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MINIREVIEWS

Recent clinical trials of cancer immunogene therapy in companion animals

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Abstract

This mini-review presents the results of veterinary clinical trials on immunogene therapy published from 2014 to 2016. A variety of tumors, among them melanoma (canine and equine), mastocytoma (canine), mammary adenocarcinoma (canine) and fibrosarcoma (feline) were treated by using diverse strategies. Non-viral vectors were usually employed to transfer genes of cytokines, suicide enzymes and/or tumor associated antigens. In general terms, minor or no adverse collateral effects were related to these procedures, and treated patients frequently improved their conditions (better quality of life, delayed or suppressed recurrence or metastatic spread, increased survival). Some of these new methodologies have a promising future if applied as adjuvant treatments of standard approaches. The auspicious results, derived from immunogene therapy studies carried out in companion animals, warrant their imperative usage in veterinary clinical oncology. Besides, they provide a strong preclinical basis (safety assays and proofs of concept) for analogous human clinical trials.

Key words: Cancer; Gene therapy; Immunotherapy; Companion animals; Comparative oncology

Core tip: Cancer immunogene therapy is a major growing area among human clinical trials. Until August 2016 there were about 2409 registered gene therapy trials, where 1554 were aimed to cancer, and among them 864 corresponded to immunotherapy. Working with veterinary cancer bearing patients can significantly speed up translational research and benefit both veterinary and human patients. New data demonstrated the safety and efficacy of different immunotherapy approaches. Following our previously published review on the subject covering from 1996 to 2014, this new mini-review is focused on veterinary cancer immunogene therapy covering published work in the field from 2014 to 2016.

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INTRODUCTION

More than 20 years elapsed from the publication of the pioneering work of Quintin-Colonna *et al*[1] in 1996 on *ex vivo* interleukin-2 (IL-2) immunogene therapy for canine melanoma and feline fibrosarcoma. As we had previously discussed in a previous review covering this subject from the early beginnings up to 2014[2], nearly all veterinary cancer gene therapy clinical trials involved the stimulation of immune responses against tumor cells. In this new review we are covering the three years period from January 2014 to December 2016. As we did in the preceding review, we focused our interest only on reports of clinical data collected from client-owned patients with spontaneously arising tumors.

Why clinical trials of cancer immunogene therapy in companion animals do actually matter? Despite some progresses treating tumors in companion animals and human patients, there is still a need of new therapeutic approaches for diverse malignancies because of the lack of long lasting effective treatments and the unwanted side effects of most of them. In addition, spontaneous tumors in companion animals offer a very useful translational model because pets display relatively large sizes, diversity of genetic backgrounds and intact immune systems. They usually offer a strong support as proof of concept in a setting similar to the population of human patients, while directly providing data about toxicity and long-term efficacy. Nowadays there is a revival of the idea that researchers should turn to canine clinical trials to advance cancer therapies[3].

Up to date we found 57 reports about veterinary immunogene therapy clinical trials: 45 canine, 7 feline and 5 equine. As seen in Table 1, the fourteen newly reported trials mainly involved non-viral gene transfer (13/14), sometimes enhanced by physical methods (10/13: 7 electrotransfer and 3 jet injection), while a viral vector was used in 1 trial (1/1: poxvirus). Reports about using genetically modified bacteria[4,5] or suicide gene expressing encapsulated mammalian cells[6] as vectors are not discussed in this mini-review.

New trials (7/14) were mainly reported from only three countries: 3 from United States (2 canine, 1 feline), 2 from Belgium (canine) and 2 from Italy (canine). The remaining five studies were: 1 from Argentina (canine), 1 from France (feline), 1 from Germany (equine), 1 from Slovenia (canine), 1 from United Kingdom (canine), 1 from South Africa (canine) and 1 multi-centric from Ukraine, Russia and Italy (canine).

Most of the current veterinary trials (6/11 canine, 1/2 feline and 1/1 equine) employed cytokine genes, mainly IL-2-and IL-12 as single (6/8) or combined genetic agents (2/8), followed by antigens (5/11 canine, 1/2 feline, 1/1 equine).

Immunogene therapy was applied alone (10/14), or combined with chemotherapy (3/14) or suicide gene (1/14). Most trials were focused on feasibility and safety (8/14) with few treated animals and often carrying diverse kind of tumors. On the other hand, larger controlled trials (4/14) allowed the determination of clinical efficacy.

CYTOKINE-BASED IMMUNOGENE THERAPY

Only five cytokine genes were assayed in veterinary clinical settings in the latest three years. Granulocyte-macrophage colony stimulating factor (GM-CSF) stimulates dendritic cells that then induce antitumor immune responses. The result is twofold: Direct destruction of the injected lesion and enhanced antigen presentation, leading to an immune response against metastatic melanoma. T-cells treated with GM-CSF have demonstrated increased antitumor responsiveness.

IL-2 is a naturally occurring glycoprotein secreted by T-cells to augment the immune response and was first used in clinical cancer studies in the early 1980s. This glycoprotein promotes T-lymphocyte proliferation and stimulates cytotoxic T-cells and natural killer cells. IL-2 has been used as immunotherapy for nearly 40 years. The treatment with the recombinant protein displays systemic cytotoxicity that deteriorates the patient's quality of life.

Interferon-β (IFN-β) simultaneously displays antiproliferative and anti-angiogenic effects as well as immunomodulatory activities. It was among the first cytokines approved for clinical use. Even though clinically effective, the completion of IFN-β therapy is often impaired by its inherent systemic toxicity that negatively affects the patient's quality of life.

IL-12 and IL-18 exhibit different immune regulatory roles including activation of cytotoxic T-lymphocytes and natural killer cells, production of interferon-γ (IFN-γ). In addition they have antiangiogenic properties and induce apoptosis in tumor cells. Although they produce from moderate to severe side effects, significant antitumor activity was demonstrated in clinical trials for recombinant IL-12 and IL-18 proteins.

Combination with suicide gene therapy and whole tumor cells extracts for canine melanoma

Spontaneous canine melanoma is a highly aggressive tumor. It appears mainly in the oral cavity, footpads, digits and mucocutaneous junctions, displaying a clinical behavior analogous to aggressive human melanoma. Both diseases are resistant to chemotherapy, radiotherapy and treatments with modulators of biological responses. They share comparable location selectivity and metastatic phenotypes.

As a continuation of encouraging data obtained with a surgery adjuvant treatment that consisted of complete or cytoreductive surgery followed by a combination of suicide gene therapy with a subcutaneous vaccine[7], a

Table 1 Veterinary cancer immunogene therapy trials 2014-2016

No.	Genes	Tumor	Vector	Mode	Results	Ref.
1	hIL-2 + hGM-CSF cIFN-β + HSV-tk	MEL	Plasmid lipofection: HSV-tk + IFN-β + GCV. Plasmid lipofection: hIL-2 and hGM-CSF	*In vivo* (SG) + (IFN-β) - i.t. *In vivo* (hIL-2 + hGM-CSF) + (TV) - s.c./+ SX	Combined treatment (n = 301)/surgery controls (n = 162). Complete surgery arms: Local disease-free patients: 83%/11%. Metastasis-free patients: 89%/44%. Median survival: > 2211 d/109 d. Metastasis-free median survival: > 2211 d/134 d	Finocchiaro *et al*[8]
2	tthIL12	SCC MEL	Plasmid/EGT	*In vivo* - i.t. EGT	(n = 4) Dose escalation and safety. Two confirmed local and distant responses. No severe side effects	Cutrera *et al*[9]
3	cIL-12	AMB, PCY, SCC, STS	Naked plasmid + bleomycin/ gemcitabine EGT	*In vivo* - i.t./+ CHT	(n = 13) 2 CR, 4 PR, 5 SD, 2 PD. No severe side effects	Cutrera *et al*[12]
4	hIL12	MCT	Naked plasmid + cisplatin or bleomycin electrotransfer	*In vivo* - i.t./+ CHT	(n = 18) 13 CR, 2 PR, 1 SD, 2 PD. No severe side effects	Cemazar *et al*[14]
5	hIL12	FSA, MAC, MCT, OSA, SCC, SCH	Plasmid/EGT	*In vivo* - i.t.	(n = 9) Safety studies and demonstration of immunogenic and anti-angiogenic effects. No significant clinical benefits	Cicchelero *et al*[10]
6	hIL12	ADC, FSA, MEL, OSA, SCH	Plasmid/EGT + metronomic cyclophosphamide	*In vivo* - i.t. EGT/+ metronomic CHT	(n = 6) No significant unwanted side effects. Combined therapy slowed down tumor progression and improved QOL	Cicchelero *et al*[13]
7	fIL-2	FSA	Canary pox virus	*In vivo* - p.t.	Combined treatment (n = 25)/ Surgery + brachytherapy controls (n = 23). Disease-free patients: 41%/72%. Median survival: > 730 d/287 d	Jas *et al*[11]
8	eIL-12 + eIL-18 hgp100 htyr	MEL	Plasmid like	*In vivo* - i.m./p.t.	(n = 27) No differences between eIL-12 + eIL-18 without (n = 9) or with either hgp100 (n = 9) or htyr (n = 9). Tumor size reduction on day 120 approximately 20%	Mählmann *et al*[15]
9	CSPG4	MEL	Plasmid/EGT	*In vivo* - i.m./ + SX	(n = 14) VAC/(n = 13) CTR Median survival: VAC 532 d/ CTR 205 d	Riccardo *et al*[23]
10	hp62	MAC	Plasmid	*In vivo* - i.m.	(n = 7) 5PR (50%-75% reduction) 2 SD. Safety verified	Gabai *et al*[22]
11	htyr	MEL	Naked plasmid - Jet injection	*In vivo* - i.m./ + SX ± RX	Retrospective study (n = 38). Responding patients median survival: 870 d. Non-responding patients median survival: 240 d	McLean *et al*[19]
12	htyr	MEL	Naked plasmid - Jet injection	*In vivo* - i.m./ + SX ± RX	Retrospective study (n = 32). Median survival: 335 d. Progression-free median survival: 160 d	Treggiari *et al*[20]
13	CSPG4	MEL	Plasmid/EGT	*In vivo* - i.m./ + SX	(n = 23) VAC/(n = 19) CTR Median survival: VAC 684 d/ CTR 220 d	Piras *et al*[24]
14	htyr	MEL	Naked plasmid - Jet injection	*In vivo* - i.m./ + SX ± RX	Retrospective study (n = 24). Eleven percent of manageable post-vaccination adverse effects. Safety verified. Forty-two percent died of unrelated causes or still alive	Sarbu *et al*[18]

The No. 1-8 genes are cytokines; the No. 9-14 genes are antigens; the tumors in No. 1-6 and 9-13 are canine; the tumors in No. 7 and 14 are feline; the tumors in No. 8 is equine. ADC: Adenocarcinoma; AMB: Acanthomatous ameloblastoma; FSA: Fibrosarcoma; MAC: Mammary adenocarcinoma; MCT: Mast cell tumor; MEL: Melanoma; PCY: Plasmocytoma; OSA: Osteosarcoma; SCC: Squamous cell carcinoma; SCH: Schwannoma; STS: Soft tissue sarcoma; CSPG4: Chondroitin sulfate proteoglycan-4; GM-CSF: Granulocyte macrophage colony-stimulating factor; gp100: Glycoprotein 100; HSV-tk: Herpes simplex thymidine kinase; IFN-β: Interferon-β; IL: Interleukin; p62: Protein 62; tyr: Tyrosinase; CR: Complete response; CTR: Control; GT: Gene therapy; PR: Partial response; SD: Stable disease; GCV: Ganciclovir; SG: Suicide gene; TV: Tumor vaccine; VAC: Vaccinated; i.m.: Intramuscular, i.t.: Intratumoral; p.t.: Peritumoral; s.c.: Subcutaneous; CHT: Chemotherapy; RX: Radiotherapy; SX: Surgical excision; c: Canine; e: Equine; f: Feline; h: Human; tt: Tumor targeted.

new local and vaccine formulation was assayed[8]. This new trial involved the injection (at the end of surgery) of DMRIE/DOPE lipoplexes carrying two therapeutic genes: Canine IFN-β and herpes simplex virus thymidine

kinase. In parallel, a subcutaneous genetic vaccine made of lipoplexes carrying human GM-CSF and IL-2 genes and tumor formalized extracts was periodically administrated. Taking surgery-only-treated (ST) as controls, it was found that the combined treatment (CT) that followed complete surgery (CS) significantly increased the portion of local distant metastases-free (M0) from 44% to 89% and disease-free canine patients from 11% to 83%. Even in the case of cytoreductive surgery (CR), CT had a better control of the systemic disease (M0: 82%) than ST (M0: 48%). Furthermore, taking the ST group as control, CT displayed a considerable > 17-fold (CS) and > 13-fold (CR) increase of metastasis-free survival (MFS), and 7-fold (CS) and 4-fold (CR) increase of overall survival. The remarkable rise of CS-recurrence-free survival (RFS) and CS-MFS (both > 2251 d) and CR-MFS (> 1321 d) revealed that CT was transforming a fast terminal disease into a chronic one. Finally as a surgery adjuvant, this CT was able to considerably postpone or preclude distant metastasis and postsurgical recurrence, while it extended disease-free and overall survival, and preserved the quality of life. The long-term safety and efficacy of this treatment was supported by the high number of canine patients involved and the wide follow-up (> 6 years) with negligible or null toxicity. Besides, this work confirmed that the most advantageous clinical situation is to implement this approach as a surgery adjuvant acting on the minimal residual disease.

Single treatments for canine and feline solid tumors

Electrotransfer was the preferred method for introducing IL-12 plasmids into cells. In a first report, the peptide-cytokine VNTANST-IL12 could successfully target expressed cell-surface vimentin while displaying the antitumor effects of cytokine IL-12. So, a tumor-targeted IL-12 plasmid DNA (ttIL12 pDNA) was safely delivered at clinical levels by electrotransfer to four tumor bearing canine patients, inducing antitumor immune responses in both treated and untreated tumors including metastatic lesions[9].

In a parallel study, nine dogs with spontaneous cancer were subjected to three consecutive intratumoral IL-12 electrogene therapy (EGT) sessions to assess their clinical, anti-angiogenic and immunological effects[10]. Serum and tumor tissue displayed transitory increases of IFN-γ and IL-12. Intratumoral IL-12 EGT did not produce clinically significant results, even though some anti-angiogenic and immunostimulatory activities appeared as well as transitory objective responses did. It was not possible to get a sustained tumor regression during the trial. Without severe side effects, safety was verified.

Fibrosarcoma is a lethal disease in cats that is frequently found at standard vaccine injection sites. Because of the high local recurrence rates after surgery alone, the addition of adjuvant treatments has been under investigation for the last few years. A randomized, controlled clinical study was performed to determine the safety and efficacy the of a recombinant canarypox virus carrying the feline IL-2 gene (ALVAC IL-2)[11]. Additionally to surgery and post-surgical brachytherapy and as adjuvant treatment, the vector was transferred to cats with a first occurrence of feline fibrosarcoma. The pet patients were distributed at random to a control group (23 cats), low dose injected group (25 cats) and high dose injected group (23 cats). The treatment consisted of six successive intratumoral injections of ALVAC IL-2. With limited mild local reactions, it was well tolerated. Injected animals displayed a significantly longer median time to relapse (> 730 d in the low dose injected group) than in the control group (287 d). The risk of relapse of treated cats displayed a considerable decrease: 56% at one year (injected groups compared to control group) and 65% at two years (low dose injected group compared to control group).

Combination with electro-chemotherapy for canine solid tumors

In a further report, a study demonstrated the efficacy and safety of recurring cycles of *IL-12* gene therapy plus chemotherapy for long-term treatment of aggressive tumors[12]. In this case 13 canine patients were subjected to various rounds of electro-chemo-gene therapy (ECGT) with canine IL-12 plasmid DNA (pDNA) and either gemcitabine or bleomycin. This approach was versatile, being effective for many histotypes, and allowed fast eradication or debulk of large tumors (sarcoma excluded). It affected both treated tumors and non-treated metastatic tumors. Without severe adverse events reported, even after multiple treatment cycles, ECGT with IL-12 pDNA proved to be a very valuable approach for many types of spontaneous cancers including refractory, large and multiple tumor loads. Being this phase Ⅰ trial designed for dose escalation/safety assessment, no data about long term quality of life and progression free survival could be obtained.

By combining with metronomic cyclophosphamide, the outcomes of three successive intratumoral IL-12 electrogene transfer treatments were explored in six dogs with spontaneous tumors[13]. This treatment induced erythema and swelling of the tumor, a transient increase in IL-12 levels, and a continuous increase in IFN-γ and thrombospondin-1 concomitant with a continuous decrease in vascular endothelial growth factor. While the treatment improved the quality of life and weight, it slowed tumor progression in most of the patients (4/6).

Mast cell tumors (MCTs) are common cutaneous neoplasms in dogs, accounting for up to 21% of all canine skin tumors. Peritumoral *IL-12* gene transfer was combined with electro-chemotherapy (cisplatin or bleomycin) targeting mast cell tumors in 18 canine patients[14]. Without emerging side effects, one month after the therapy a considerable local tumor control was evidenced. The complete responses rate increased up to 72% during the observation period. *IL-12* gene electrotransfer produced detectable serum IL-12 and/or IFN-γ levels in 78% of patients. This study showed a high antitumor efficacy of the combined treatment that also prevented recurrences or distant metastases.

In addition the safety and feasibility of this approach was demonstrated.

Combination with antigens for equine melanoma tumors

Equine melanoma has a high incidence in grey horses. In a clinical trial[15], 27 melanoma-bearing grey horses were allocated into 3 groups (n = 9) and intramuscularly vaccinated on days 1, 22, and 78 with lipoplexes carrying DNA plasmids bearing equine *IL-18* and *IL-12* genes alone or combined with either human glycoprotein 100 or tyrosinase. In all groups, by day 120 the relative tumor volume significantly decreased about 20%. The combination of the two cytokines was safe, but the addition of DNA vectors encoding the human melanoma antigens did not potentiate their effects.

ANTIGEN-BASED IMMUNOGENE THERAPY

As it happens in dogs, in cats malignant melanoma appears as locally aggressive and highly metastatic regardless of the primary site of origin. The standard modalities used for treatment are surgery and radiotherapy, with poor results in the long-term. Following the studies about the use of xenogeneic antigen vaccination for spontaneous canine malignant melanoma[16,17], new data about intramuscular needle free jet injection of a plasmid containing human tyrosinase gene and its expression as a melanoma xeno-antigen against feline and canine melanoma were reported.

In a retrospective study the safety of the canine melanoma DNA vaccine (Oncept®) following surgery in 24 feline patients was assessed[18]. The vaccine appeared to be well tolerated, with a low number of reported adverse events. Since most cats finally died from this disease, controlled prospective studies are necessary to determine the immunogenicity and efficacy of this vaccine originally designed for canine melanoma in cats with melanoma.

Furthermore, two additional independent retrospective analyses on the use of this DNA vaccine for canine melanoma[19,20] were in agreement on safety issues with previously published results[16,17]. In the first case 38 canine patients were evaluated: The median survival time of responding patients (42%) was 870 d, while it was 240 d in the case of non-responding ones (58%)[19]. Even though a relatively small number of patients was analyzed, melanotic oral melanoma appeared to give survival advantage as compared to its amelanotic counterpart. As expected, surgery with clean margins also improved the treatment outcome. In the second case, 32 oral melanoma patients received the DNA vaccine after surgery displaying a median survival time of 335 d[20]. No significant differences were found when compared to other adjuvant treatments. Because of the low statistical power due to small number and group heterogeneity, the authors proposed controlled studies to assess whether the addition of any adjuvant treatment to surgery, including immunogene therapy, is able to significantly prolong survival in cases of canine oral melanomas. A very recent paper[21] analyzing the outcome of 69 canine melanoma patients after human tyrosinase DNA vaccination, reported a median survival time of 455 d, a value that is similar to those published earlier[19,20]. Since the authors observed responses in patients with macroscopic disease, they suggest that the vaccine could be considered as palliative treatment in dogs with remaining tumors or recurrence.

Mammary tumors are the most common tumors of unspayed female dogs with a prevalence of 40% of all tumors, and about half of them are malignant. The p62 protein (SQSTM1) is a major player in selective macro-autophagy and acts as a signaling hub for many signal transduction pathways. It is noteworthy that p62 is expendable for normal tissues, but critical for development and survival of tumors. The effects of intramuscular p62 DNA vaccine on mammary tumors of seven dogs were tested[22]. Locally advanced lesions decreased (5/7) or stabilized (2/7) their growth while the overall toxic effects of p62 were absent. The observed antitumor activity was associated with lymphocyte infiltration and tumor encapsulation *via* fibrotic reaction.

Chondroitin sulfate proteoglycan-4 (CSPG4) is an early cell surface progression marker associated with tumor cell migration, invasion and proliferation. In a first trial, the immunogenicity, safety and therapeutic efficacy of a human CSPG4 DNA-based vaccine were evaluated[23]. Canine patients with stage Ⅱ-Ⅲ surgically excised CSPG4-positive oral malignant melanoma were treated by adjuvant intramuscular electrotransfer with human CSPG4-encoded plasmid from 6 to 20 mo. Overall (653 d *vs* 220 d) and disease-free (477 d *vs* 180 d) survival times were significantly longer in 14 vaccinated dogs as compared with 13 non vaccinated controls. No clinically relevant local or systemic side effects were found. This suggested that xenogeneic electrovaccination against CSPG4 can prevail over the host tolerance to the "self" antigen, being successful in treating canine malignant melanoma.

In a second trial, the same treatment was performed in 23 dogs with surgically excised CSPG4-positive oral canine melanoma. In parallel, 19 control dogs with CSPG4-positive tumors were subjected only to surgery[24]. Vaccinated dogs displayed a one year survival rate of 74%, with a median survival time of 684 d, and one year disease-free interval rate of 48%. Non-vaccinated dogs showed one year survival rate of 26%, with a median survival time of 200 d, and a one-year disease-free interval rate of 26%. The involvement of a fair amount of canine patients together with the relatively wide follow-up (2 years) with absent or minimal adverse side effects, added to the results of the previous report[23], assure the significant efficacy and the long-term safety of this treatment.

CONCLUSION

The suitability of comparative oncology using large animal naturally occurring cancer models to test new

cancer drugs, particularly gene medicines, was widely discussed[2,25].

This approach can primarily benefit pets that do not have efficient treatments for some malignancies and ultimately human patients. The proof of concept and safety results obtained in companion animals can be readily used for designing clinical trials against the corresponding human malignancies.

Some immunogene therapy approaches similar to those presented here were also tested in human patients before 2014: Xenogeneic tyrosinase[26] and gp100[27], IL-2[28], GM-CSF[29] and IL-12[30]. On the other hand, encouraging results were recently reported about a clinical trial of *AdCD40L* gene therapy combined with cyclophosphamide chemotherapy for human melanoma[31] and p62 protein (SQSTM1) gene therapy for some human solid tumors[32]. These trials were respectively based on previous veterinary trials involving canine melanoma[33] and canine mammary adenocarcinoma[22] patients. In addition, our Unit is currently involved in a phase I clinical trial for human advanced melanoma based on the safety and efficacy of our previous long lasting veterinary clinical trials[7,8].

Because of safety reasons and the cost of producing the suitable vectors, most of the veterinary gene therapy protocols were done with non viral vectors, being electrotransfer mainly used in the latest period (2014-2016).

Four controlled trials[8,11,23,24] showed statistically significant tumor control and survival time increase, while maintaining a good quality of life. These results strongly support further developments in immunogene therapy. Meanwhile, new immune-mediated gene therapy approaches are emerging for treating equine melanoma with a bacterial antigen[34] and canine lymphoma with a chimeric antigen receptor[35]. A different gene therapy approach is also under investigation for feline oral squamous cell carcinoma with RNAi oligonucleotides targeting feline CK2α and CK2α′ (TBG-RNAi-fCK2αα′)[36].

As we discussed earlier[2], proper cancer models involving companion animals spontaneously induce the investigators to work in the duality between the veterinary perspective and the potential application to human medicine. However, these are not contradictory objectives, and more trials in humans based on the equivalent trials in pets will possibly come soon.

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