Cancer Letters 400 (2017) 205–218

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

Original Articles

Immunological, anti-angiogenic and clinical effects of intratumoral interleukin 12 electrogene therapy combined with metronomic cyclophosphamide in dogs with spontaneous cancer: A pilot study

Laetitia Cicchelero [a], Sofie Denies [a], Katrien Vanderperren [b], Emmelie Stock [b], Leen Van Brantegem [c], Hilde de Rooster [d, e, 1, *], Niek N. Sanders [a, e, 1, **]

[a] Laboratory of Gene Therapy, Department of Nutrition, Genetics and Ethology, Faculty of Veterinary Medicine, Ghent University, Heidestraat 19, B-9820 Merelbeke, Belgium
[b] Department of Medical Imaging of Domestic Animals, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium
[c] Department of Pathology, Bacteriology and Poultry Diseases, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium
[d] Small Animal Hospital, Department of Medicine and Clinical Biology of Small Animals, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium
[e] Cancer Research Institute Ghent (CRIG), Ghent University, Belgium

ARTICLE INFO

Article history:
Received 21 July 2016
Received in revised form
8 September 2016
Accepted 13 September 2016

Keywords:
Angiogenesis
Contrast-enhanced ultrasound
Immune stimulation
Gene transfer
Interleukin 12
Metronomic cyclophosphamide

ABSTRACT

The immunological, anti-angiogenic and clinical effects of metronomic cyclophosphamide and 3 consecutive intratumoral interleukin (IL)-12 gene therapy (electrogene therapy (EGT)) treatments were evaluated in 6 dogs with spontaneous cancer. In all dogs, a decrease in peripheral leukocytes 2 days after IL-12 EGT coincided with erythema and swelling of the tumor. In the tumor, a transient increase in IL-12 levels was measured, whereas a continuous increase in interferon γ (IFNγ) and thrombospondin 1 (TSP-1) were determined in contrast to a continuous decrease in vascular endothelial growth factor (VEGF). In the serum, a transient increase in IL-12 and IL-10 levels were noted in contrast to a transient decrease in VEGF and TSP-1. The treatment resulted in a significant anti-angiogenic effect. Although all primary tumors continued to progress in time, this progression was slower than before treatment according to the contrast-enhanced ultrasound data. Besides the encouraging immunostimulatory and anti-angiogenic effects observed in all dogs we also noticed in 4 out of 6 dogs clinically relevant improvements in quality of life and weight. These results hold great promise for combinatorial strategies of IL-12 EGT and metronomic chemotherapy with conventional antitumor (immuno)therapies.

Abbreviations: CBC, complete blood count; CEUS, contrast-enhanced ultrasound; CP, cyclophosphamide; DC, dendritic cell; DPBS, Dulbecco's Phosphate-Buffered Solution; EGT, electrogene therapy; GMP, good manufacturing practice; hGH, human growth hormone; IFN, interferon; IL-12, interleukin 12; IL-12 receptor beta 2, IL-12Rβ2; IP-10, Inducible Protein 10; IV, intravenous; LN, lymph node; MDSCs, myeloid-derived suppressor cells; MIG, Monokine Induced by Gamma IFN; NK, natural killer; NSAID, non-steroidal anti-inflammatory drug; PBMC, peripheral blood mononuclear cells; pDNA, plasmid DNA; PE, peak enhancement; ROI, region of interest; SAP, serum alkaline phosphatase; TAN, tumor-associated neutrophil; TSP-1, thrombospondin 1; TTP, time-to-peak; Treg, regulatory T cell; US, ultrasound; UTR, untranslated region; VEGF, vascular endothelial growth factor.

*Corresponding author. Small Animal Hospital, Department of Medicine and Clinical Biology of Small Animals, Faculty of Veterinary Medicine, Ghent University, Salisburylaan 133, B-9820 Merelbeke, Belgium.
**Corresponding author. Laboratory of Gene Therapy, Department of Nutrition, Genetics and Ethology, Faculty of Veterinary Medicine, Ghent University, Heidestraat 19, B-9820 Merelbeke, Belgium.
E-mail addresses: hilde.derooster@ugent.be (H. de Rooster), Niek.Sanders@ugent.be (N.N. Sanders).
[1] Hilde de Rooster and Niek Sanders are co-last authors.

http://dx.doi.org/10.1016/j.canlet.2016.09.015
0304-3835/

Introduction

The development of cancer is recognized by the body's immune system as an anomaly. On a genetic level, many control mechanisms are in place to detect and destroy aberrant cell copies. Similarly, immune surveillance plays an important role in the prevention of neoplastic spread [1]. Unfortunately, despite these checkpoints for abnormal cell development, cancer cells are able to escape their control resulting in the development of a tumor [2]. The relationship between cancer and the oncologic patient's immune system is very complex and continuously evolving [3]. It is therefore unlikely that one immunotherapeutic agent will be powerful enough to produce an effective antitumoral immune response. The more cancer promoting pathways that are simultaneously targeted, the higher the chances of successfully decreasing tumor escape [4]. We propose to combine the antitumoral effects of interleukin 12 (IL-12) with metronomic cyclophosphamide (CP) as a multi-targeted and complementary approach.

IL-12 is a powerful immunostimulatory cytokine that links innate and adaptive immune responses. By stimulating the immune response, IL-12 is able to activate key immune cells such as natural killer (NK) and T cells to recognize and kill cancer cells [5]. Furthermore, IL-12 decreases the suppressive function of myeloid-derived suppressor cells (MDSCs) [6] and inhibits the differentiation of T cells to regulatory T cells (Tregs) as well as the expansion of Tregs [7,8].

Metronomic chemotherapy, i.e. the continuous administration of low-dose chemotherapeutics, has much potential in combinatorial treatments [9] and possibly synergizes with other immunomodulatory or anti-angiogenic antitumoral therapies [10]. The aim of metronomic chemotherapy is not to kill cancer cells directly but indirectly, by influencing the microenvironment of the cancer cells. Cyclophosphamide is a well-known alkylating chemotherapeutic that has been mainly used in a cytotoxic dose to kill rapidly multiplying cells such as cancer cells [9]. When given in metronomic doses, CP is able to selectively decrease the amount of circulating Tregs and has anti-angiogenic properties [11].

There are multiple advantages to the combination of intratumoral IL-12 electrogene therapy (EGT) and metronomic CP as both therapies decrease immunosuppression and angiogenesis. Whereas metronomic CP has shown to selectively decrease the amount of Tregs [12–14], IL-12 decreases the suppressive function of immunosuppressive MDSCs [6], which could compensate for the CP-induced MDSC expansion [13]. The anti-immunosuppressive effects of metronomic CP restores NK cell effector functions and thus facilitates the generation of innate and eventually adaptive immune responses induced by IL-12 [15]. Indeed, the combination of a low dose of CP and IL-12 induced a stronger Th1 response than IL-12 alone in mice [16]. Furthermore, multiple complementary angiogenesis pathways are simultaneously targeted through this combinatory approach. IL-12 inhibits neo-angiogenesis via induction of interferon γ (IFNγ) and its cascade products Inducible Protein (IP) 10 and Monokine Induced by Gamma interferon (MIG) [17]. On the other hand, metronomic CP causes apoptosis of capillary endothelial cells by up-regulating the endogenous angiogenesis inhibitor thrombospondin 1 (TSP-1) [18]. In turn, TSP-1 binds not only to the CD36R on endothelial cells inducing apoptosis [19], but also to the CD47-part of the vascular endothelial growth factor receptor (VEGFR), thus blocking the proangiogenic effects of VEGF on endothelial cells [20].

Because of severe systemic toxicity, systemic injection of recombinant IL-12 (rIL-12) is not recommended [21]. Local (intratumoral) injection of rIL-12 has been considered, but requires frequent injections due to the short half-life of rIL-12 (12 h) [22]. Therefore, intratumoral IL-12 gene therapy, using plasmid (p)DNA electroporation, has been evaluated as it offers a prolonged delivery of IL-12 and resulted in very promising (pre)clinical results [23–27]. Electroporation is the short administration of electrical pulses to ensure the formation of transient pores in the plasma membrane of cells thereby facilitating the intracellular delivery of large molecules such as pDNA [28]. Once the pDNA reaches the cell's nucleus, the production of the protein encoded on the plasmid ensues [29,30]. In vivo electroporation does not cause any severe adverse events [31], and several canine and human clinical trials showed the safety and clinical efficacy of electroporation-mediated intratumoral delivery of a pDNA encoding IL-12 [23–26].

In this article, the effects of intratumoral IL-12 EGT in combination with metronomic CP on tumor angiogenesis, amount of circulating Tregs and shifts in the tumor microenvironment were studied in 6 dogs with various tumor types and sizes. To our knowledge, this is the first article that describes the combinatorial effects of intratumoral IL-12 EGT and metronomic CP (IL-12-CP treatment) in dogs with spontaneous tumors.

Materials and methods

Animal selection

Client-owned dogs with spontaneous malignant neoplasms were enrolled between April 2015 and October 2015 if they met the following inclusion criteria: a histologically confirmed neoplasia accessible for application of electrodes, normal cardiovascular function, good general health status and a biochemistry profile within reference range. Patients included in the study were not eligible for surgery, either due to recurrent disease for which conventional surgery was already exhausted, or due to the impractical location of the tumor. Other conventional therapies such as chemotherapy or radiation were not given prior to IL-12-CP treatment.

Table 1
Signalment, tumor type, tumor size and metastasis status of the dogs treated by intratumoral IL-12 gene therapy and metronomic cyclophosphamide.

Dog	Breed	Sex	Age	Tumor type (localization)	Tumor size	Metastases
1	Golden Retriever	Fn	14 y	Amelanocytic melanoma (intra-oral)	50.24 cm^3	Lymph node cytology and liver US positive
2	Golden Retriever	Mn	14 y 10 m	Schwannoma (dorsal to carpus)	360.00 cm^3	Lymph node cytology and thoracic free
3	Stabyhoun[a]	Fn	7 y	Adenocarcinoma (anal sac)	NA	Abdominal metastasized lymph nodes resected, hypogastric lymph node cytology positive, thoracic free
4	Malinois[b]	M	11 y	Fibrosarcoma (dorsal to carpus)	43.96 cm^3	Lymph node cytology and thoracic free
5	French Bulldog	Mn	8 y 11 m	Osteosarcoma (costal)	201.00 cm^3	No, locally invasive in thorax
6	Beagle	M	10 y 2 m	Fibrosarcoma ((rostro)mandibular)	14.13 cm^3	Lymph node cytology and thoracic free

F: female, Fn: female neutered, M: male, Mn: male neutered, NA: not applicable, US: ultrasound.
[a] No contrast-enhanced ultrasound was performed in this dog.
[b] This dog received a second IL-12-CP treatment 5 months after the first IL-12-CP treatment cycle.