblast population (**Figure 1**) (**Table 1**).

Using Cre recombinase activated by the gene promoter of Paired related homebox 1 (Prx1-Cre) (72) that deletes LoxP flanked alleles in the early limb mesenchyme (multipotential cells), 22% of mice with P53-mediated heterozygosity developed OS. Not surprisingly, homozygous deletion of P53 had a threefold increase in OS incidence over the heterozygous animals. In contrast, the deletion of Rb in the mesenchymal Prx expressing progenitors did not produce any OS tumors (65, 66). Interestingly,

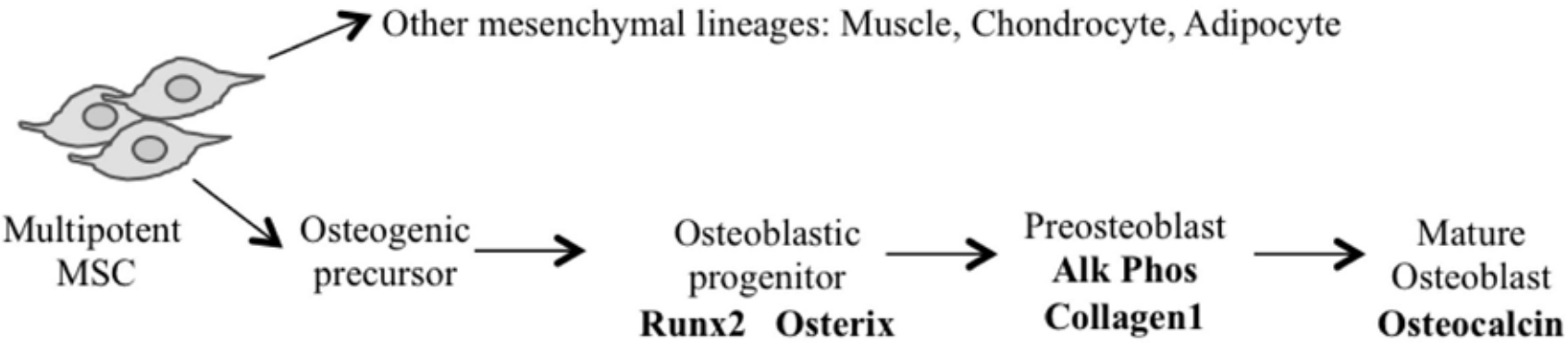

Figure 1. *Model of osteoblast differentiation and putative stage of Cre expression is shown.*

the highest incidence (92%) of OS occurred with the combined deletion of one allele of Rb with homozygous P53 deletion (66). Homozygous deletion of both genes resulted in more non-specific tumor formation with only 18% OS tumors and the remainder being poorly differentiated soft tissue sarcomas (PD-STS) and lymphoma (65, 66).

For a more restricted deletion of genes in the osteoblast lineage, promoters of genes ranging from those expressed early in the commitment of progenitors as Osterix 1 and Collagen1 α1–3.6 to those expressed in more lineage-restricted osteoblast precursors such as Collagen1 α1–2.3 and osteocalcin (Og2) have been used. Development of OS with a penetrance of 100% (67, 68) has been observed following osteoblast specific deletion of P53 using Osterix-mediated Cre expression (Osx-Cre) (73). As with mesenchymal progenitors, Rb deletions have no effect and combined deletion of Rb and P53 in osteoblasts once again generated fibroblastic or undifferentiated OS with high penetrance (100%) (67, 68). Potential translational utility is the existence of short-latency spontaneous metastatic OS similar to human tumors in which cells are arrested in their differentiation (67, 68). Although the greatest proportion of tumors was OS when P53 was conditionally deleted, neuroendocrine tumors and hibernomas were also reported to be generated in several mice (67, 68). However, Walkley et al. enriched the C57BL/6 background of the mouse strain and the percentage of hibernomas was reduced, suggesting a possible impact of mouse strains in the phenotype observed (69). A recent study in mice that expressed SV40 T/t antigen (Tag) in mature osteoblasts under the Og2 (74) showed OS with complete penetrance (71) and 90% incidence of lung metastases. Further analysis of the tumors derived from this model revealed a recurrent genomic deletion of the Prkar1a gene in a specific subset also in human OS. Transgenic shRNA has been used to specifically knock down P53 (rather than delete) using the Osx-Cre transgene (69). These mice develop osteoblastic OS with a 100% penetrance, and although they have a longer latency to tumor onset, they more often develop in long bones and are highly metastatic (lung and liver), features similar to human OS. This model has not developed any non-OS tumors.

Independent of the stage of development in which Cre becomes active, the latency of OS is essentially the same when comparing either P53 alone or in combination with Rb. The use of Cre in more primitive cells (Prx), however, leads to the development of tumors of other mesenchymal lineages at higher frequency.

Cell	Cre	Gene	OS penetrance (%)	Other tumors	Metastatic disease
MSC/skeletal	*Prx-1*	$p53^{fl/+}$	22 (65)		
Progenitors		$p53^{fl/fl}$	61 (65); 62(66)	PDS (32%), LY (3%), LPS (3%); RMS (15%), PDS (12%)	Yes (24%)
		$p53^{fl/fl}$-$Rb^{fl/+}$	92 (66)	RMS (9%), PDS (18%), HIB (4%)	
		$p53^{fl/fl}$-$Rb^{fl/fl}$	18 (65); 29 (66)	PDS (57%), LY (14%); RMS (12%), PDS (3%), HIB (91%)	
Pre-osteoblasts	*Osx*	$p53^{fl/fl}$	100 (67); 100 (68)		Yes (32%); yes (40%)
		$p53^{fl/fl}$-$Rb^{fl/+}$	53 (67); 100 (68)		
		$p53^{fl/fl}$-$Rb^{fl/fl}$	72 (67); 100 (68)	Multiple tumors per animal; concurrent HIB (20–25%)	Yes (37%)
		shp53	100 (69)	0%	Yes (83.33%)
		shp53-$Rb^{fl/+}$	100 (69)	0%	Yes (58.82%)
		shp53-$Rb^{fl/fl}$	100 (69)	0%	Yes (85.72%)
	Col1α 1–3.6	$p53^{fl/fl}$	60 (70)		
Osteoblasts	*Col1α 1–2.3*	$p53^{fl/fl}$	85 (65)		
	Og2	SV40 Tag	100 (71)		Yes (90%)

LPS, liposarcoma; LY, lymphoma; RMS, rhabdomyosarcoma; PDS, poorly differentiated sarcoma; HIB, hibernomas.

Table 1. *Summary of genetically modified OS murine models.*

Possibly providing insight into the initiating events of OS (70), a prominent cellular feature of conditional inactivation of P53 in osteoblastic progenitors is the hyperproliferation of osteoblasts prior to tumor formation. Rb has been proposed to have a role in influencing late osteoblast differentiation by interacting with Runx2 (75). However, a number of independent studies have shown that the removal of Rb alone is not sufficient to induce OS. The different experimental approaches strongly suggest that mutation in the p53 pathway can serve as an initiating event in OS, with a subsequent mutation in the Rb pathway strongly accelerating tumor development.

These engineered mouse models of OS reproduce many features of human OS including similar gene-transcription signatures (76) and cytogenetic complexity. However, the sites of primary tumor formation in Cre–loxP mice do not recapitulate the spontaneous human disease. The majority of lesions (85%) arise in axial skeletal sites (mandibule, maxilla, rib/vertebra, skull, sternum) while on 13.6% of tumors developed from the appendicular skeleton (hind leg, front leg) (68). This contrasts with the anatomic distribution of OS diagnosed in humans, with the distal femur, proximal tibia, and proximal humerus being the most common sites involved and only 10% develop in the

axial skeleton, most commonly the pelvis (5). Only in one study (69) did the tumor arise primarily in long bones. In addition, the observed frequency of distant metastases was comparatively low when compared to human disease except for the P53 knockdown model (69). As opposed to a complete deletion of P53, the primary tumor cells proliferated slower and the animals did not have to be sacrificed for local tumor size prior to completion of the metastatic process. Furthermore, the primary site of metastases in human OS is predominantly the lung parenchyma while in Cre–loxP mice, sites of metastases were more diverse with both the lung and liver being affected in almost equal proportions.

Other genes such as C-FOS (77, 78), TWIST (79), p14ARF (80), p16INK4a (81), PRKAR1A (71), and p21CIP (82) have also been implicated in OS pathogenesis based on studies of human OS samples. Their mutation appears to complement the defects in the P53 and RB pathways, and their involvement in osteosarcomagenesis is also demonstrated from genetically engineered mouse models. They provide important information regarding the genetics of OS, but the long latency combined with low penetrance makes utilization of these models less practical.

Targeted Therapies in OS

Osteosarcoma is very resistant to therapy and therefore there is an urgent need to effectively treat affected patients. The emergence of new anti-cancer drugs and the small number of patients eligible for early-phase clinical trials present another challenge in the clinical testing of novel compounds for OS treatment. As discussed earlier, xenotransplantation models have provided the greatest utility for preclinical screening of drugs with tumoricidal potential. To this end, the National Cancer Institute (NCI) has implemented the Pediatric Preclinical Testing Program (PPTP), a consortium of institutions across the United States and in Australia. Its objective is to identify agents with significant activity in panels of mouse xenograft models representing the most common pediatric cancers including OS (83). The program has been successful, leading to Phase I and II clinical trials for cixutumumab, sorafenib, and rapamycin for OS treatment. (84–86). In each case, these agents demonstrated high levels of response in the PPTP and were well-tolerated with promising anti-tumor activity in some adult and pediatric patients.

The use of spontaneous and transgenic OS models for high throughput screening of anti-OS drugs is hampered due to practical considerations associated with the cost and time of generating sufficient numbers of animals for statistically meaningful data. This is due to variations in disease onset as well as tumor heterogeneity, incidence, and progression. However, the recent generation of transgenic animals expressing shRNAs to knock down P53 (69) represents a potential breakthrough with respect to preclinical screening. Unlike conventional Cre-mediated gene deletion approaches, P53 knock down mice exhibited 100% penetrance for osteoblastic OS (the most common form of the disease). Moreover, the tumors were most frequently present in long bones and preferentially disseminated to the lungs, consistent with human OS.

Another consideration for preclinical testing in in vivo models is the accurate measurement of the disease burden at non-accessible sites. The use of in vivo imaging offers the opportunity to detect and monitor the development and progression of the disease. However, imaging systems are costly and not always widely accessible for many researchers. OS has the advantage that the primary tumor in genetically engineered mouse models appears in long bones and is therefore more accessible than abdominal tumors. The monitoring/visualization of micrometastases represents a greater challenge due to their small size. Inaccurate evaluation of metastatic spread in preclinical studies potentially

leads to disappointing results in clinical trials. Consequently, there is great interest in refining the methods to enable reproducible and ultrasensitive detection of metastases at the single cell level. The main focus therefore is on techniques, which allow the detection of tumor cells in vivo, such as microcomputer tomography (micro-CT), positron emission tomography (PET), bioluminescence, or fluorescence imaging.

Conclusion

Our understanding of human OS biology is hindered by its rapid onset, low prevalence, and absence of predisposing conditions or precursor lesions. With limited human tissue available for study, animal models provide a valuable tool to investigate the underlying mechanisms driving tumor initiation, progression, metastatic events, and therapeutic interventions. While these models have yet to faithfully recapitulate all aspects of OS, there is no doubt that the study of OS animal models has enabled insight into the genetics of tumor initiation as well as the cellular and molecular profiles of tumor growth and metastasis. In particular, gene knockout studies have been instrumental in identifying genetic mutations that promote OS tumor initiation (P53), as well as co-operative mutations that increase disease incidence (RB, c-FOS). With the use of cell lineage specific markers, it is now possible to introduce genetic mutations by sequential targeting from early precursor (multipotent mesenchymal cell) to more mature osteoblastic cells (osteoblast to osteocyte) to investigate OS incidence and tumor pathology. With this strategy, Prx1 and Osx have been used to identify mesenchymal and osteoprogenitor cells, respectively, following conditional mutation of P53. It remains to be seen, however, whether these populations are truly distinct, as Prx1 could be co-expressed with Osx in a certain subpopulation of cells. Another consideration particularly relevant in OS is its tumor heterogeneity among patients, which suggests that multiple cell types could act as cell of origin. Additionally, this concept of heterogeneity calls into question the utility of models exploiting single gene manipulation. Its consideration may permit a more systematic analysis of the genetic lesions involved in OS initiation and progression and could serve as a platform for the identification of early disease biomarkers. Cell of origin identification may also have important implications in the prevention of relapse and elucidate key molecular pathways and driver mutations that could lead to new therapeutic approaches to prevent the disease.

Thus, although for now, conventional orthotopic and subcutaneous transplantation models will remain indispensable to continue the study of OS in vivo, new models of spontaneous OS need to be developed to further our understanding of OS biology. Models that accurately reproduce the establishment of spontaneous micrometastases are necessary to investigate novel anti-metastatic agents, as this clinical scenario is most often the lethal event for patients with this form of cancer.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Citation: Guijarro MV, Ghivizzani SC and Gibbs CP (2014) Animal models in osteosarcoma. *Front. Oncol.* 4:189. doi: 10.3389/fonc.2014.00189

Received: 31 May 2014; Accepted: 07 July 2014; Published online: 18 July 2014.

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Genetically modified mouse models of cancer

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Tabla de contenido:

Tres suplementos alimentarios que ayudan a mantener la función cardíaca

La adición de determinados suplementos aminoacídicos y componentes derivados de aminoácidos a la dieta de las mascotas puede ayudar al tratamiento de determinadas patologías. Además, la deficiencia de algunos de ellos puede ser la causa directa de enfermedades del corazón.

(R) evolution

PEQUEÑOS ANIMALES

Taurina

La taurina es uno de los aminoácidos libres más abundantes. Se encuentra en elevadas concentraciones en los tejidos del músculo cardiaco, músculo esquelético, sistema nervioso central y plaquetas. Actúa en numerosos procesos metabólicos, ejerciendo diversas funciones:

- Antioxidación
- Actividad en las células fotorreceptoras de la retina
- Estabilización de las membranas neuronales
- Desarrollo del sistema nervioso
- Reducción de la agregación plaquetaria
- Reproducción
- Actividad miocárdica[1]:
  - Modulación de las concentraciones de calcio en los tejidos y su disponibilidad.
  - Inactivación de los radicales libres y cambio de la osmolaridad celular.
  - Efectos en la osmorregulación del miocardio.
  - Otros mecanismos específicamente relacionados con la función miocárdica incluyen la N-metilación de los fosfolípidos de la membrana celular, efectos directos en las proteínas contráctiles e interacciones con el sistema renina-angiotensina-aldosterona.

¿Qué ocurre en casos de deficiencia de taurina?

En **gatos**, la taurina es un aminoácido esencial y su deficiencia puede causar miocardiopatía dilatada (MCD), degeneración de la retina y anomalías reproductivas. Existen evidencias de que la MCD causada por su deficiencia puede ser reversible con la suplementación de este aminoácido[2,3].

En **perros**, hasta hace unos años, la taurina no se consideraba un aminoácido esencial ni se conocía su papel en el desarrollo de la MCD[4]. Sin

CALIER

embargo, diversos estudios han demostrado que sí lo es en perros alimentados con dietas restrictivas en proteína y que, al igual que los gatos, pueden desarrollar MCD secundaria a la deficiencia de taurina[5].

L-carnitina

La L-carnitina es un derivado aminoacídico que se obtiene de la proteína de la dieta o por síntesis endógena en el hígado, siendo la lisina y la metionina los aminoácidos precursores. La síntesis requiere hierro, vitamina C y vitamina B6 como cofactores. El músculo esquelético y el cardiaco son los lugares donde se almacena hasta el 95-98% de la carnitina del cuerpo.

Entre las funciones de la carnitina, la más importante es la de cofactor de algunas enzimas necesarias para el transporte de ácidos grasos de cadena larga al interior de las mitocondrias, donde se oxidan para la generación de energía para el corazón, que obtiene de esta manera aproximadamente el 60% de su producción de energía total.

¿Qué es la miocardiopatía dilatada (MCD)?

Se trata de una enfermedad del corazón muy habitual, progresiva y, en gran medida, irreversible, que puede conducir a fallo cardiaco congestivo o muerte súbita. Es la segunda enfermedad cardiaca más habitual en perros, con una prevalencia superior al 50% en algunas razas10. La nutrición está actualmente aceptada como un importante adyuvante a la terapia médica en perros y gatos con MCD.

¿Qué ocurre en casos de deficiencia de L-carnitina?

La deficiencia de L-carnitina puede ser un trastorno primario o secundario.

- Las deficiencias primarias pueden aparecer por defectos genéticos en la síntesis, transporte, absorción o degradación. En personas se han asociado con cardiomiopatías.
- Las deficiencias secundarias son más comunes en pacientes que siguen dietas restrictivas

Se ha demostrado en perros que la deficiencia de L-carnitina puede favorecer el desarrollo de MCD en perros. Además, varios estudios[6,7,8,9,10] han puesto de manifiesto que suplementar con carnitina mejora el tiempo de supervivencia de perros con MCD.

Hidrolizado de levadura de cerveza

La levadura de cerveza es un subproducto de la industria cervecera que puede ser un ingrediente beneficioso en la alimentación de las mascotas, ya que aporta el contenido nutricional que necesitan los perros y gatos[11]. Esterilizada y sin poder leudante, es una levadura inactiva compuesta por el organismo unicelular Saccharomyces cerevisiae.

Su administración tiene efectos beneficiosos sobre la salud intestinal y la función inmune de los perros, estimulando las respuestas Th1 y, en consecuencia, la inflamación. Además, mejoran la palatabilidad de las dietas[12]. Esto resulta especialmente útil para los perros con poco apetito a consecuencia de una enfermedad crónica.

Es una fuente proteínica rica en aminoácidos esenciales y vitaminas del grupo B:

- Los aminoácidos ayudan a la mascota a construir y mantener sus músculos, huesos, sangre, órganos, sistema inmunitario y pelaje y uñas. En particular, la arginina es un aminoácido esencial que reacciona con el oxígeno para producir óxido nítrico. El óxido nítrico relaja los músculos lisos de los vasos sanguíneos y reduce la presión arterial[13]. La hipertensión puede contribuir a las cardiopatías y a la insuficiencia cardíaca crónica, por lo que es conveniente controlar la tensión arterial de cualquier perro sospechoso de padecer una cardiopatía.
- Las vitaminas del grupo B contribuyen a la función cerebral, la fuerza muscular, la producción de glóbulos rojos y la digestión de los animales.

Por otro lado, se ha demostrado que estimula la producción de determinados marcadores de defensa antioxidantes, lo que ayuda a mejorar la salud cardiovascular de los animales[14].

- A medida que progresa la insuficiencia cardiaca congestiva, aumenta el daño a las células cardiacas por la formación de radicales libres. Los estudios realizados en perros con insuficiencia cardíaca congestiva han demostrado que estos pacientes presentan un aumento de oxidantes reactivos y una disminución de antioxidantes a medida que progresa la enfermedad[15].
- En perros con fallo cardiaco, la oxigenación y el metabolismo celular no funcionan de forma apropiada, lo que conlleva la producción de elevadas cantidades de radicales libres. Los radicales libres son responsables de los principales daños celulares, lo que se denomina estrés oxidativo[16].

Adicionalmente, la levadura de cerveza contiene sodio, calcio, magnesio y potasio. Muchos de los medicamentos utilizados para tratar las cardiopatías disminuyen los niveles sanguíneos de potasio y magnesio.

o Unos niveles inadecuados de potasio y magnesio pueden favorecer las arritmias cardiacas y debilitar las contracciones del músculo cardiaco[17].

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