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Evaluation of Toxicity following Electrically Mediated Interleukin-12 Gene Delivery in a B16 Mouse Melanoma Model

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Abstract

Purpose: Interleukin-12 (IL-12) has potential as an immunotherapeutic agent for the treatment of cancer but is unfortunately associated with toxicity. Delivery of a plasmid encoding IL-12 with electroporation induces an antitumor effect in the B16 mouse melanoma model without serious side effects. To translate this observation to the clinic, an evaluation of toxicity was done in the mouse model.

Experimental Design: Weight change, tumor response, blood chemistry and hematology values, and serum IL-12 levels were evaluated. Multiple tissues were analyzed histopathologically.

Results: A pronounced reduction in tumor volume, including a large percentage of complete regressions, was observed after electrically mediated gene therapy. No significant increases in serum IL-12 levels were detected. Tumor-bearing mice showed an increased number of atypical hematology values when compared with normal naive controls. Statistically significant differences in chemistry and hematology values were observed sporadically in most of the standard chemistry and hematology categories in all groups. The only histopathologic abnormality specific to the animals receiving both plasmid and electroporation was inflammation associated with the kidney at the last time point.

Conclusions: In general, mice that received both plasmid and electroporation showed the least abnormal histopathologic findings and were found to be in the best health, reflecting the reduced burden of disease. No significant toxic effects due to the IL-12 gene therapy were observed.

Interleukin-12 (IL-12) is a versatile cytokine and has great potential as an immunotherapeutic agent for the treatment of cancer. Recombinant protein therapy has had some success, but unfortunately it has been associated with toxicity. When given systemically in a phase I clinical trial, recombinant IL-12 induced multiple serious adverse effects, including renal and systemic toxicity (1, 2). High-dose levels were linked to temporary immune suppression, which would be unfavorable for effective immunotherapy. A successful anticancer treatment protocol should be one that initiates an extensive, rapid immune response without inducing these toxic side effects. Delivery of IL-12 in the form of gene therapy for melanomas has shown some success without serious adverse side effects (3, 4) and has reached clinical trials (5).

Electroporation is a physical method that facilitates uptake of molecules by increasing the permeability of the cell membrane. *In vivo* electroporation has been used to deliver chemotherapeutic agents, such as bleomycin, in both animal studies and clinical trials (6–14). The same rationale for using *in vivo* electroporation to deliver chemotherapeutic agents is valid for the delivery of larger molecules, such as plasmid DNA. Electrically mediated delivery of several cytokine cDNAs has been shown in the B16 melanoma model (15–22).

Many of the preclinical IL-12 studies examining delivery of DNA encoding the IL-12 cDNAs as a single treatment for melanoma have been conducted in the poorly immunogenic and metastatic B16 murine melanoma model. Significant inhibition of tumor growth was observed after adenoviral delivery (17) as well as delivery mediated by Semliki Forest virus (23). Plasmids encoding the cDNAs have been delivered therapeutically in this model using the gene gun (3), electroporation (17, 18, 21, 22), injection alone (24), or a chemical carrier (25). The majority of studies have shown inhibition of tumor growth or limited tumor regression. IL-12 gene therapy has also resulted in the regression of established tumors and protection against lung metastasis following challenge in this model (22, 26, 27).

Complete regression of established B16-F10 melanomas has only been observed after DNA delivery by *in vivo* electroporation (21, 22) or when electroporation was used to deliver IL-12 plasmid in combination with bleomycin (28). Intratumor injection of a plasmid encoding IL-12 followed by electroporation results in an 80% cure rate of established B16-F10

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tumors in mice (22). Twelve of 12 cured mice were resistant to subsequent challenge with B16-F10 cells. In addition, i.m. injection of plasmid encoding IL-12 followed by electroporation significantly reduced the formation of lung colonies following i.v. injection of B16-F10 cells. Electrically mediated IL-12 plasmid delivery resulted in both a therapeutic response to existing tumors and a prophylactic effect against metastases. To translate this to the clinic, the following study was done to fully characterize the potential toxicity following treatment by intratumor *in vivo* electroporation of IL-12 plasmid DNA.

Materials and Methods

This study was approved by the University of South Florida Institutional Animal Care and Use Committee (Tampa, FL). This committee follows USPHS Policy on Humane Care and Use of Laboratory Animals (www.grants.nih.gov/grants/olaw/olaw.htm#pol).

Cells lines and propagation. B16-F10 murine melanoma cells (CRL 6475, American Type Culture Collection, Rockville, MD) were maintained as monolayers in 90% McCoy's medium with 10% fetal bovine serum and 1% gentamicin. The cells were removed from flasks using trypsin-EDTA (0.05% trypsin-0.53 mmol/L EDTA; Mediatech), collected by centrifugation, and washed, and cell viability was assessed by trypan blue exclusion dye method. Cells with a viability of >90% were resuspended in sterile PBS at 2×10^7/mL for injection.

Tumor induction. Tumors were established in the flank of 6- to 7-week-old male and female C57Bl/6 mice (National Cancer Institute, NIH, Bethesda, Maryland) by the s.c. injection of 0.05 mL (10^6) B16-F10 cells. Tumors were allowed to grow for 7 to 10 days to a volume of 40 to 50 mm^3 before treatment.

Plasmid. pUCMV3-mIL-12 (Aldevron, Fargo, ND), containing the murine p35 and p40 IL-12 cDNAs under the control of the cytomegalovirus promoter, was prepared by the manufacturer. Endotoxin levels were <100 endotoxin units/mg. Before use, plasmid DNA was diluted in 0.9% sterile injectable saline to the appropriate concentration for each experiment.

Tumor treatment. Treatments were done on days 1, 5, and 8 after tumor growth. Briefly, mice were initially anesthetized in an induction chamber that was infused with a mixture of 3% isoflurane and 97% O_2 for several minutes then fitted with a standard rodent mask supplied with 2% isoflurane in O_2. Group 1 animals received only an intratumor 0.05 mL saline injection. Group 2 animals received an intratumor injection of 0.05 mL 0.1 mg/mL pUCMV3-mIL-12 only. In Group 3, injection of 0.05 mL 0.1 mg/mL pUCMV3-mIL-12 was followed by application of six 0.1 ms pulses with field strength of 1,300 V/cm from a MedPulser Electroporation System (Inovio Biomedical Corporation, San Diego, CA) delivered using a six needle array. Group 4 received an intratumor injection of 0.05 mL 1 mg/mL pUCMV3-mIL-12 only, whereas group 5 received an intratumor injection of 0.05 mL 1 mg/mL pUCMV3-mIL-12 followed by pulses.

Tumor monitoring. Tumors were monitored every 2 to 3 days; not all data points were shown. Tumors were monitored using a digital caliper. Tumor volume was calculated by the standard formula $v = \pi ab^2/6$, where a is the longest diameter and b is the next longest diameter perpendicular to a.

Sample collection. Each mouse was monitored for body weight and general condition on a daily basis beginning the day before the first treatment. The general condition of the mice included examination of the coat, demeanor, and health of the animal. The evaluation of demeanor and health of the mouse was determined by observing if the mouse was eating, drinking, and moving without discomfort.

On days 9 (the day after the third treatment), 11, 16, 23, and 30 after treatment, 10 mice from each group (5 male and 5 female) were euthanatized. Blood was collected from each animal in heparinized tubes, and tissue samples taken in order: brain, spleen, kidney, liver, lung, heart, lymph nodes, then finally tumor, and skin around the tumor. Different sets of instruments were used to avoid cross-contamination.

Histologic analysis. Tissue samples from groups 1, 4, and 5 on days 9, 16, and 30 were blinded and analyzed histologically. Each specimen obtained for analysis was submitted entirely for histologic examination. Radial and sagittal sections were taken. After fixation, sections were stained with H&E (Richard-Allan Scientific, Kalamazoo, MI) using standard histologic techniques.

Results

Treatments were done on days 1, 5, and 8 after tumor growth (see Materials and Methods). Group 1 animals received only an intratumor 0.05 mL saline injection. Group 2 animals received a low-dose (0.005 mg pUCMV3-mIL-12) intratumor injection only. In group 3 animals, a low-dose intratumor plasmid injection was followed by application of pulses as described. Group 4 received a high-dose (0.05 mg pUCMV3-mIL-12)

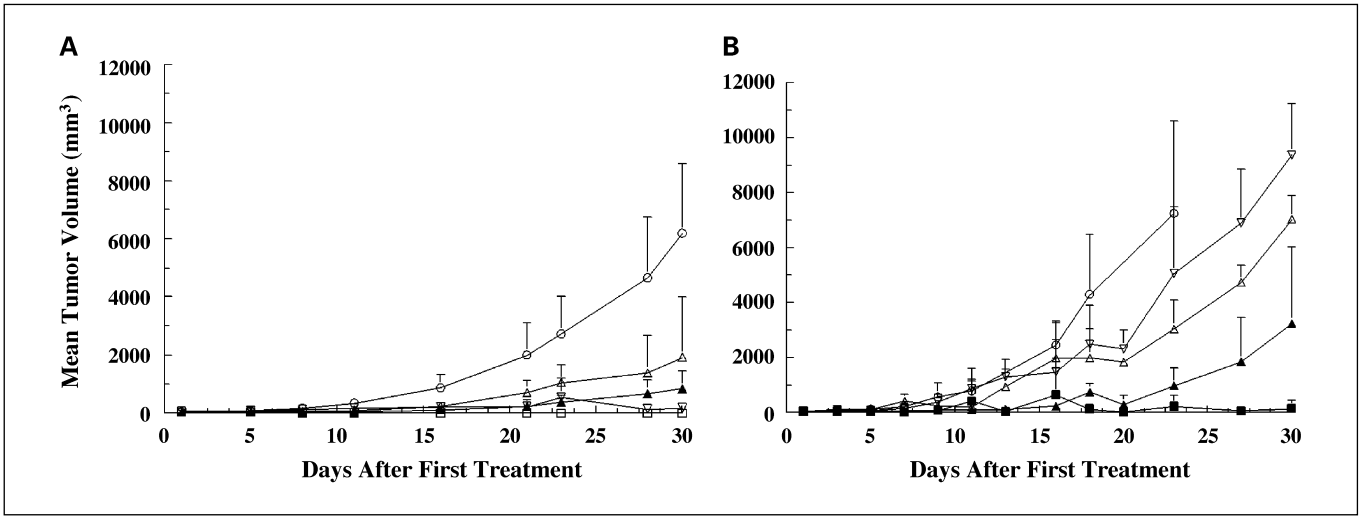

Fig. 1. Tumor growth in females (*A*) and males (*B*). ○, group 1; Δ, group 2; ▲, group 3; ▽, group 4; □, group 5. Error bars, standard deviation of the mean.

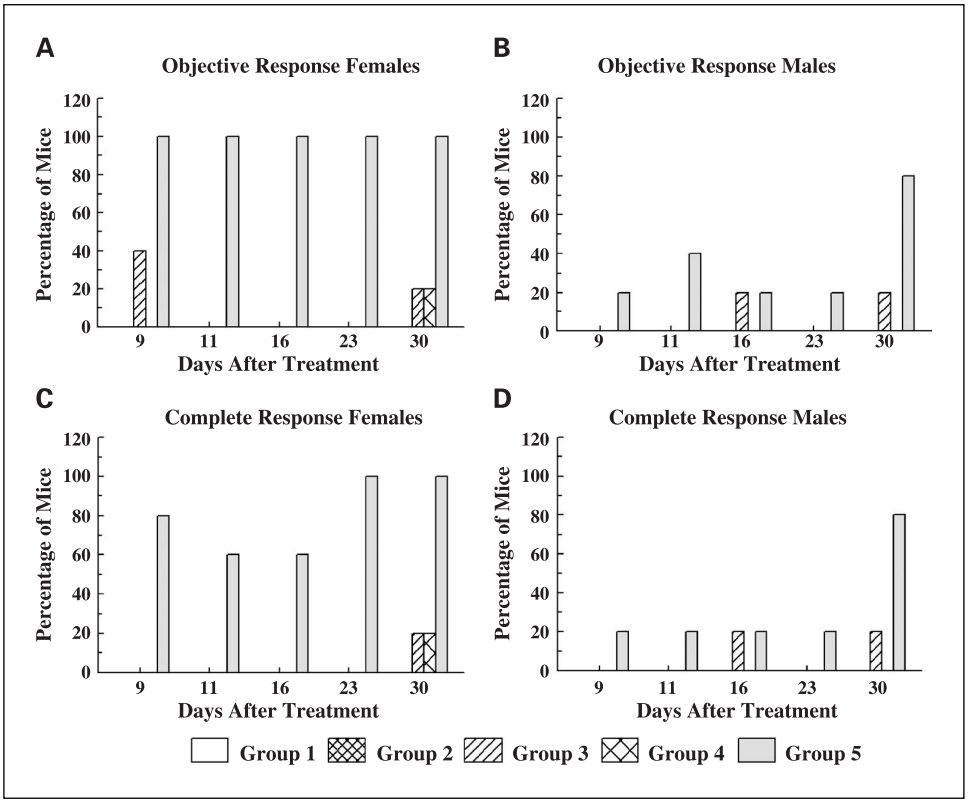

Fig. 2. Response rates. *Columns,* percentage of mice that had an objective response (*A* and *B*) or complete response (*C* and *D*). There were five mice at each time point for each group, except for group 1 on day 30 (zero male and three female survivors) and group 4 on day 30 (four male and five female survivors).

intratumor injection only, whereas group 5 received a high-dose intratumor plasmid injection followed by pulses.

General health status and survival. No significant weight loss was observed in any of the treatment groups when compared with the group that received saline injection alone (group 1). Five of the group 1 males, two of the group 1 females, and one group 4 male did not survive to the final time point due to unimpeded tumor growth. Overall, mice seemed to have a generally healthy appearance with the exception of the tumor growth.

Table 1. Complete blood count values for normal, healthy mice

	Females		Males	
	Mean	SD	Mean	SD
WBC	4.72	2.03	6.19	2.15
%Neutrophils	10.11	2.99	21.01	12.36
%Leukocytes	84.37	4.29	70.52	15.48
%Macrophages	4.31	1.4	6.81	3.26
%Eosinophils	0.1	0.15	0.09	0.05
%Basophils	1.11	0.56	1.58	0.65
RBC	8.6	0.66	9.34	0.57
Hemoglobin	13.6	1.17	14.42	0.97
Hematocrit	40.74	3.31	43.81	3.13
MCV	189.2	2.54	187.64	3.36
MCH	63.24	1.35	61.72	1.08
MCHC	133.64	2.01	131.56	1.71
RDW	56.16	3	57.32	4.06
Platelets	1035.6	115.85	1342.8	119.9
MPV	18.63	0.39	18.88	0.5
Pack crit	0.48	0.05	0.63	0.05
PDW	62.64	1	62.84	0.79

Abbreviations: MCV, mean corpuscular volume; PDW, platelet distribution width.

Tumor growth or regression. Tumor growth in most male groups and in all female treated groups was significantly delayed when compared with group 1 (Fig. 1). Tumors continued to grow in group 1 mice with no objective or complete responses observed. In males, all treated groups showed a significant reduction in tumor growth from day 13 to 23, with the exception of group 4, which showed no significant difference from group 1 during this time period. In females, all treated groups showed a significant reduction in tumor growth from day 11 to 23. Day 30 (the final day tested) was significantly different in group 5 only. This pronounced reduction in tumor volume included a large percentage of complete regressions, particularly at the later time points.

The regression status of all mice was observed at the time points the mice were euthanatized for toxicity studies (Fig. 2). Complete regressions were observed in males in groups 3 and 5, whereas complete regression was observed in females in groups 3, 4, and 5. All deaths before euthanasia were due to disease progression. Metastatic disease was observed on necropsy in six males (groups 1 and 4) and two females (group 1).

Chemistry and hematology findings. Blood chemistry and hematology values were established in 10 male and 10 female naive, healthy mice (Tables 1 and 2). At 9, 11, 16, 23, and 30 days after the initial plasmid delivery, whole blood from five males and five females in each group was tested and compared with the established normal values.

Table 2. Mean blood chemistry for normal, healthy mice

	Glucose	BUN	Creatinine	Albumin	Total bilirubin	Alkaline phosphatase	Aspartate aminotransferase	Alanine aminotransferase
Females								
Mean	209.6	16.2	0.26	2.08	0.16	154.4	186.9	44.4
SD	40.58	1.75	0.07	0.13	0.11	12.95	96.33	12.29
Males								
Mean	253.6	16.5	0.22	1.91	0.1	111.5	121.1	34.5
SD	23.64	1.96	0.04	0.19	0	25.95	40.72	8.7

Group 1 mice showed an increased number of atypical hematology values when compared with normal naive controls, although blood chemistry values were not more likely to be atypical than in the treated groups. Statistically significant differences ($P < 0.01$) were observed sporadically in most of 17 standard hematology categories (Table 3). Male values tended to be significantly decreased or increased more often than female values. In both males and females, group 1 mice displayed increasingly aberrant values with time. In males, RBCs, hemoglobin, and hematocrit in particular were reduced at later time points. In females, hemoglobin and hematocrit were reduced, whereas an increased mean platelet volume (MPV) was observed at later time points. Females in groups 2, 3, and 4 did not show trends in significantly different values, whereas females in group 5 showed a decreased mean corpuscular hemoglobin (MCH) concentration (MCHC) over time. Males in group 2 tended to have an increased RBC distribution width (RDW). Males in group 3 showed increased RBCs and hemoglobin only at the early time points. Group 4 males showed decreased RBCs, hemoglobin, and hematocrits at later time points. Untreated mice showed progressive lymphocytopenia, anemia, and neutrophilia. These changes were seen but to a lesser extent in the injection-only groups. No significant abnormalities were seen in the high-dose electroporation group. These results suggest that the combination of plasmid injection and electroporation was not associated with bone marrow suppression.

In female untreated mice, progressive azotemia and hypoalbuminemia was seen with progressive melanoma (Table 4). These changes were observed also in the injection-only group of females but not in females that received high dose of plasmid and electroporation. In the male mice, these abnormalities were

Table 3. Complete blood count values differing from mean of normal, healthy mice ($P < 0.01$)

Group	Day 9		Day 11		Day 16	
	Increased	Decreased	Increased	Decreased	Increased	Decreased
Females						
I	None	MCV	%B	Hemoglobin	%M, MPV	%L, RBC, hemoglobin, hematocrit
II	MCHC	MCV	None	MCH, MCHC	None	None
III	Platelets, pack crit	MCV	None	MCH, MCHC	MPV, pack crit	None
IV	MPV	None	MPV	MCHC	MPV	None
V	%E	MCV	MPV	MCHC	None	MCH, MCHC
Males						
I	MCV	None	%B, MCV	MCHC, PDW, RBC, hemoglobin, hematocrit	%E	RBC, hemoglobin, hematocrit
II	%E	None	Pack crit	None	%E, %B, RDW	None
III	RBC, hemoglobin, hematocrit, platelets, pack crit	None	RBC, hemoglobin, hematocrit, platelets, pack crit	None	RBC, hemoglobin, platelets, pack crit	MCV
IV	%B, RBC, hemoglobin, hematocrit, platelets, pack crit	None	None	None	MCHC	Hematocrit
V	None	WBC, MCHC	None	None	MCHC	None

seen, and, in addition, hypoglycemia was also observed in the untreated males. In males that received injection of plasmid and electroporation, these changes were also observed but to a lesser extent.

Serum was also assayed for levels of IL-12 following each treatment. No significant serum IL-12 levels were seen in any group (data not shown).

Histopathology. In general, the untreated animals comprised the most profound histopathologic abnormalities. The predominant tissue that showed metastatic spread was the skin around the tumor. Tumor growth in the skin was found in 31% of group 1 mice, whereas only 12% of group 4 mice and 8% of group 5 mice were positive. It should be noted that the tumor growth in the skin observed in group 5 mice occurred predominately at an early time point (day 9). Metastatic spread was also particularly observed to the lymph nodes and liver in groups 1 (12% to both tissues) and 4 mice (17% lymph nodes and 12% liver). Lymph nodes (4%) and liver (2%) involvement was greatly decreased in group 5 mice, although, in one mouse in this group, kidney and spleen metastases were observed. No metastases were observed in the lungs, heart, or brain in any group. In addition, acute and chronic inflammation in the skin around the tumor was observed particularly in groups 1 (12% to in both tissues) and 4 (17% in lymph nodes and 12% in liver).

No abnormalities were seen in brain or heart tissue. Lungs were congested, and focal acute and chronic inflammation was observed in all mice. Most mice showed extramedullary hematopoiesis, which is a common and normal phenomenon in the spleen of C57Bl/6 mice (29). Focal inflammation in the kidney was not observed in group 1 females but was observed in two of five group 4 females by day 16 and one of five by day 30. Two of five group 5 females also showed focal inflammation in the kidney at day 16, and the level of inflammation increased to four of five by day 30. In males, kidneys in groups 1 and 4 males were normal, but three of five of the group 5 males showed focal glomerulosclerosis at the later time points.

Discussion

IL-12 is a cytokine that shows promise as an anticancer agent. In human phase I clinical trials, systemic infusions of recombinant IL-12 protein were toxic but showed clinical activity. Since then, multiple investigators have shown that, in animal models, direct delivery of IL-12 to the tumor site maintains its anticancer activity but avoids much of the toxic side effects (4, 26, 30). Recent phase I melanoma clinical trials using localized IL-12 gene therapy alone have reported local production of IL-12 via injection of naked plasmid DNA (5) or of IL-12 producing transduced autologous cells (31, 32). Recent preclinical data suggest that the efficacy of IL-12 gene therapy can be dramatically increased by electrically mediated gene transfer (22, 21).

Pulsed electric fields (electroporation) are effective means of enhancing intracellular delivery of molecules *in vivo*. Electrochemotherapy, which combines an antitumor drug with electroporation, is an effective localized treatment for a variety

Table 3. Complete blood count values differing from mean of normal, healthy mice ($P < 0.01$) (Cont'd)

Day 23		Day 30		Total, *n* (%)
Increased	Decreased	Increased	Decreased	
%N, %M, MCH, MCHC, RDW, MPV	%L, RBC, hemoglobin, hematocrit, platelets, pack crit	WBC, %N, %M, %B, RDW, MPV, pack crit, PDW	%L, hemoglobin, hematocrit, MCV, MCH	21/68 (30.9)
None	RBC, hemoglobin	%N, RDW, MPV	%L, RBC, hemoglobin, hematocrit	13/85 (15.3)
None	RBC	WBC, %N, %B, platelets, pack crit	%L	14/85 (16.5)
None	RBC, hemoglobin, hematocrit	None	RBC, hemoglobin, hematocrit	10/85 (11.8)
None	MCH, MCHC	None	%M, MCH	10/85 (11.8)
%N, MCV, RDW, MPV	%L, RBC, hemoglobin, hematocrit, platelets			34/85 (40)
%E, MCH, RDW	RBC, hemoglobin, hematocrit	WBC, %N, RDW, MPV	%L, RBC, hemoglobin, hematocrit	19/85 (15.3)
%E	None	%E	MCH	18/85 (21.2)
%N, %E, %B, MCV, platelets, MPV, PDW	%L, RBC, hemoglobin, hematocrit, RDW, pack crit	%N, %M, platelets, MPV, PDW	%L, RBC, hemoglobin, hematocrit, RDW, pack crit	32/85 (37.6)
None	MCHC	RBC, hematocrit	MCH	7/85 (8.2)

Table 4. Blood chemistry values differing from mean of normal, healthy mice ($P < 0.01$)

Group	Day 9		Day 11		Day 16		Day 23		Day 30		Total (%)
	Inc	Dec	Inc	Dec	Inc	Dec	Inc	Dec	Inc	Dec	
Females											
I	BUN	None	None	ALKP, ALT	BUN	ALKP	BUN	Albumin, ALKP	BUN	ALKP	10/40 (25)
II	BUN	None	None	None	None	None	BUN	Albumin, ALKP	BUN	Albumin, ALKP	7/40 (17.5)
III	BUN	None	None	None	BUN	None	BUN	None	None	ALKP, ALT	5/40 (12.5)
IV	None	None	BUN	None	None	None	BUN	None	BUN	ALKP	4/40 (10)
V	None	None	BUN, ALKP	None	ALKP	None	BUN, ALKP	Glucose	None	None	6/40 (15)
Males											
I	None	None	AST	None	AST	Glucose, ALKP	BUN, AST	Glucose, ALKP			8/32 (25)
II	None	None	BUN	Glucose	AST	Glucose, ALKP	AST	Glucose, ALKP	AST	Glucose, ALKP	11/40 (27.5)
III	BUN, ALT	Glucose	None	Glucose	BUN	Glucose	AST	Glucose, ALKP	AST	Glucose, ALKP	12/40 (30)
IV	None	None	BUN	None	BUN	ALKP	AST	Glucose, ALKP	AST	ALKP, glucose, Albumin	10/40 (25)
V	None	None	None	None	None	Glucose	None	None	BUN	Glucose	3/40 (7.5)

Abbreviations: Inc, increased; Dec, decreased; ALKP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

of tumor types (33). This electrically enhanced drug delivery has progressed to several successful clinical trials (34, 35). Because electroporation is a physical method that facilitates uptake of molecules by increasing the permeability of the cell membrane, it can be used to effectively deliver a variety of molecules to a broad range of cell types. The main limitation to *in vivo* electroporation is the ability to access the tissue to inject the molecule to be delivered and properly place the electrodes. Although electric fields have been used to deliver chemotherapy (electrochemotherapy) to s.c. tumors, they have not been used to introduce DNA constructs in humans. The use of electric pulses for the *in vivo* delivery of genes is in an earlier stage of development.

Several studies have indicated that gene delivery could potentially achieve a similar success as drug delivery (30). Studies accomplished thus far have shown that *in vivo* electroporation could enhance the delivery of plasmid DNA to several tissue types. Of interest is the ability to control the expression levels and kinetics of expression by carefully choosing the electroporation variables (30, 36).

The electroporation conditions (field strength, pulse duration, and electrodes) proposed to be used for the IL-12 electrogene transfer study are essentially the same as those used in the electrochemotherapy clinical studies. These conditions were well tolerated in the electrochemotherapy trials. Although electrochemotherapy has had tremendous success in inducing tumor regression, this effect was localized to the treated lesion. Therefore, the effect could be even higher for gene delivery, as this therapy could potentially elicit systemic effects. Results presented in this report as well as in previous studies have shown the potential of this delivery system as part of an antitumor regimen. Work in the B16-F10 model produced strong evidence of this potential. Complete long-term responses can be obtained following treatment of established melanoma tumors using this system to deliver a plasmid encoding IL-12. The majority of mice that had durable long-term regressions were also resistant to challenge (21, 22). In addition, intramuscular delivery produced high serum cytokine levels, which prevented the formation of lung lesions following tail vein injection of B16-F10 cells (22).

Although the potential efficacy of this approach has been shown in preclinical animal studies, it was necessary to evaluate potential toxicity of this approach to translate this delivery approach to the clinic. Although a previous study showed that there was minimal toxicity associated with direct delivery (intratumor injection of plasmid) of IL-12 plasmid in the B16 mouse melanoma model (4), electrically mediated delivery had not been evaluated. Therefore, this study was designed to assess toxicity associated with this therapeutic approach. The results of this study showed that there was no significant toxicity associated with the electrically mediated delivery of a plasmid encoding IL-12. The only abnormality specific to animals receiving both plasmid and electroporation was inflammation associated with the kidney at the late time point. We did not, however, observe creatinine elevation in these mice. In human renal disease, focal glomerulosclerosis can manifest with asymptomatic proteinuria or edema (37). Therefore, we would recommend monitoring of renal function in human

IL-12/electroporation trials and should include monitoring of blood urea nitrogen (BUN)/creatinine, urine sediment, and hypotension. It should also be pointed out that, in general, these mice showed the least abnormal histopathologic findings and were found to be in the best health, which, we believe, reflects a reduced burden of disease.

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