SHORT COMMUNICATION

Regression of subcutaneous B16 melanoma tumors after intratumoral delivery of an IL-15-expressing plasmid followed by *in vivo* electroporation

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In vivo electroporation has been used to efficiently deliver drugs and 'therapeutic' genes to tumors, including melanoma lesions. This study reports on the effect of intratumoral delivery of an optimized DNA plasmid expressing interleukin-15 (pIL-15) on established murine melanoma tumors. IL-15 has been demonstrated to have a pivotal role in the function of memory CD8 + T cells and natural killer cells, which are critical for tumor immunosurveillance. In this study, C57BL/6 mice were injected with B16.F10 melanoma cells and randomized into different experimental groups: untreated (P–V–E–), treated with pIL-15 (P+) or backbone plasmid (V+), with or without electroporation (E+ or E–). Treatment was performed intratumorally with 50 µg of plasmid on days 0, 4 and 7 and tumor volume/size, tumor regression and long-term survival were measured. At day 100 after initiation of treatment, the percentage of mice surviving with complete tumor regression in the P–V + E+, P + V–E–, P + V–E+ and P–V–E– treatment groups were 0, 12.5, 37.5 and 0%, respectively. These results demonstrate the ability of pIL-15 to mediate B16 melanoma regression, with the effect being significantly enhanced by electroporative delivery. This is the first description of the ability of a naked DNA plasmid expressing IL-15 to alone mediate complete regression of B16 melanoma tumors and underscores the potential clinical use of these plasmids for the treatment of malignant tumors when delivered with *in vivo* electroporation.

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Introduction

Electroporation is a method for delivery of molecules into cells utilizing electrical fields to increase membrane permeability, which allows for the entry of normally impermeable molecules into the cytoplasm. By applying an electric field to tissues that have received DNA plasmids coding for specific proteins, the expression of the proteins has been increased significantly compared to that expressed in the absence of electroporation.¹ These findings suggest that this method could be utilized for the effective delivery of genes expressing 'therapeutic' proteins, including cytokines, with the potential of enhancing a clinical effect.

Studies have demonstrated that electrically mediated delivery of plasmids encoding therapeutic molecules can be directed to different tumor types including melanomas.^{2,3} Examples involving experimental melanoma treatment demonstrate that delivery of plasmids encoding tumor antigens as well as some cytokines elicit an antitumor effect.³ This approach has recently been reviewed and underscores the potential for the delivery of plasmids expressing cytokines with antitumor activity through *in vivo* electroporation.⁴

Interleukin-15 (IL-15) is a 15 kDa cytokine protein that uses the gamma and beta chains of the IL-2 receptor complex with a unique alpha chain to signal T cells.⁵ It stimulates memory CD8 + cells in contrast to IL-2, which inhibits memory CD8 + T-cell proliferation. In addition, IL-15 also inhibits IL-2-mediated activation-induced cell death (AICD) associated with self-tolerance. Likewise, in addition to stimulating memory CD8 + T cells, IL-15 also stimulates the activation, proliferation and cytotoxicity of natural killer (NK) cells.⁶ Because of the roles of CD8 + memory T cells and NK activity in immunity against tumors, IL-15 has been targeted as an antitumor cytokine

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with potential advantages over IL-2.⁶ It was therefore hypothesized that IL-15, when delivered as a DNA plasmid through electroporation, could mediate anti-tumor activity. This study summarizes the first reported analysis of the therapeutic potential of intratumoral delivery, through electroporation, of an IL-15-expressing plasmid into established B16 murine melanoma tumors. The conclusions of the study indicate the ability of an IL-15-expressing DNA plasmid to mediate complete regression of subcutaneous B16.F10 melanoma tumors, with the incidence of regression being significantly enhanced when delivered by *in vivo* electroporation.

Materials and methods

Mice, cell lines and plasmids

The human IL-15 expression plasmid (pIL-15) used was optimized for maximal expression and was 80-fold more efficient than standard pcDNA3-based plasmids. The cloning and generation of this plasmid has been described previously.⁷ In addition, this human IL-15-expressing plasmid was demonstrated to be approximately 70% homologous to murine IL-15 and was shown to enhance antigen-specific CD8⁺ immune responses in mice.⁷ Also, it has been shown that the human IL-15, generated from the plasmid, did not induce murine anti-human IL-15 antibodies after injection into mice.

Briefly, the strategy for plasmid optimization involved the insertion and replacement of the existing Kozak sequence with a stronger Kozak sequence as well as removing upstream inhibitory AUGs through primer design. In addition to these changes, the native long signal peptide sequence was replaced by an optimized leader sequence, which had been shown to enhance secretion and expression of the protein. Subsequently, the optimized IL-15 plasmid was inserted into a cloning vector, which contains a ubiquitous and constitutively active promoter. In the experiments reported here, the optimized IL-15 plasmid has been designated pIL-15. All of the DNA generated for use in these experiments was produced using endotoxin-free Clontech Giga (Clontech, Palo Alto, CA) kits.

C57BL/6 mice, the murine strain syngeneic for the B16F10 melanoma tumor cell line, were used in this study and were purchased from the National Cancer Institute. Mice were housed and maintained during this study in accordance with Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) guidelines. The B16.F10 murine melanoma cell line clone (CRL 6475) was originally purchased from ATCC and was maintained for studies as monolayers in culture in 90% McCoy's medium supplemented with 10% fetal bovine serum. For the preparation of the single-cell suspension for tumor induction, monolayers of cells were detached from flasks using trypsin-ethylenediaminetetraacetic acid.

Tumor induction and measurement

Tumors were induced by the subcutaneous injection of 10⁶ B16.F10 cells (greater than 90% viability by Trypan

blue exclusion) into the left flanks of C57BL/6 mice. Tumors were permitted to grow to an average size (i.e. volume) of 40 mm³ before initiation of the treatment regimen. This approximate tumor volume has been determined to be an ideal minimal size for intratumoral injection as the administered treatment volume is retained effectively within the lesion with no significant leakage, providing confidence that the entire dose had been administered. This mean tumor volume for initial therapeutic intratumoral injection has been used in previous studies.³ Tumor volumes were determined before and at periodic intervals following treatment, using a digital caliper by measuring the longest diameter (a) and the next longest diameter (b) perpendicular to (a). Using these measurements, the tumor volume was calculated by the formula: $V = ab^2 \times \pi/6$. The mice were followed in the experiments for 100 days or until tumor volume was determined to be 1300 mm³ at which point any mice had usually succumbed to tumor burden or were requisitely and appropriately euthanized owing to the size of the tumor.

Intratumoral plasmid treatment and in vivo electroporation

Female C57BL/6 mice, 6–7 weeks old were injected with the B16.F10 melanoma cells as indicated above and tumors were allowed to grow to the required size. Tumors were then treated intratumorally with either 50 µg of the pIL-15 or the backbone plasmid vector. Subsequently (i.e. within 1 min), tumors from the appropriate groups were subjected to *in vivo* electroporation using a custom-made applicator, containing six penetrating electrodes that was inserted into the tissue around the tumor and six pulses that were 100 µs long at a field strength of 1500 V/cm were administered using a BTX T820 pulse generator (BTX Harvard Apparatus, Hollister, MA) and autoswitcher (Genetronics, San Diego, CA). These electroporation conditions have been previously described in other studies by our group and were selected because of their less stringent parameters coupled with the ability to elicit a significant biological effect.³

As indicated, treatments were administered on days 0 and 4 with pIL-15 at a dose of 50 µg. For the treatment, groups P+ or P– indicates with or without treatment with the pIL-15 plasmid and E+ or E– indicates with or without electroporation, respectively. V+ designates the control 'backbone' vector, at a dose of 50 µg, which was delivered with electroporation. The treatment groups were as follows: P–V–E– = no treatment, P–V+E+, P+V–E– and P+V–E+. The results presented are the mean results of a total of 16 mice for each group from two separate experiments.

The mean tumor volumes were calculated for each group at selected time points after the treatment regimen up to day 100 after the initiation of the treatment regimen. Additional quantitative measurements made were fold increase in tumor volume compared to day 0 as well as percent of mice undergoing complete tumor regression coupled with long-term survival.

Expression of intratumoral IL-15 after treatment with pIL-15

In order to access the effect of *in vivo* electroporation on intratumoral expression of IL-15 after delivery of pIL-15, an enzyme-linked immunosorbent assay (ELISA) assay was utilized. Briefly, three groups of four C57BL6 mice each were injected with 10^6 B16.F10 cells as described above. The tumors were allowed to develop to the appropriate size (i.e. 40 mm^3). One group was untreated, whereas the second and third groups were treated with $50 \mu\text{g}$ of pIL-15 with or without concomitant *in vivo* electroporation respectively. Thirty-six hours later, animals were killed and tumors were removed and homogenized by sonication in phosphate-buffered saline containing a protease inhibitor cocktail. The rationale for the 36 h time point was based upon *in vitro* expression studies with pIL-15 in other tumor cell lines, which indicated that expression of IL-15 peaked at 36–48 h.⁷ IL-15 levels were then measured in the tumor homogenates/lysates with a human Duo IL-15 ELISA kit (R and D Systems Inc., Minneapolis, MN) and expressed as specific pg of IL-15/mg tumor. Data indicated are the mean of four quadruplicates. In addition, sera samples were collected from treated mice and assayed for IL-15 expressed from pIL-15.

Histological analysis of sections from pIL-15-treated tumors

An additional study was performed, which examined tumor sections from mice treated with pIL-15. In this study, four groups of mice ($n=6$) that differed in the treatment regimens (P–V–E–, P+V–E–, P–V+E+ and P+V–E+) were treated on days 0, 4 and 7. Forty-eight hours after the final treatment, the mice were killed after which the tumors were excised, fixed in 10% formalin and sectioned. The sections were stained for histological analysis with hematoxylin and eosin by standard methods and examined microscopically for the presence of tumor cells, necrosis as well as lymphocytic infiltration.

Statistical analysis

Among the different treatment groups, the mean tumor volume was used in the calculation of mean fold increase in tumor volume for the selected time-point assessment as compared to day 0. Statistical analysis of any treatment differences, as measured by mean fold tumor volume increase, was made using Student's *t*-test methods.

Results

The initial experiment reported here addressed the hypothesis that intratumoral electroporation of subcutaneous B16 melanoma tumors will enhance the expression of IL-15 from an IL-15 DNA expression plasmid. As indicated above, $50 \mu\text{g}$ of the pIL-15 was injected intratumorally into subcutaneous B16.F10 melanoma tumors of the appropriate tumor volume in either the

absence (P+V–E–) or presence (P+V–E+) of subsequent intratumoral electroporation. Thirty-six hours later, tumors were excised and homogenized and IL-15 concentrations were measured and expressed as pg IL-15/mg tumor. The background IL-15 concentration in tumor homogenates of untreated (i.e. no pIL-15 or electroporation) was 6.0 pg IL-15/mg tumor. Figure 1 shows intratumoral IL-15 concentrations of 9.4 and 28.1 pg/mg tumor in the P+V–E– and P+V–E+ groups, respectively. This result indicates that *in vivo* electroporation enhanced intratumoral expression of IL-15 approximately 4.7-fold, compared to non-treated controls, and threefold compared to pIL-15 treatment without electroporation. In this study, the levels of IL-15 measured in both the P+V–E– and P+V–E+ groups were statistically elevated ($P<0.05$ by the Student's *t*-test) when compared to the level measured in the P–V–E– group. Also, and importantly, the intratumoral IL-15 level measured in the P+V–E+ group was significantly elevated compared to that measured in the P+V–E– group. These results demonstrate the ability of the pIL-15 plasmid to be expressed within the tumor after intratumoral delivery with additional significant enhancement of expression through delivery by *in vivo* electroporation. However, at this 36 h post-treatment time point, IL-15 expressed from this plasmid was not measurable in sera samples from mice from the appropriate treatment groups (data not shown).

It was subsequently relevant to determine the potential therapeutic efficacy of intratumoral delivery of pIL-15 and whether *in vivo* electroporation could enhance any antitumor therapeutic effect of pIL-15. Therapeutic end points in these experiments are 'slowing' of tumor growth, as measured by tumor volume, as well as by the incidence of complete regressions of tumors coupled with the long-term survival. In this experiment, C57BL/6 mice, as described in the Materials and methods section, were injected subcutaneously with B16.F10 melanoma cells.

Figure 1 Measurement of expression of IL-15 in B16 melanoma tumor lysates after intratumoral delivery of pIL-15 with or without electroporation. The key for the letter and +/- designations for the treatment groups are provided in the Materials and methods section. The mean concentrations of tumoral IL-15 are expressed as pg/mg tumor.

When tumors had attained the appropriate volume mice were separated into four groups ($n = 16$ each) and treated. In untreated controls, the tumors grew rapidly, which is characteristic of the B16.F10 clone, and all of the tumors reached a volume of approximately 1000 mm^3 by day 18.

In Table 1, data for the other groups at day 18 after the initiation of treatment are summarized. The day 18 measurement was selected because at this time point at least 50% of the mice in each of the treatment groups were still alive (i.e. had not succumbed to tumor burden) allowing for a meaningful analysis. The untreated control (P–V–E–), as indicated above, had undergone an average 22-fold increase (i.e. from 46.5 to 1026.8 mm^3) in tumor volume at day 18 compared to day 0. By contrast, in the P+V–E+ group, the mean fold tumor volume increase from day 0 to day 18 was only 1.2 (i.e. from 39.9 to 49.6 mm^3). In addition to these findings, there appeared to be some tumor growth slowing/attenuation effect in the other treatment groups as well at day 18 compared to day 0. That is, the fold increase in tumor volume from day 0 to day 18 in groups P–V+E–, P–V+E+ and P+V–E– was 12.1, 4.2 and 15.6, respectively. These results indicate an initial ‘nonspecific’ vector backbone and electroporation effect on tumor growth. However, the growth attenuation effect in the P+V–E+ group, receiving the IL-15 expressing plasmid, was significantly greater in terms of fold increase in tumor volume, compared to any of the other treatment groups when accessed by a Student’s *t*-test ($P < 0.05$). The growth attenuation effect noted with control vector treatment or electroporation alone has been noted previously and has been attributed to effects of CpG motifs within the vector and an initial inflammatory/toxic effect of the electroporation.⁸ It is relevant to point out, however, that at day 18 the percent of mice in the P+V–E+ group, which had undergone tumor regression, was significantly higher (i.e. 62.5%) than any of the other treatment groups.

Ultimately, the endpoint for this study with the most relevant clinical significance is complete tumor regression coupled with long-term survival of the mice. Time-point measurements of percent mouse survival with complete tumor regression within the different treatment groups were performed up to 100 days after the initiation of the experiment. For the B16.F10 murine melanoma tumor model, complete tumor regression and animal survival 100 days post-tumor cell injection has been generally

accepted as the benchmark for ‘curative’ therapeutic regimens. That is, maintenance of complete tumor regression 100 days after the initiation of treatment can be considered to be a long-term ‘cure’. These data are summarized in the Kaplan–Meier survival curves shown in Figure 2. The data summarized in the graph indicates that at the day 100 time point only the P+V–E– and P+V–E+ treatment groups had surviving animals with complete tumor regressions. Mice in all of the other groups had succumbed to tumor burden by that time point. In the P+V–E– group, 2/16 (12.5%) of the mice survived until at least the day 100 measurement. In contrast, the P+V–E+ group had 6/16 (37.5%) of the treated animals surviving to at least day 100. This was a threefold enhancement of the therapeutic effect compared to pIL-15 treatment without electroporation. This enhancement was statistically significant at the 0.05 level when measured by a Student’s *t*-test. Importantly, in the control groups that were treated with the vector backbone with or without electroporation, none of the animals survived long term with complete tumor regressions. In addition, no tumor-bearing mice that received electroporation alone (data not shown) survived long term with complete tumor regression. These results demonstrated that the pIL-15 treatment was able to mediate complete tumor regression/long-term survival in B16 melanoma bearing C57BL/6 mice. Also, the end point therapeutic effect of pIL-15 was also enhanced by electroporation, indicating the clinical potential of this delivery method.

As indicated in the Materials and methods section, an additional study was performed, which examined tumor sections from the various groups histologically 48 h after the final treatment. This was carried out in order to access for the presence of melanoma tumor cells. Results of the histological analysis indicated that in the P–V–E– control group there was evidence of tumor in all of the

Table 1 Initial effects of pIL-15 plus electroporation on tumor growth

Group	Day 0 TV ^a (mm)	Day 18 TV ^a (mm)	Fold increase in TV ^a (D0–D18)	Day 18 %R ^b
P–V–E–	46.5	1026.8	22.1	0
P–V+E–	39.4	479.9	12.1	0
P–V+E+	40.0	166.8	4.2	6.25
P+V–E–	36.2	564.2	15.6	18.75
P+V–E+	39.9	49.6	1.2	62.5

^aTV = Tumor volume.

^b%R = Percent of mice with complete tumor regression.

Figure 2 Kaplan–Meier survival curves for C57BL/6 mice in treatment groups injected with pIL-15 or control plasmid with or without electroporation. Groups of mice (P–V–E–, P–V+E–, P–V+E+, P+V–E– and P+V–E+) were treated as described in the Materials and methods section and were followed for tumor regression and long-term survival (up to 100 days after initiation of the treatment regimens).

mice, whereas in the P+V-E+ group 83% of the mice failed to demonstrate histologic evidence of melanoma. In the P-V+E+ and P+V-E- groups, only 17% of the mice in each group failed to demonstrate evidence of tumor. These data further establish the therapeutic efficacy of treatment with pIL-15 and *in vivo* electroporation.

An extension of the regression/long-term survival study reported here was performed in which long-term survivors were challenged subcutaneously with 10⁶ B16.F10 melanoma cells. This experiment was conducted in order to determine whether mice cured of their initial tumors through treatment could resist re-challenge with the B16.F10 cell line. Resistance to re-challenge would likely assume that an immunological mechanism (e.g. T-cell immune response) was operant and which putatively could be involved in protection. In this study, surviving mice from the study described in Figure 2 were 'challenged' shortly after the day 100 time point with the melanoma cells and were accessed over time for the development of tumors. The results demonstrated that 60% of mice re-challenged from the P+V-E+ group remained tumor free for at least 50 days, whereas likewise one of the two mice (i.e. 50%) from the P+V-E- group failed to develop a tumor in this time span. The results from this limited re-challenge study, while not statistically significant, can be considered to be biologically significant because of the aggressive nature of the B16.F10 melanoma cell line. That is, naive mice injected with the number of B16.F10 melanoma cells used in these experiments would normally succumb to tumor burden within approximately 20 days. As such, these findings suggested that pIL-15 treatment mediated an immunological response that protected a proportion of the surviving/cured mice from tumor re-challenge.

Discussion

It has been demonstrated that IL-2 and IL-15 have opposing roles on lymphocytes with IL-2 mediating the death of self-reactive lymphocytes to yield self-tolerance and IL-15 favoring the survival of memory CD8+ T cells. IL-2 is also involved in AICD, which is a process that results in the elimination of self-reactive T cells with facilitation of the induction of tolerance to self.² This characteristic, however, may result in the death of T cells that recognize, as self, antigens expressed on the tumor cells. For this reason, even though IL-2 has been approved by the Food and Drug Administration for the treatment of patients with renal cell carcinoma and melanoma and has been demonstrated to have some efficacy, IL-15 may potentially be superior for the treatment of cancer. In fact, IL-15-expressing vaccinia virus vaccines have been shown to be superior to those expressing IL-2 in terms of induction of long-lasting CD8+ cytotoxic T-lymphocyte-mediated immunity⁹ and likewise it appears that IL-15 may be superior to IL-2 in stimulating NK cell activity.¹⁰ Some of these effects could have been responsible for the ability of IL-15 to function as an adjuvant therapy in enhancing significantly the

cytotoxic effects against a B16-derived murine melanoma of an IL-12-transduced B16 cell vaccine.⁶ Taken together, these studies demonstrate the potential efficacy of IL-15 as an antitumor agent.

The data presented in this report demonstrate the therapeutic antitumor potential of the IL-15-expressing plasmid when delivered intratumorally into established subcutaneous B16 melanoma tumors in C57BL/6 mice. In addition, it was also demonstrated that delivery of pIL-15 with *in vivo* electroporation significantly enhanced the antitumor activity of this expression plasmid, which was associated with an approximate threefold and 4.7-fold increase in expression of IL-15 when delivered with electroporation as compared to treatment without electroporation or no treatment, respectively. Also, it appeared that the plasmid backbone vector, when delivered by electroporation resulted in an initial temporary attenuation of tumor growth due likely owing to immune stimulatory effects of the CpG motifs contained in the plasmid as well as an inflammatory response from the electroporation procedure. However, only treatment with pIL-15 resulted in any complete tumor regressions with long-term survival, indicating specificity in mediating this relevant endpoint therapeutic response. It is anticipated that further studies with electroporative delivery of pIL-15 will allow maximization (i.e. at least an 80% complete tumor regression/long-term survival rate) of the therapeutic response. Therapeutic maximization strategies include modulation of the dose as well as the number and intervals of treatments.

Re-challenge with B16 melanoma cells of mice that had been 'cured' of the initial melanoma tumors by treatment with pIL-15 plus electroporation resulted in resistance to tumor challenge in a large proportion of the mice. This suggested that a mechanism resulting in immunological memory mediated the resistance to tumor cell challenge even though preliminary analysis at the 36 h post-treatment time point failed to demonstrate sera levels of expressed IL-15. Future studies in this area will be aimed at further examining the tumoral and sera IL-15 expression levels after treatment with pIL-15 plus electroporation as well as the measurement of antigen-specific memory T cells or NK cell activity as possible immunological mechanisms for mediating the antitumor activity of pIL-15.

The tumor induction and challenge model utilizing the B16.F10 cell line, as reported in this study, is particularly relevant for several reasons: (a) the B16.F10 melanoma is a highly invasive, metastatic and poorly immunogenic tumor, which is very difficult to 'cure', (b) the treatment regimen used in this study was administered to established tumors rather than injected concomitantly with tumor cells or before malignant lesions had visibly formed and (c) the ultimate therapeutic end point was complete tumor regression and long-term survival rather than simply attenuation of tumor growth. This is relevant as the majority of other studies with this tumor cell line have either administered treatments before the development of visible tumors or accessed tumor growth attenuation as the therapeutic end point.

In sum, the results presented in this report further confirm the potential utility of naked DNA plasmids expressing therapeutic cytokines such as IL-15 as anti-cancer therapeutics. In addition, the enhancement of the delivery, expression and therapeutic index for these molecular reagents through intratumoral electroporation has been established.

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