Cancer Electrogene Therapy with Interleukin-12

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Abstract: Electrogene therapy combines administration of plasmid DNA into tissue followed by local application of electric pulses. In electrogene therapy with *interleukin-12* (*IL-12*), different routes of administration, different doses of plasmid DNA and different protocols for delivery of electric pulses were evaluated in numerous preclinical studies. Antitumor effectiveness was tested in different types of primary tumors, distantly growing tumors and induced metastases. Intratumoral *IL-12* electrogene therapy has been proved to be very effective in local tumor control, having also a systemic effect. Intramuscular and peritumoral *IL-12* electrogene therapy had also a pronounced systemic effect and when combined with other treatment strategies resulted in tumor cures. Antitumor effectiveness of *IL-12* electrogene therapy is due to the induction of adaptive immunity and innate resistance and anti-angiogenic action. Translation of preclinical studies into clinical trials in human and veterinary oncology has started with encouraging results that would hopefully lead to further investigation of this therapy, also in combination with other cancer treatment modalities.

Keywords: Interleukin-12, electroporation, electrogene therapy, gene electrotransfer, clinical studies, melanoma.

INTRODUCTION

Interleukin-12 (IL-12) is a soluble cytokine that is produced by phagocytes and dendritic cells in response to pathogens, activated T cells and component of inflammatory extracellular matrix [1]. IL-12 was discovered in 1989. It was isolated from the phorbol diester-induced Epstein-Barr virus-transformed human B lymphoblastoid cell line RPMI 8866 and was named "natural killer cell stimulatory factor" [2]. In this first paper, the three activities of IL-12 were already recognized; induction of IFN-γ production, augmentation of NK cell-mediated cytotoxicity and enhancement of mitogenic response of T cells. It was also determined that the protein is a heterodimer composed of two subunits, a 35 kDa light chain (also known as p35 or IL-12α), and a 40 kDa heavy chain (known as p40 or IL-12β). Just one year later, an independent discovery of the "cytotoxic lymphocyte maturation factor" was published and in 1991 it was proposed that this newly discovered cytokine should be given the designation IL-12 [3, 4]. Shortly thereafter, human and murine genes for IL-12 were cloned and this stimulated further studies on the therapeutic efficacy of recombinant IL-12 in different tumor models including metastases [5, 6]. IL-12 antitumor effectiveness is multifactorial and is still not fully elucidated. Briefly, it consists of induction of IFN-γ production that induces infiltration of $CD8^+$ T lymphocytes and NK cells into tumors, which exhibit cytolytic activities. In addition, IL-12 has antiangiogenic action through activation of IFN-γ induction of interferon inducible protein-10 (IP10) and monokine Mig induced by IFN-γ. Furthermore, IL-12 augments the $CD4^+$ Th1 response, leading to activation of a specific B cell response. IL-12's potent antitumor and antimetastatic activity was shown on many preclinical tumor models [1, 7-17]. Preclinical studies encouraged clinical trials, where the safety and antitumor efficacy of recombinant human IL-12 (rh-IL-12) were examined [18-25]. The first clinical phase I escalation study on patients with different malignancies was published only 8 years after discovery of IL-12 [18]. Mainly patients with renal carcinoma and melanoma were included. They were treated with intravenously injected human recombinant IL-12. Only a minor tumor response was obtained; one transient complete response in a melanoma patient and a partial response in a renal carcinoma patient. Unfortunately, the first reports of phase I and II clinical trials demonstrated toxic side-effects of recombinant human IL-12 protein at doses which barely resulted in any antitumor effectiveness [26]. In the most effective study, treatment with rhIL-12 resulted in a partial or complete response in 56% of patients with cutaneous T-cell lymphoma [25].

Gene therapy introduced an advanced route of administration and improved action of IL-12. Recent studies have evaluated the antitumor effectiveness and safety of intratumoral *IL-12* gene therapy. The delivery systems for *IL-12* gene therapy are various and include transfer of naked plasmid DNA alone [27, 28], adenoviral [29-33] or other viral vectors [34-36], as well as gene gun [37, 38], electroporation [39-42] and other non-viral vectors [43, 44]. Clinical studies in human and also in veterinary oncology were initiated and the first results showed that *IL-12* gene therapy is a safe treatment with some beneficial clinical effect [28, 45-55].

As mentioned, IL-12 is a very potent cytokine with diverse biological activities. It has profound antitumor efficacy that was demonstrated on a variety of different types of tumors and metastases in preclinical as well as clinical studies. In recent years, several excellent reviews describing the activities of IL-12, the preclinical and clinical use of recombi-

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nant IL-12 and gene therapy with *IL-12* have been published [1, 8, 11, 45, 56, 57]. On the other hand, 10 years have passed since the first paper on combination of plasmid DNA encoding for IL-12 injected intramuscularly with electroporation was published [39]. There are also several reviews describing the basics of electroporation as well as its clinical use in electrochemotherapy and electrogene therapy [58-62]. Therefore, the aim of this review is to wrap-up studies on electrogene therapy with *IL-12* that culminated in the first published clinical study of electrogene therapy with *IL-12* for treatment of subcutaneous melanoma metastases [46] and therefore this therapy shows great promise to further translate this therapy into human and veterinary oncology.

PRECLINICAL STUDIES WITH *IL-12* ELECTROGENE THERAPY

The use of electroporation for transfection of cells dates back to 1982, when Neumann *et al.* demonstrated increased uptake of plasmid DNA into mouse lyoma cells using electroporation with a high-intensity electric field [63]. They demonstrated that when linear or circular plasmid DNA containing the *herpes simplex thymidine kinase* (*TK*) gene was added to a suspension of mouse L cells deficient in the *TK* gene and the cells were then exposed to electric fields, stable transformants were formed that survived in the HAT selection medium. After this first demonstration that electroporation can be used for delivery of plasmid DNA, the method gained a lot of attention since it represented a promising alternative to viral and chemical methods for introduction of genes of interest into cells. Gene electrotransfer to tissues was introduced by Titomirov *et al.* in 1991. The skin of newborn mice was transfected with plasmid DNA. The skin was then excised and NEO-resistant colonies were found in primary cell cultures obtained from the treated skin [64]. After this first study, the use of gene electrotransfer to tissues grew rapidly. Optimization studies aiming to optimize the parameters of electric pulses for application of gene electrotransfer to different tissues, as well as studies dealing with the therapeutic effect of gene electrotransfer were performed. Therapeutic applications of gene electrotransfer were focused mainly on two fields: DNA vaccination against infectious disease, and cancer gene therapy [65, 66]. The first use of electrogene therapy (a term used to describe gene therapy in which transfection of cells is achieved by means of electroporation) for treatment of cancer was published in 1999. Niu *et al*. demonstrated, that electrotransfer of a *Stat3* variant with dominant-negative properties to melanoma subcutaneous tumors induced in C57Bl/6 mice suppressed the growth of transfected tumors by inducing apoptotic cell death [65]. Protection against the lethal influenza virus was achieved by intramuscular electrotransfer of plasmid DNA encoding for neuraminidase from different subtype-A viruses [66].

The first evaluation of the effects of combined use of IL-12 and electroporation involved the delivery of plasmid DNA encoding *IL-12* (50 μg) by electrotransfer to skin. Local transfection of skin of BalbC mice resulted in increased serum concentration of interferon-γ (IFN-γ), whose production and secretion is induced by the presence of IL-12. The serum concentration of IFN-γ reached a peak of ~50 pg/ml two days after treatment. The electric pulse parameters that were used for skin electrotransfection with IL-12 plasmid DNA were the same as the ones used in electrochemotherapy protocols: 8 pulses, duration 100 μs, amplitude 1200 V, interelectrode distance 8 mm. In this study, besides an electric pulse protocol consisting of high voltage pulses of short duration (1500 V/cm voltage over interelectrode distance, 100 μs duration), another protocol consisting of low voltage pulses of longer duration (100 V/cm, 20 ms duration) was also tested [39]. Higher transfection efficiency was obtained in the skin with high voltage electric pulses of short duration. Later that year, the same group reported that electrotransfection of *IL-12* plasmid DNA to mouse muscle resulted in systemically measurable IL-12 and IFN-γ. The serum concentration of IL-12 reached a peak 4 days post-transfection (~150 pg/ml) and returned to baseline values ~3 weeks after the treatment. The peak of IFN-γ concentration lagged behind that of IL-12 and was reached ~1 week after electrotransfer (~250 pg/ml) and returned to an almost baseline level ~3 weeks after treatment [67]. Serum concentrations of IFN-γ after gene electrotransfer in muscles were 5 times higher than after electrotransfer in skin. Interestingly, for electrotransfer in muscles, low voltage electric pulses of longer duration (100 V/cm voltage over interelectrode distance, 20 ms duration) proved to be more effective and resulted in a higher level of gene expression and in long-term expression (at least 3 weeks post-transfection) [67]. These first two studies were performed on healthy, non-tumor bearing mice and provided evidence that *IL-12* electrotransfer to skin and muscle is an efficient delivery method which results in an increased systemic concentration of IL-12 and its effector molecule IFN-γ, and that it could be further used in immunotherapy protocols.

The first *IL-12* electrogene therapy study demonstrating antitumor effectiveness was performed by intratumoral injection of *IL-12* plasmid DNA (100 μg) followed by application of 10 electric pulses of 150 V amplitude (4 mm interelectrode distance) and 50 ms duration [68]. The growth of treated mouse hepatocellular carcinoma was significantly inhibited. In addition, the growth of distant non-treated tumors was also reduced, but to a lesser extent. Furthermore, development of spontaneous lung metastases was inhibited in mice treated with *IL-12* electrogene therapy. The mechanicsms of antitumor effectiveness of intratumoral *IL-12* electrogene therapy were evaluated and were found to be the same as indicated for other types of therapy involving the use of IL-12: increased IL-12 and IFN-γserum levels, tumor infiltration of NK and $CD3^+$ T cells and reduced microvascular density [68]. The peak in IL-12 serum concentration of 450 ng/ml was reached 5 days after treatment, while the peak in IFN-γ concentration of 100 pg/ml was reached on day 7. Compared to studies performed by Heller *et al.*, higher serum concentrations of IL-12 were achieved, while the concentrations of IFN-γ were in a similar range. The reason for the observed differences in IL-12 concentrations probably lies in the different plasmid concentrations injected as well as in transfection of different tissues (skin, muscle, and tumor). It seems that higher concentrations of IL-12 do not reflect in increased IFN-γlevels, demonstrating a saturation effect. Another *IL-12* electrogene therapy was preformed on a mouse squamous cell carcinoma (SCCVII) [42]. In this study, intramuscular *IL-12* electrotransfer was performed with a low dose of plasmid DNA (10 μg) and only 2 electric

pulses of 20 ms duration at 375 V/cm (voltage over interelectrode distance). Similarly to previous studies, increased serum levels of IL-12 and IFN-γ were obtained. However, the inhibition of growth of the established tumor was smaller than in previous studies. The authors also tested the prevention of tumor growth by electrogene therapy with *IL-12* first performed on the same day as when the SCCVII were inoculated and then repeated three more times at one-week intervals after inoculation. Tumors did not develop in 40% of treated animals, thus demonstrating that electrogene therapy with *IL-12* has an effect on the establishment of tumors [42]. In the same year, other researchers performed *IL-12* electrogene therapy in mouse melanoma B16 tumors [40, 41] demonstrating that *IL-12* electrogene therapy is also effective on a melanoma tumor model, resulting in significantly prolonged tumor growth delay. In addition, *IL-12* electrotransfer was compared to adenoviral delivery by measuring tumor and serum levels of IL-12. Tumor IL-12 levels were comparable, while serum concentrations of IL-12 were much higher after adenoviral delivery. Furthermore, apparent toxicity of adenoviral *IL-12* delivery was observed, which was demonstrated by animal weight loss, apathy and splenomegaly. Although the authors did not compare the antitumor effectiveness of the two different delivery methods for *IL-12*, they concluded that *IL-12* electrogene therapy has great therapeutic potential as it results in a high local cytokine concentration without systemic increase which could lead to systemic toxicity also observed in adenoviral IL-12 gene therapy [41]. Another study performed on B16 melanoma compared the antitumor effectiveness of IL-12 gene therapies performed either intratumorally or intramuscularly [69]. The intratumoral IL-12 electrogene therapy resulted in better antitumor effectiveness than the intramuscular IL-12 electrogene therapy. In this study, 47% of mice were cured after intratumoral electrogene therapy, while in previous studies on electrogene therapy with IL-12 performed on a melanoma B16 tumor model, no tumor cures were obtained. This discrepancy is most probably due to different electric pulses used in these studies (high voltage microsecond pulses compared to low voltage millisecond pulses) and also to the different promoters used. Both, Lohr *et al.* and Lucas *et al.* used the CMV promoter that can be methylated and thus inactivated, thus leading to downregulation of downstream genes in *in vivo* conditions [70]. However, they used different electric pulse parameters. Kishida *et al.* used a CMV immediate enhancer/β-actin (CAG) promoter that was shown to lead to sustained expression [71], but they used electric pulses of very low amplitude (50 V). In a recent study, a detailed evaluation of different electric pulse parameters was done on B16 melanoma. In this study, it was demonstrated that both electropermeabilization and electrophoresis are involved in gene electrotransfer [72]. Therefore, one can speculate that low antitumor effectiveness obtained in studies of Lohr *et al.* and Kishida *et al.* was due to low transfection efficiency as they used a small number of electric pulses that produce an electrophoretic force on DNA without sufficient electropermeabilization. On the other hand, Lucas *et al.* used electric pulses that mainly produce electropermeabilization [40, 69]. In the case of B16 melanoma that have a soft consistency with large cells and minimal content of the extracellular matrix [73], high voltage electric pulses of microsecond duration resulted in better transfection efficiency compared to electrophoretic electric pulses, therefore leading to better antitumor effectiveness. Furthermore, Lucas *et al.* demonstrated that intratumoral *IL-12* electrogene therapy leads to high local IL-12 and IFN-γ levels, tumor infiltration of lymphocytes and a reduced number of vessels in the tumors. On the other hand, intramuscular *IL-12* electrogene therapy resulted in increased IL-12 and IFN-γ levels in serum but not in tumors and no tumor cures were obtained. In addition, the effects on tumor vascularity and $CD4^+$ and $CD8^+$ T lymphocytes were less pronounced compared to intratumoral gene therapy [69]. In the same study, intratumoral *IL-12* electrogene therapy was also performed in athymic nude mice, which lack T lymphocytes. The antitumor effect was minimal compared to pertinent control groups, demonstrating a pivotal role of T lymphocytes in antitumor effectiveness of *IL-12* electrogene therapy. The observed minimal antitumor effect in nude mice could be due to either antiangiogenic properties of IL-12 or to its effect on stimulation of NK cells and the humoral immune response, but this was not elucidated in the study. It is important to note that the immunocompetent mice which were cured were challenged with B16 tumor cells and 71% of mice did not develop tumors for 100 days, thus demonstrating that antitumor immunity was developed following *IL-12* electrogene therapy [69]. Antitumor immunity was also demonstrated in another study on effectiveness, where intratumoral *IL-12* electrogene therapy was performed 3 times and resulted in 80% of tumor cures [74]. These mice were rechallenged with tumor cells and none of them developed a tumor. In addition, it was demonstrated that intratumoral gene therapy has an effect on growth of distant tumors which were induced on the day of treatment of the primary tumors – tumors did not develop in 56% of mice. Furthermore, the occurrence of induced lung metastases was also reduced by intramuscular treatment on the day of intravenously injected B16 tumor cells and repeated on day 4 post-tumor cell injection. Only 37.5% of treated mice developed lung metastases [74]. However, the effect of intratumoral *IL-12* electrogene therapy on development of lung metastases was not evaluated.

The antitumor effectiveness of intratumoral *IL-12* electrogene therapy was demonstrated also in other types of cancer, such as colon carcinoma, renal cancer, lymphoma, breast carcinoma and sarcoma mouse tumor models [75-80]. Treatment of CT26 colon carcinoma tumors with 3 times repeated intratumoral *IL-12* electrogene therapy resulted in significant inhibition of tumor growth. However, no tumor cures were observed. Prolonged therapy (7 times every two days) resulted in 80% tumor cures. Rechallenge of the 4 cured mice with tumor cells was performed 50 days after therapy. Tumors in rechallenged mice grew slower compared to naïve mice and in one mouse no tumor developed, demonstrating that these mice developed antitumor immunity. In the same study, subcutaneous renal carcinomas were treated with intratumoral *IL-12* electrogene therapy. Tumors were treated twice at a 1-week interval and significant antitumor effectiveness was achieved [75], but no tumor cures were obtained. Lee *et al.* evaluated intramuscular *IL-12* electrogene therapy on three different subcutaneous tumor models, lymphoma 38C13, colon carcinoma CT-26 and melanoma B16F1 only three days after tumor inoculation. Intramuscular *IL-12* electrogene therapy was very effective in these

small tumors. The most pronounced effect was on lymphoma tumors with ~70% tumor cures, followed by CT-26 colon carcinoma with ~55% tumor cures, while no tumor cures were obtained in B16F1 melanoma, which confirmed the study of Lucas *et al.* [69, 76]. However, intramuscular *IL-12* electrogene therapy was less effective for treatment of induced lung metastases of B16F1 and CT-26 tumors, resulting in a reduced number and size of metastases compared to control groups but without eradication of metastases. Although the authors did not address this difference in antitumor effectiveness, one can speculate that intravenously injected tumor cells that colonize the lungs have a better vascular supply and can thus seed and grow quicker compared to tumor cells that are injected into subcutaneous tissue. Therefore, the IL-12-induced antitumor mechanisms attack a smaller number of tumor cells in the case of subcutaneous tumors as opposed to lung metastases. This notion is also supported by a study by Lucas *et al.*, who treated induced lung metastases of B16 tumor cells on the same day and demonstrated that 37.5% of mice did not develop metastases [74]. Furthermore, Tevz *et al.* showed in a different tumor model, SA-1 sarcoma, that prophylactic treatment with intramuscular *IL-12* electrogene therapy (1 day before injection of tumor cells) resulted in a 90% reduction of lung metastases, while treatment 24 h after intravenous injection of tumor cells resulted in only a minor effect (30% reduction compared to control untreated group) [78]. In the same study, 3 times repeated intramuscular *IL-12* electrotransfer resulted in 27% SA-1 and 14% LPB subcutaneous sarcoma tumor cures. The effects of peritumoral and intratumoral *IL-12* electrogene therapy were compared on the same tumor model, the SA-1 sarcoma [79]. This was the first study which addressed the antitumor effectiveness of *IL-12* electrogene therapy applied to skin in the vicinity of established tumors – peritumoral electrogene therapy. As expected, the intratumoral *IL-12* electrogene therapy resulted in better antitumor effectiveness in this tumor model (it produced 90-100% tumor cures) than peritumoral therapy. This direct antitumor effectiveness was even better than in the reports on the effectiveness of *IL-12* gene therapies employing either viral or non-viral gene delivery methods [54, 81, 82]. Peritumoral *IL-12* electrogene therapy resulted in 16% tumor cures with a significant growth delay of the remaining tumors, which is comparable to the effect after 3 times repeated intramuscular therapy [78]. Furthermore, both intratumoral and peritumoral therapies resulted in a detectable increase in tumor and serum IL-12 and IFN-γ levels that did not differ between the routes of administration. In line with these results, the growth of distant untreated tumors was delayed to the same degree regardless of the therapy used. As shown with other tumor models, the animals that were rechallenged with SA-1 tumor cells after intratumoral *IL-12* electrogene therapy did not develop tumors in 61% of cases [79]. The pronounced antitumor effectiveness of the studies on sarcomas can be ascribed to several facts. One is that the SA-1 tumor model is highly immunogenic [83] and the other is that electric pulse parameters used in these studies were optimized for tumors, muscles and skin respectively [72, 84, 85], and therefore high transfection efficiencies were obtained that translated in pronounced antitumor effectiveness. Another tumor model breast adenocarcinoma BJMC3879, was treated with intratumoral *IL-12* electrogene therapy performed once a week for 8 weeks. The treatment resulted in significant subcutaneous tumor growth delay and in reduction of lymph node and lung metastases [77].

Most of the studies of *IL-12* electrogene therapy were performed in mice. Recently, two studies were published that evaluated gene transfer of *IL- 12* in beagle dogs. Pavlin *et al.* evaluated different electric pulse parameters for intramuscular gene transfer to determine an effective protocol for further clinical use. Besides reporter gene (*green fluorescent protein*) to determine local transfection efficiency, IL-12 was also used to determine whether intramuscular gene electrotransfer results in a systemically measurable increase in IL-12 and IFN-γ. Electric pulse protocols that resulted in the highest local transfection (tested with reporter gene expression) were tested with *IL-12* gene electrotransfer. The serum level of IL-12 was increased in only one dog (out of 6), while IFN-γ levels were increased in half of the treated dogs. Blood biochemistry values were all within the reference values up to 4 weeks after electrotransfer to muscles. The only side- effect that was observed and was connected to the therapy was tissue swelling at the site of electroporation, but was only transitory [86]. Another study on beagle dogs evaluated the effectiveness of intratumoral *IL-12* electrogene therapy on a transplantable canine transmissible venereal tumor. Significant growth inhibition was observed which even led to complete responses. In addition, a systemic antitumor effect was achieved that prevented growth of new tumors and cured an established tumor grown at a distant site [87].

In summary, preclinical studies of *IL-12* electrogene therapy have proved that this therapy is a feasible and effective approach to treatment of established tumors and metastases of many different histologies. Furthermore, rechallenge experiments demonstrate that antitumor immunity developed after *IL-12* electrogene therapy. Variability in tumor response can be ascribed to different models used in the studies, to different route of plasmid DNA administration (intratumoral, peritumoral, intramuscular), different doses of plasmid DNA and also to different electric parameters as well as different electrode designs. Overall, intratumoral *IL-12* electrogene therapy was the most effective treatment, followed by intramuscular and peritumoral administration. The intratumoral route of administration produced a high level of IL-12 and IFN-γ in tumors with minimal or no increase in serum IL-12 and IFN-γ levels. Systemic toxicity was not observed in any of the studies. A comprehensive toxicity evaluation was performed after intratumoral *IL-12* electrogene therapy in B16 melanoma tumor-bearing mice [88]. No significant toxic effects were observed in animals treated with *IL-12* intratumoral electrogene therapy and the only histopathological abnormality found in these mice 30 days after treatment was inflammation associated with kidneys. Otherwise, these animals showed less abnormalities compared to other non-treated tumor-bearing mice, which was due to the reduced tumor burden [88].

Mechanisms of antitumor effectiveness of *IL-12* electrogene therapy were addressed in many studies. The antitumor action of IL-12 is multifaceted and involves activation of innate resistance as well as adaptive immunity. Increased local and systemic levels of IL-12 and IFN-γ were demonstrated; these levels can be controlled by the dose of the

plasmid DNA, the route of administration, the parameters of electric pulses and the number of applications. Intratumoral *IL-12* electrogene therapy induced infiltration of immune cells $CD8^+$ T lymphocytes, active iNOS+ macrophages and NK cells. Infiltration of immune cells, especially $CD8^+$ T lymphocytes, were absent or much less pronounced after intramuscular therapy. Tumor-specific cytotoxic T lymphocytes (CTL) activity measured in lymphocytes isolated from spleens was higher after intratumoral than after intramuscular *IL-12* electrogene therapy. Furthermore, studies performed in nude mice lacking T lymphocytes demonstrated the main role of T lymphocytes in tumor eradiation for successful *IL-12* electrogene therapy. Depletion of $CD4^+$ T lymphocytes from immunocompetent mice did not affect tumor eradication, while depletion of NK cells partially reversed *IL-12* electrogene therapy demonstrating an important role of NK cells in the antitumor action of IL-12 [68, 69, 77, 89]. Antiangiogenic effects were also demonstrated for *IL-12* electrogene therapy. Immunohistological staining of CD31 in tumors following *IL-12* electrogene therapy demonstrated reduced microvessel density and electron microscopy also revealed apoptosis of endothelial cells [68, 69, 77, 89, 90]. In addition, the expression of VEGF was decreased in tumors treated by *IL-12* electrogene therapy. IP-10 and Mig, chemokines that are regulated by IFN-γ and are involved in inhibition of angiogenesis, were upregulated in tumors treated by *IL-12* electrogene therapy [90]. Furthermore, a microarray study showed that Mig, Stat1 and IRF7 were the three genes that were the most altered after *IL-12* electrogene therapy. IRF7 accumulated in the nuclei upregulated the expression of Mig and Stat1 after intratumoral *IL-12* electrogene therapy to a larger extent than after intramuscular *IL-12* electrogene therapy. Additional analysis demonstrated involvement of Mig in induction of $CD4^+$ and $CD8^+$ T cell infiltration in tumors and that a lack of Stat1 expression inhibited IL-12-mediated induction of IP10 [91].

Taken together, the antitumor action of *IL-12* electrogene therapy is pleotropic and involves different elements, infiltration of effector immune cells and antiangiogenic action. It appears that for complete tumor eradication, which was mainly observed after intratumoral *IL- 12* electrogene therapy, tumor infiltration with $CD8^+$ T cells is necessary, while prevention of tumor establishment, growth of distant tumors and metastases, is also regulated by NK cells, a protective B cell response and the antiangiogenic action mediated by IFN-γ induction of IP10 and Mig (Fig. (**1**)).

IL-12 ELECTROGENE THERAPY IN COMBINATION WITH OTHER THERAPIES

Electrogene therapy with *IL-12* was not curative in certain tumor types when injected intratumorally or in most cases when it was injected into the muscle or into the skin. Therefore, several studies address the possibility of combining *IL-12* electrogene therapy with other treatments. Some of the studies combined it with electrochemotherapy, which is another method using electroporation that is already in clinical practice with the chemotherapeutic drugs cisplatin and bleomycin. The other studies combined it with other therapeutic genes, and some with standard established cancer treatments, such as radiotherapy.

The combination of electrochemotherapy with bleomycin and electrogene therapy with IL-12 was addressed in two studies. In 2003, treatment effectiveness of intratumoral injection of a mixture of bleomycin with plasmid DNA encoding *IL-12* followed by application of low voltage electric pulses (125 V/cm voltage over interelectrode distance) of 50 ms duration was evaluated in B16 melanoma tumors. Significant tumor growth delay and reduction of induced lung metastases with 37.5% cured mice were observed [92]. Another study was performed in squamous cell carcinoma SCC-VII and high-grade malignant mammary 4T1 tumors. Increased antitumor effectiveness was obtained with 60% of 4T1 mammary tumor cures and 100 % SCCVII tumor cures. In addition, inhibition of metastatic tumor growth and prevention of redevelopment of tumors was also achieved [93]. The authors studied the contribution of single agents (bleomycin or IL-12) to antitumor effectiveness of a combined treatment with both agents. The vascular-disrupting effect was attributed to the action of bleomycin and stimulation of the immune response to IL-12. The vascular effect of electroporation and, specifically, the vascular-disrupting effect of electrochemotherapy with bleomycin are well-known [94], supporting the results obtained by Torrero *et al.* [93].

Improvement of the therapeutic effectiveness of *IL-12* electrogene therapy was also achieved by additional treatment of melanoma B16 tumors with *IL-18* plasmid DNA that was mixed with *IL-12* plasmid DNA. Repetitive intratumoral treatment (4-times treatment on days 0, 2, 10, 12) with both cytokines resulted in ~70% tumor cures, while the treatment with *IL-12* electrogene therapy alone was not curative [40]. Combined intratumoral electrogene therapy with plasmid DNA encoding *IL-12* and *Herpes simplex virus thymidine kinase* was performed in B16 and CT-26 tumor models, combining immunotherapy with the suicide gene. The therapy was repeated twice, four or six times. The number of CT26 tumor cures increased with the increased number of therapy repetitions and reached 91%, when the therapy was repeated 6 times in 2-day intervals. In B16 tumors, 4 times repeated therapy resulted in 92% growth inhibition [95]. Electrogene therapy combining the costimulatory molecule *B7.1*, which was shown to activate $CD4^+$ and $CD8^+$ T lymphocytes [96], and *IL-12*, was tested in squamous cell carcinoma SCCVII, which lacks B7.1, and the prostate carcinoma TRAMP tumor model. The effect was more pronounced in SCVII tumors with 80% tumor cures. The cured mice also developed antitumor immunity [97]. A high complete response rate (100%) was also obtained in CT-26 tumors treated with a combination of *IL-12* and *IL-27* electrogene therapy. This high complete response rate was only obtained when *IL-12* electrogene therapy was performed 10 days before *IL-27* electrogene therapy. The response of the highly malignant 4T1 tumor was less pronounced, resulting in 33% tumor cures [98]. An interesting study was performed on the mouse mammary tumor D2F2 using combined intratumoral electrogene therapy with *IL-12* and *tetanus toxin fragment C.* This therapy was effective only in mice with depleted regulatory T lymphocytes (Treg) resulting in 63% tumor cures, while in mice without depletion, this therapy only had a minor effect on tumor growth. These results are in contradiction with other published reports on *IL-12* electrogene therapy. The reason for this discrepancy was not addressed by

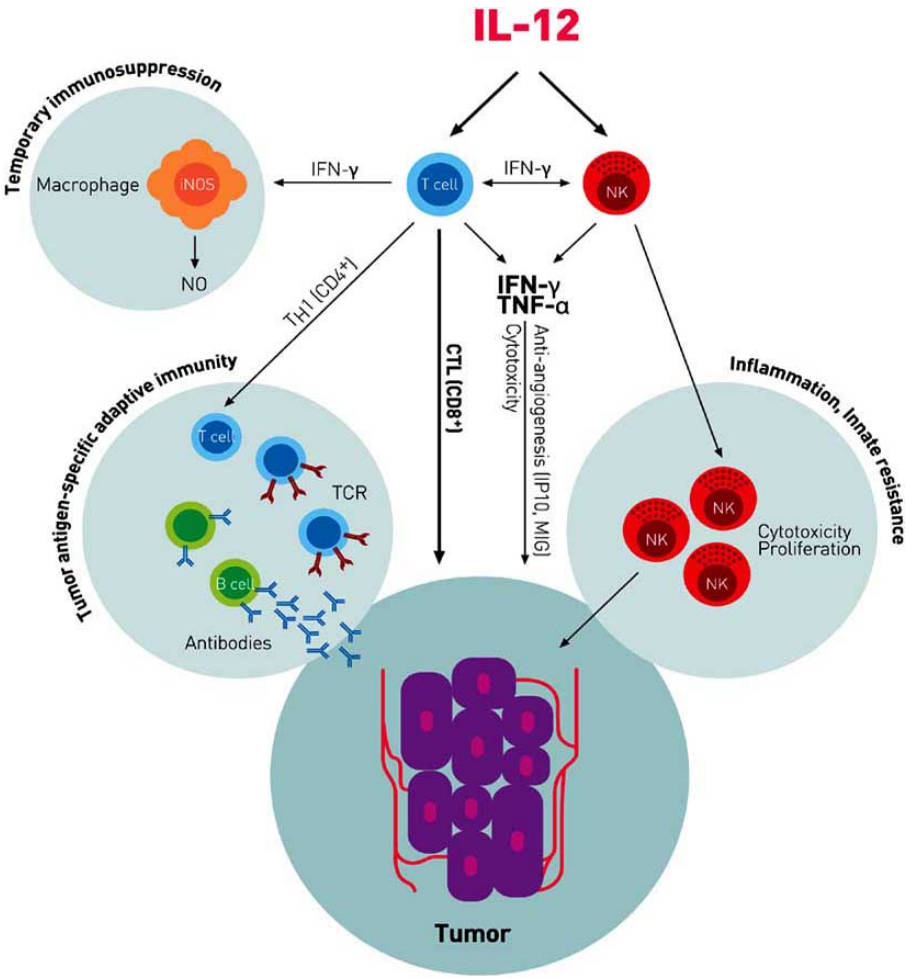

Fig. (1). Mechanisms involved in antitumor effectiveness of IL-12.

the authors and remains to be elucidated [80]. Recently, intratumoral *IL-12* electrogene therapy combined with small interference RNA (siRNA) targeting microphthalmia-associated transcription factor (Mitf) was evaluated in B16 melanoma [99]. Mitf is a transcription factor regulating various genes associated with the melanin synthesis pathway and malignant transformation of melanocytes into malignant melanoma [100]. Combined treatment with intratumorally injected siRNA duplexes against Mitf (20μg) mixed with 5μg of plasmid DNA encoding *IL-12* and followed by application of electric pulses to the tumor resulted in pronounced antitumor effectiveness. It was more effective than single treatments with *IL-12* electrogene therapy alone or siRNA against Mitf alone. However, no tumor cures were obtained [99].

A radiosensitizing effect of recombinant protein IL-12 and local adenoviral gene therapy was indicated when combined with radiotherapy in previous studies [101-105]. To date, only intramuscular electrogene therapy with *IL- 12* was tested in combination with local radiotherapy. Combined treatment was evaluated on established subcutaneous sarcoma tumors SA-1 and LPB and on induced SA-1 lung metastases. *IL-12* electrogene therapy repeated three times with one tumor irradiation applied 24 h after the first electrogene therapy resulted in ~45% SA-1 tumor cures and 100 % LPB tumor cures. The effect on induced lung metastases was also determined. Prophylactic electrogene therapy performed 24 h before irradiation resulted in almost complete reduction of SA-1 lung metastases. A 1.3 fold dose-modifying factor was determined when electrogene therapy was combined with graded irradiation doses [78].

Results from studies combining *IL-12* electrogene therapy with other treatments pointed out that boosting the immune response of the organism by IL-12 is very efficient when *IL-12* electrogene therapy is combined with standard treatments, such as chemotherapy or radiotherapy. These treatments resulted in a high percentage of complete eradication of primary tumors and induced lung metastases also leading to development of antitumor immunity. Combined therapies with other gene therapies were less effective. Further studies on evaluation of toxicities associated with *IL-12* electrogene therapy combined with standard treatment are warranted to translate these effective treatments into clinical studies.

CLINICAL STUDIES WITH *IL-12* ELECTROGENE-THERAPY

Gene therapy with *IL-12* showed remarkable antitumor activity in different tumor models at the preclinical level, and has progressed to a number of clinical trials in both human

and veterinary medicine. In these studies, different delivery methods for *IL-12* were used: naked plasmid DNA injection, adenoviral and retroviral vector and electroporation [28, 46, 48, 50-54, 106-108].

In veterinary oncology, three preliminary studies were reported in the congress proceedings [106-108]. Canine mast cell tumors, which are the most common malignant cutaneous tumors in dogs, with extremely variable biological behavior, which can make the proper staging of disease and therefore choice of appropriate therapy very challenging, were treated with intratumoral *IL-12* electrogene therapy. Eight dogs with 11 cytologically confirmed tumors were included in the study. A good local antitumor effect with significant reduction of treated tumors' size, ranging from 15% to 83% (mean 52%) of the initial tumor volume was obtained (Fig. (**2**)). Additionally, a change in the histological structure of treated nodules was seen as reduction in the number of malignant mast cells and inflammatory cell infiltration of treated tumors. Furthermore, systemic release of IL-12 and IFN-γ in treated dogs was detected, without any noticeable local or systemic side-effects. Again, the data suggest that intratumoral *IL-12* electrogene therapy could be used for controlling local as well as systemic disease [107, 108]. In horses, sarcoid represents a spontaneous model of tumor which is frequent in equine oncology and is in many cases a real therapeutic challenge. The combination of electrochemotherapy with intratumoral cisplatin and intratumoral *IL-12* electrogene therapy was performed in 6 sarcoids with poor prognosis.The clinical response, increased mRNA IFN-γ levels present in lymphocytes and the presence of $CD4^+$ and $CD8^+$ T cells in the immunohistochemically-stained tumor section, demonstrated good antitumor effectiveness of this approach [106].

The first clinical study on *IL-12* electrogene therapy was published in 2008. A phase I dose escalation study of intratumoral *IL-12* electrogene therapy was carried out in 24 patients with malignant melanoma subcutaneous metastases. Patients were treated 3 times on days 1, 5 and 8. The maximum dose of plasmid per tumor nodule was 1.6 mg, which resulted in a cumulative dose of 3.8 and 5.8 mg in two patients with multiple nodules. Fine needle aspiration biopsy was performed before, whilst excisional biopsy was performed after the treatment to assess histology of tumors, immune cell infiltration and to determine the levels of IL-12 in tumors. Reponses to treatment were evaluated by the modified Response Evaluation Criteria in Solid Tumors (RECIST). The response to therapy was observed in treated as well as in distant non-treated tumor nodules. In 53% of patients a systemic response was observed resulting in either stable disease or an objective response. The major adverse side-effect was transient pain after application of electric pulses. In post-treatment biopsies, tumor necrosis and immune cell infiltration was observed. This first human clinical trial with IL-12 electrogene therapy in metastatic melanoma proved that this therapy is safe and effective. It also forms a firm foundation for further clinical evaluation of this approach [46].

CONCLUSIONS

The literature review demonstrates that many preclinical and clinical studies were done and are still going on in gene therapy of cancer. Although the vast majority of studies were performed with viral vectors, non-viral approaches are gaining interest, due to their safety and simplicity in plasmid preparation. In addition, one of the major advantages of electrogene therapy with *IL-12* is that it does not result in systemic toxicity. Neither intratumoral nor intramuscular or peritumoral *IL-12* electrogene therapy resulted in any adverse effects that were previously demonstrated for antitumor treatments with recombinant IL-12 protein or adenoviral approaches. Many of the studies have dealt with *IL-12* electrogene therapy as a means to target local or systemic disease, because IL-12 is a potent inductor of antigen-specific adaptive immunity and innate resistance. Although clinical

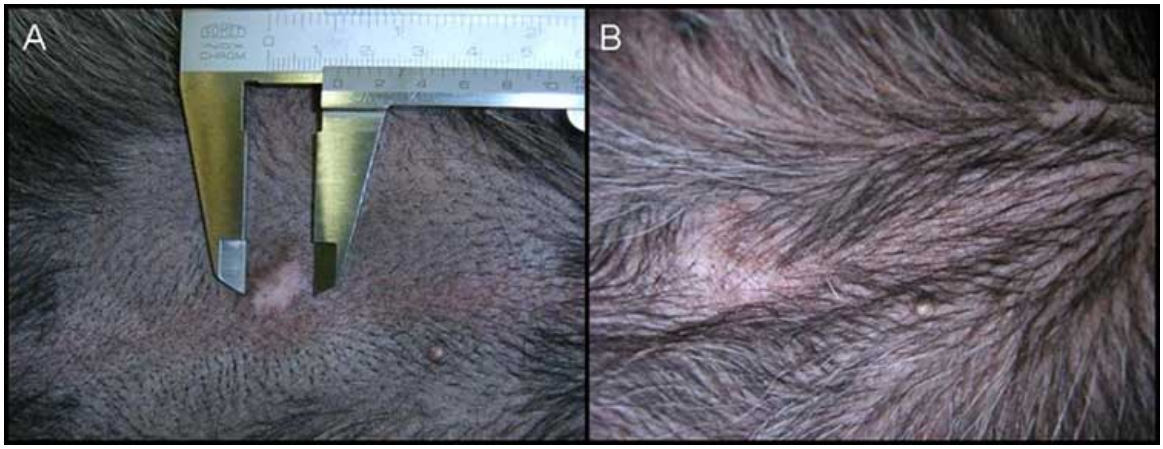

Fig. (2). Pictures of mast cell tumor in dog treated with intratumoral *IL-12* electrogene therapy. Tumor was located on linea alba. Due to the inconvenient location of tumor for surgical intervention, electrochemotherapy with cisplatin was performed. Initial size of the tumor was 1.9 x 1.4x 0.7 cm. Electrochemotherapy was performed by intrautmoral injection of 4 mg of cisplatin followed by application of electric pulses. Needle electrodes were used with interelectrode distance 4 mm (420 V, 100 μs). One month afer therapy, fine needle aspiration was performed that confimed the presence of residual malignant cells **(A)**. Therefore, intratumoral *IL-12* electrogene therapy was performed on the remaining tumor nodule. Electrogene therapy consisted of injection of plasmid DNA encoding for IL-12 (1 mg) followed by two application of electric pulses (360 V amplitude, 5 ms duration, plate electrodes with 6 mm interelectrode distance,). Tumor was completely eradicated within 1 month **(B)** and has been currently in complete response for more than 1 year.

Table 1. Preclinical Studies in Mice of *IL-12* Electrogene Therapy as a Single Antitumor Treatment

Tumor Type	Type of Therapy	Treatment Outcome	Reference
Hepatocellular carcinoma	Intratumoral	Inhibition of growth of treated tumors Inhibition of growth of distant non-treated tumors Inhibition of development of lung metastases	[68]
Squamous cell carcinoma	Intramuscular	Inhibition of tumor growth Inhibition of tumor establishment	[42]
Melanoma	intratumoral	Inhibition of tumor growth	[40, 41]
Melanoma in immunocompetent mice	Intratumoral and intramuscular	47% TC[1] after intratumoral therapy No TC after intramuscular 71% mice resistant to rechallenge	[69]
Melanoma in nude mice	intratumoral	No TC Importance of immune system demonstrated	[69]
Melanoma	3 times intratumoral	80% TC 100% mice resistant to rechallenge Inhibition of growth of distant non-treated tumors	[74]
Melanoma	2 times intramuscular	Inhibition of development of induced lung metastases	[74]
Colon carcinoma	3 or 7 times intratumoral	80% TC after 7-times repeated therapy Development of antitumor immunity	[75]
Renal carcinoma	2 times intratumoral	Inhibition of tumor growth	[75]
Melanoma (<3 mm)	intramuscular	No TC	[76]
Colon carcinoma (<3 mm)	intramuscular	55% TC	[76]
Lymphoma (<3 mm)	intramuscular	70% TC	[76]
Breast carcinoma	8 times intratumoral	Inhibition of tumor growth Reduction of lung and lymph node metastases	[77]
Sarcoma	Intramuscular	90% reduction of induced lung metastases 14-28% TC	[78]
Sarcoma	Intratumoral	90-100% TC Reduction of growth of distant non-treated tumors 61% mice resistant to rechallenge	[79]
Sarcoma	peritumoral	16% TC	[79]

[1]TC = tumor cures.

Table 2. Preclinical Studies in Mice of *IL-12* Electrogene Therapy in Combination with Other Treatment Modalities for Treatment of Tumors

Tumor Type	Type of Therapy	Treatment Outcome	Reference
Melanoma	Intratumoral *IL-12* + bleomycin	37.5% TC[1] Inhibition of growth of lung metastases	[92]
Squamous cell carcinoma	Intratumoral *IL-12* + bleomycin	80% TC Inhibition of growth of lung metastases 40% mice resistant to rechallenge	[93]
Mammary carcinoma	2-times intratumoral *IL-12* + bleomycin	60% TC Inhibition of growth of lung metastases	[93]
Melanoma	4-times Intratumoral *IL-12* + intratumoral *IL-18*	70% TC	[40]

(Table 2) contd…..

Tumor Type	Type of Therapy	Treatment Outcome	Reference
Melanoma	2, 4 and 6-times intratumoral *IL-12* + Herpes virus thymidine kinase	Inhibition of tumor growth after 4-times repeated therapy	[95]
Colon carcinoma	2, 4 and 6-times intratumoral *IL-12* + Herpes virus thymidine kinase	91% TC after 6-times repeated therapy	[95]
Squamous cell carcinoma	Intratumoral *IL-12* + B7.1	80% TC 75% mice resistant to rechallenge	[97]
Prostate carcinoma	Intraumoral *IL-12* + B7.1	No TC, inhibition of tumor growth	[97]
Colon carcinoma	Intratumoral *IL-12* + *IL-27*	100% TC	[98]
Mammary carcinoma	Intratumoral *IL-12* + *IL-27*	33% TC	[98]
Mammary carcinoma	Intratumoral *IL-12* + tetanus toxin fragment C	63% TC in Treg depleted mice	[80]
Melanoma	Intratumoral *IL-12* + siRNA against Mift	No TC, inhibition of tumor growth	[100]
Sarcoma	Intramuscular *IL-12* + radiotherapy	45-100% TC	[78]

[1]TC=tumor cures.

studies with recombinant IL-12 were a disappointment, gene therapy with *IL-12*, due to its controlled release and lower toxicity, has gained a lot of interest and has the potential to be translated into the clinic.

The studies have demonstrated that *IL-12* gene electrotransfer is as effective approach as local treatment by intratumoral gene electrotransfer, or as systemic treatment by muscle gene electrotransfer. Furthermore, peritumoral treatment has also shown both local and systemic effects. All these approaches can target accessible tumor nodules by a direct intratumoral approach or disseminated disease in the case of intramuscular gene electrotransfer. Due to the still limited effectiveness of *IL-12* gene electrotherapy, combined approaches with established treatments such as radiotherapy, electrochemotherapy were performed. Synergistic or additive effects were observed.

Antitumor effectiveness, local and loco-regional, of intratumoral *IL-12* gene electrotransfer was already demonstrated in a clinical trial on melanoma metastases. The first studies are also being undergone in veterinary oncology, supporting the clinical experience in human medicine and demonstrating that *IL-12* electrogene therapy can be successfully used in the treatment of primary tumors and metastases in dogs and horses.

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