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IL-12 Plasmid Delivery by *in Vivo* Electroporation for the Successful Treatment of Established Subcutaneous B16.F10 Melanoma

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Interleukin-12 (IL-12) has been used in numerous immunotherapy protocols against melanoma. However, delivery of IL-12 in the form of recombinant protein can result in severe toxicity, and gene therapy has had limited success against B16.F10 murine melanoma. The purpose of this study was to examine the effectiveness of *in vivo* electroporation for the delivery of plasmid DNA encoding IL-12 as an antitumor agent against B16.F10 melanoma. We treated mice bearing established B16.F10 melanoma tumors with intratumoral (i.t.) or intramuscular (i.m.) injections of a plasmid encoding IL-12, followed by *in vivo* electroporation. For i.t. treatments, we used an applicator containing six penetrating electrodes to deliver 1500-V/cm, 100-μs pulses. We administered i.m. pulses with an applicator containing four penetrating electrodes delivering 100-V/cm, 20-ms pulses. The i.t. treatment resulted in the cure of 47% of tumor-bearing mice, and 70% of cured mice were resistant to challenge with B16.F10 cells. The i.m. treatment did not result in tumor regression. We found that i.t. treatment resulted in increased levels of IL-12 and interferon-γ (IFN-γ) within the tumors, the influx of lymphocytes into the tumors, and reduction in vascularity. Neither i.m. nor i.t. treatment was successful against B16.F10 tumors in a nude mouse model, supporting a role for T cells in regression of this tumor model.

Key Words: IL-12, electroporation, melanoma, plasmid DNA, B16.F10, gene therapy

INTRODUCTION

The antitumor potential of IL-12 has been reported in numerous immunotherapy studies [1–11]. The proposed antitumor mechanisms of IL-12 include effects on the immune system such as the induction of IFN-γ, upregulation of T cells, and proliferation of natural killer (NK) cells. In addition, IL-12 inhibits angiogenesis, the formation of new blood vessels [1,10]. This wide range of effects on the immune system as well as antiangiogenic properties results in a potentially potent antitumor treatment.

Unfortunately, preclinical and clinical trials using systemic administration of recombinant IL-12 demonstrated potential adverse side effects [11,12]. The use of gene therapy for the delivery of IL-12, by gene gun, resulted in fewer side effects than recombinant protein therapy [11]. Several studies using viral and nonviral gene delivery techniques have reported success in slowing and/or preventing tumor growth [6–11]. However, these studies have had limited success in demonstrating complete regression of the poorly immunogenic B16.F10 melanoma and subsequent resistance to challenge.

In vivo electroporation is a gene delivery technique that has been used successfully for efficient delivery of plasmid DNA to many different tissues [13–26]. We recently reported the expression of IL-12 and IFN-γ in the serum of mice after intramuscular delivery of a plasmid encoding IL-12 with electroporation [18], and other studies have reported the administration of *in vivo* electroporation for delivery of plasmid DNA to B16 melanomas [22–26]. Although systemic administration of recombinant IL-12 revealed its antitumor potential [1], expression of IFN-γ at the tumor site has been shown to be critical for successful tumor regression [4,9]. Systemic and local expression of a gene or cDNA encoded by a plasmid can be obtained with administration of *in vivo* electroporation. Use of *in vivo* electroporation enhances plasmid DNA uptake in tumor tissue, resulting in expression within the tumor [22–25], and delivers plasmids to

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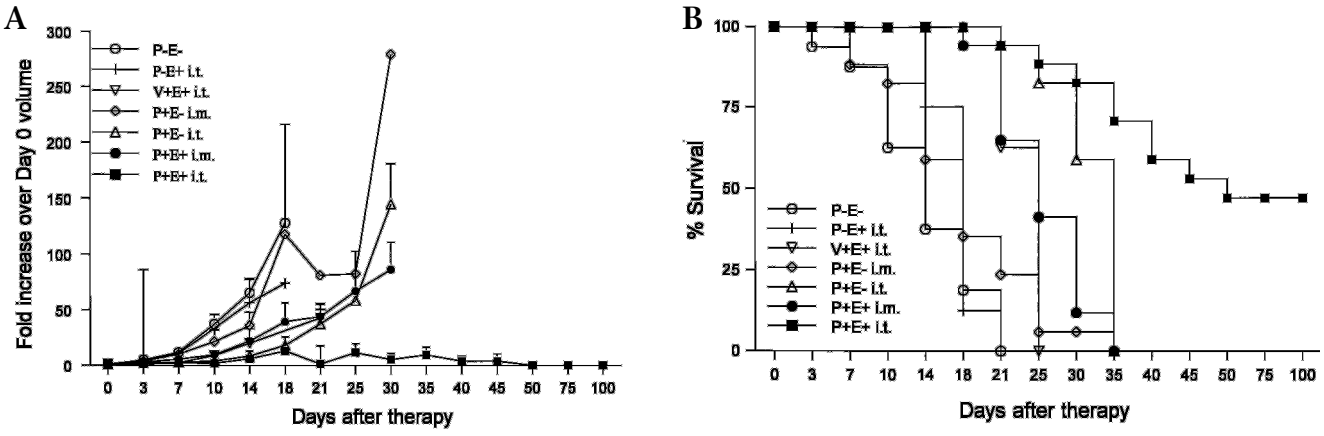

FIG. 1. Administration of plasmid DNA encoding IL-12 followed by electroporation results in complete tumor regression. (A) Fold increase over day 0 tumor volume following treatment. P, pIRES IL-12; V, control plasmid, pND2Lux; E, electroporation. Treatment mode of delivery: i.t., intratumor; i.m., intramuscular. A plus sign indicates treatment was administered; a minus sign indicates treatment was not administered. Initial treatment day is day 0; mice were treated again on day 7. Results for all groups (except P^-E^+ i.t. and V^+E^+ i.t.) represent the combined data from three replicate experiments, and error bars represent the standard error of the mean. The P^-E^+ i.t. and V^+E^+ i.t. treatment groups were tested in one experiment because existing data in our lab showed these treatments to be ineffectual. Error bars for these two groups represent standard deviation. The total number of samples for each treatment group are as follows: P^-E^-, $n = 16$; P^-E^+ i.t. and V^+E^+ i.t., $n = 8$; and for the remainder of groups, $n = 17$. Mice were killed when tumor volume exceeded 1000 mm^3. Data are expressed for surviving mice on each day. (B) Percentage survival of mice represented in (A). Mice either succumbed to disease or were killed when tumor volume exceeded 1000 mm^3.

muscle tissue, resulting in systemic cytokine expression [18].

Of the studies in the B16 melanoma model, only that of Heller *et al.* showed cures of established tumors with resistance to challenge [23]. In that study, tumor regression resulted from a combination of electrochemotherapy and gene delivery by *in vivo* electroporation using a pulse protocol delivering microsecond (μs) pulses [23]. Lohr *et al.* compared delivery by electroporation with adenoviral vectors and found that electroporation was effective in delivering plasmid coding for IL-12 and, unlike adenoviral delivery, did not result in toxic side effects [25]. In addition to these studies, Kishida *et al.* also used electroporation for delivery of IL-12 and IL-18 to a B16 model [26]. Millisecond (ms) pulses were administered in these two studies, and unfortunately, neither protocol resulted in complete regression of established tumors and long-term survival of animals [25,26].

Its wide range of effects on the immune system and its antiangiogenic properties make IL-12 an excellent candidate for use as an immunotherapeutic agent. Because of its potential toxicity, it is important to give careful consideration to the delivery method of IL-12. *In vivo* electroporation is a safe, nontoxic delivery system and has been used for efficient delivery of chemotherapeutic agents and plasmid DNA, including plasmids encoding IL-12 [18,23,27]. Therefore, we hypothesized that administration of an electroporation protocol for delivery of IL-12 will result in regression of B16.F10 melanoma tumors and long-term animal survival.

RESULTS

Intratumoral Delivery of IL-12 by Electroporation Results in Tumor Regression, Long-Term Animal Survival, and Resistance to Challenge

To explore the antitumor potential of IL-12 delivered by *in vivo* electroporation, we treated C57BL/6 mice with established subcutaneous B16.F10 melanoma by injecting 50 μg (1 μg/μl) of plasmid DNA encoding IL-12 (pIRES IL-12) in sterile saline into the tumor (i.t.) or the gastrocnemius muscle (i.m.), followed by electroporation. An applicator containing six penetrating electrodes was used to deliver 1500-V/cm, 100-μs pulses i.t.; this protocol is similar to those used in gene delivery and electrochemotherapy protocols that resulted in successful tumor regression [23,27,28]. For i.m. delivery, an applicator, specifically designed for the mouse gastrocnemius muscle and containing four penetrating electrodes, was used to administer 100-V/cm, 20-ms pulses, a protocol shown to result in high systemic IL-12 and IFN-γ expression [18]. A single treatment did not result in long-term animal survival (data not shown). Therefore, in the following experiments we administered a second treatment 7 days (day 7) after the initial treatment (day 0). Tumor size was evaluated throughout the experiment, and the results are presented as the fold increase over day 0 tumor volume for each treatment group (Fig. 1A). Treatment with pIRES IL-12 injected i.t. followed by electroporation slowed tumor growth, with nearly half (8/17) of the mice showing complete regression of their tumors. Progressive tumor growth was observed in mice receiving i.m. injections of plasmid

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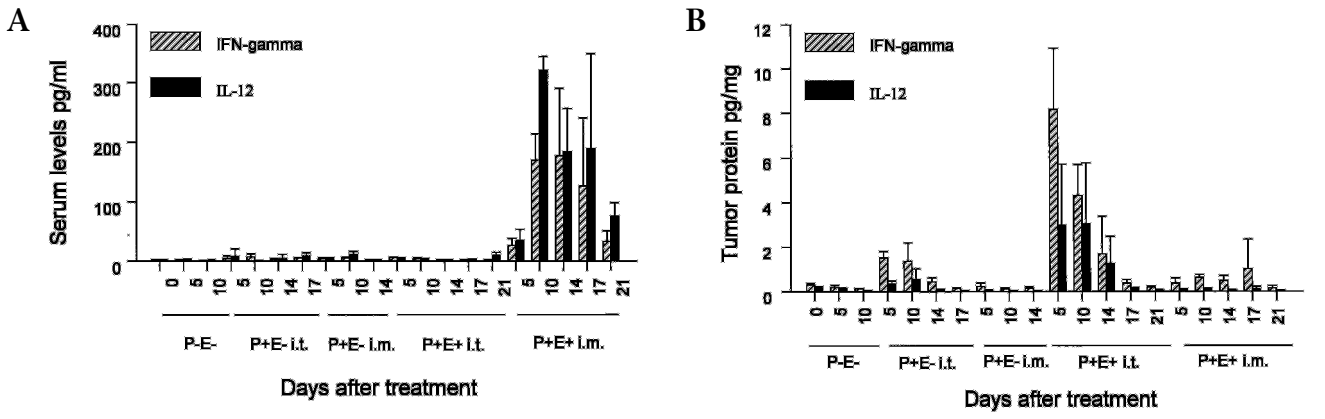

FIG. 2. Analysis of serum and tumor tissue for IL-12 and IFN-γ expression. P, pIRES IL-12; E, electroporation. Mode of delivery: i.t., intratumor; i.m., intramuscular. (A) Serum levels of IL-12 and IFN-γ in tumor-bearing mice. For each treatment group on each day tested, $n = 4$ mice. Error bars represent standard deviation. (B) Mean tumor expression of IL-12 and IFN-γ. For each treatment group on each day tested, $n = 4$ mice. Error bars represent standard deviation.

encoding IL-12 followed by electroporation. Mice not receiving electrical pulses, (P^+E^-), showed continued tumor growth until all mice were killed or succumbed to the tumor burden. Neither the administration of electroporation alone (P^-E^+) nor i.t. delivery of a control vector (pND2Lux) with electroporation (V^+E^+) decreased tumor growth. These results provide evidence that neither electrical pulses alone nor plasmid DNA is responsible for tumor regression. None of the treatment groups except the P^+E^+ i.t. group showed tumor regression, although P^+E^- i.t. did show slower tumor growth than P^-E^- through day 14 ($P < 0.05$).

Evaluation of mice 100 days after the initial treatment showed that 47% of mice (8/17) receiving i.t. delivery of IL-12 with electroporation were tumor-free (Fig. 1B). These mice were considered cured. All mice receiving i.t. treatment with IL-12 and electroporation experienced prolonged survival compared with animals in other treatment groups. None of the mice in control groups survived longer than 35 days. Specifically, if left untreated or treated with pulses alone, mice did not survive longer than 21 days.

We challenged seven of the animals that showed complete regression and remained disease-free for 50 days in the right flank with B16.F10 tumor cells. No additional treatments were administered. Of the seven challenged, five were resistant to tumor growth on the right flank, while tumors grew in 100% of naive mice. This result suggests the development of an immune memory response following treatment of the initial subcutaneous tumor established on the left flank.

Intratumoral Administration of IL-12 with Electroporation Results in Cytokine Expression within the Tumor

As mentioned earlier, IL-12 induces several effects on the immune system. To evaluate the cytokine expression induced by either i.m. or i.t. treatment, we analyzed serum and tumor levels of IL-12 and IFN-γ. Serum levels of both cytokines were highest after i.m. injection followed by electroporation (Fig. 2A). Serum IL-12 peaked at 320 pg/ml 10 days after treatment, whereas serum IFN-γ induced by IL-12 expression peaked at 177 pg/ml on day 14. Serum levels of both cytokines were significantly greater from mice treated i.m. with electroporation than other treatments on days 5, 10, and 14 ($P < 0.05$). Serum levels of these cytokines in mice treated with i.t. injection followed by electroporation were not significantly greater than expression in mice that received no treatment ($P > 0.05$).

Analysis of IL-12 and IFN-γ expression within the tumors revealed that i.t. treatment with electroporation resulted in the presence of these cytokines at the tumor site (Fig. 2B). Intratumoral IL-12 reached 3 pg/mg of tumor tissue on day 5 and remained at that level through day 10, whereas IFN-γ levels peaked at 8.16 pg/mg of tumor on day 5. Treatment with pIRES IL-12 injected i.t. followed by electroporation produced significantly higher ($P < 0.05$) IFN-γ levels than other treatment groups on days 5 and 10. Although tumor expression of IL-12 reached 3 pg/mg of tumor with i.t. treatment, as opposed to 0.64 pg/mg of tumor with i.m. treatment, these levels were not significantly greater ($P > 0.05$) as a result of a wide spectrum of expression levels in these tumors after i.t. treatment (0.5–6.9 pg/mg of tumor tissue).

Treatment with i.m. injection followed by electroporation did not result in significant ($P > 0.05$) cytokine expression within the tumors (Fig. 2B). Following i.m. treatment the highest IFN-γ expression measured was 1 pg/mg of tumor on day 17 (Fig. 2B). Therefore, treatment protocols that did not result in tumor regression also did not produce intratumoral IL-12 or IFN-γ expression. These results support previous reports on the critical need for cytokine expression within the tumor [4,9].

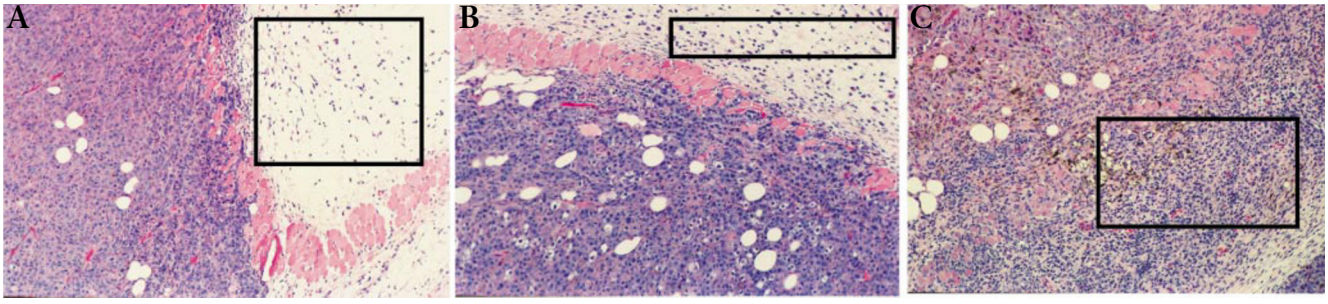

FIG. 3. Representative sections of tumor tissue, 5 days after treatment, analyzed by H&E staining for infiltrating immune cells. Three sections per tumor were examined. All sections are shown at ×250 magnification. An area containing immune cells is marked by a box. (A) No treatment. (B) Administration of IL-12 i.m. with electroporation. (C) Administration of IL-12 i.t. with electroporation.

Tumor Regression Is Characterized by Lymphocytic Infiltrate at the Tumor Site

Resistance to challenge following successful tumor regression suggests the development of an immune memory response. We examined the tumors histologically 5 days after initial treatment to evaluate the influx of immune cells to the tumor. We stained tumor sections with hematoxylin and eosin (H&E) to distinguish infiltrating immune cells from tumor cells. The H&E-stained sections showed infiltration of lymphocytes into the tumors of mice 5 days after receiving i.t. injection of pIRES IL-12 followed by electroporation (Fig. 3C). In contrast, mice not treated or receiving i.m. treatment with electroporation did not display a great influx of lymphocytes (Figs. 3A and 3B). Treatment protocols not including *in vivo* electroporation (P^+E^- either i.t. or i.m.) also did not result in the influx of lymphocytes (data not shown).

By immunohistochemical phenotyping, we demonstrated that the lymphocytes observed in tumors following i.t. treatment with IL-12 and electroporation were $CD4^+$ and $CD8^+$ T cells (Figs. 4C and 4D). In comparison, lymphocytes were observed in limited numbers in untreated tumors (Figs. 4A and 4B). Treatment of mice with i.m. injection followed by electroporation also resulted in limited lymphocytic infiltrate, similar to that characterizing the untreated control group (Figs. 4E and 4F). Additionally, mice receiving injection of plasmid encoding IL-12 (P^+E^- i.t. or i.m.) or control plasmid with electroporation (V^+E^+ i.t.) did not show infiltrating lymphocytes (data not shown).

Treatment Does Not Result in Tumor Regression in a Nude Mouse Model

To further evaluate the need for T lymphocytes in tumor regression, we used athymic nude mice deficient in T cells as the mouse model in place of C57BL/6 mice. We injected B16.F10 tumor cells subcutaneously and began treatment when tumors reached 3–5 mm in diameter. Mice received i.t. treatments as explained earlier: i.t. injections of plasmid encoding IL-12 without electroporation, i.t. injection of a control plasmid followed by electroporation, or i.t. injections of plasmid encoding IL-12 followed by electroporation. Because of the lack of successful response in C57BL/6 mice following i.m. injection, we administered only i.t. treatments. None of the treatments in the nude mouse model resulted in tumor regression (Fig. 5A). In addition, no mice in any treatment group survived longer than 30 days (Fig. 5B). This observation further suggests the necessity of a T-cell response for successful regression of B16.F10 melanoma tumors.

Intratumoral Administration of IL-12 with Electroporation Results in an Antiangiogenic Effect

Another potential role of IL-12 on tumor regression is its effect on angiogenesis. To assess the antiangiogenic role of IL-12 on B16.F10 tumors in C57BL/6 mice, we stained representative sections of three tumors from each treatment group with anti-CD31 antibodies, marking endothelial cells. Five different areas of highest vascularity were examined at a magnification of ×400 for each group (Fig. 6). A representative section of the vessels in an untreated tumor on day 0 is shown in Fig. 6A. Figures 6B and 6C show the large number of vessels present within untreated tumors or tumors from mice receiving i.m. injection followed by electroporation on day 5. In contrast, Fig. 6D shows the reduction of blood vessels after i.t. injection and electroporation on day 5. Tumors from mice receiving injection of plasmid encoding IL-12 without electroporation (P^+E^- i.t. or i.m.) or control plasmid with electroporation (V^+E^+) did not show a reduction in vasculature (data not shown).

In addition, we counted vessels in each of the three tumors excised from untreated mice, mice receiving i.m. IL-12 and electroporation, and mice receiving i.t. IL-12 and electroporation. Table 1 shows the number of blood vessels counted in the field of highest vascularity at a magnification of ×400 for each of the three excised tumors. Only i.t. injection followed by electroporation (P^+E^+ i.t.) resulted in significant ($P < 0.05$) vessel reduction compared with untreated animals. Although an antiangiogenic effect was observed following i.t. treatment with electroporation, the lack of response in the nude mouse model

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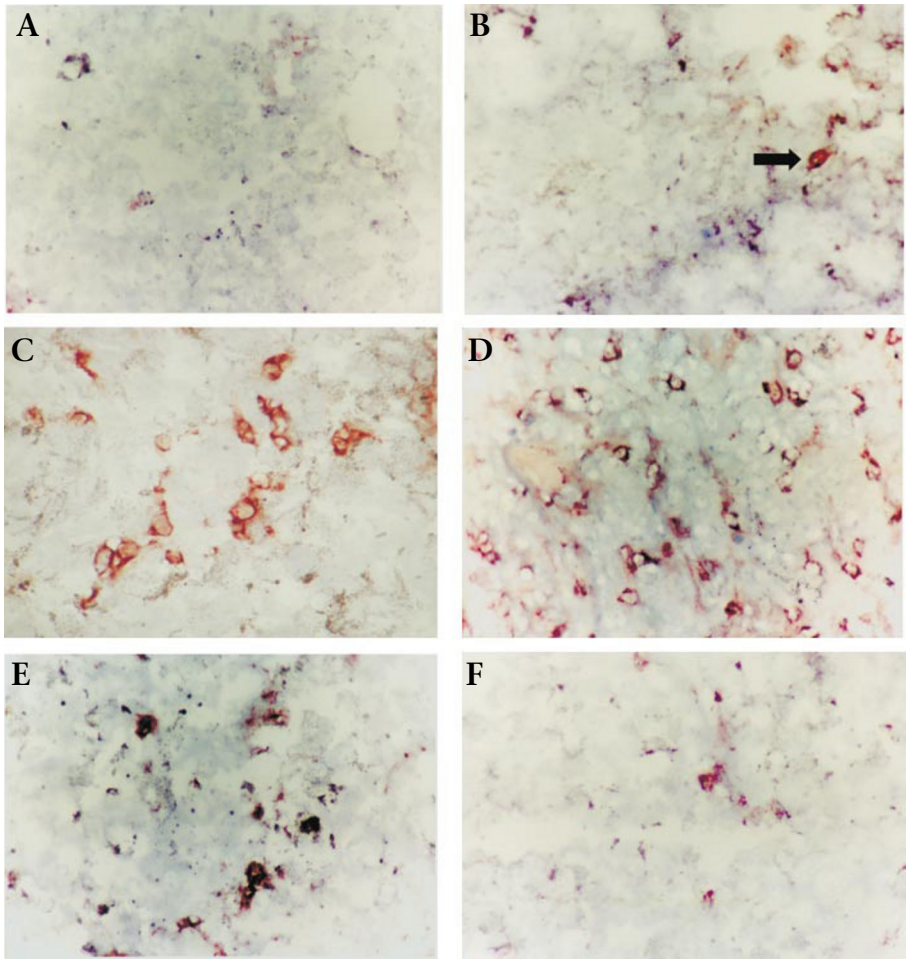

FIG. 4. Representative sections of tumor tissue, 5 days after treatment, analyzed by immunohistochemistry for the presence of $CD4^+$ and $CD8^+$ lymphocytes. Three sections per tumor were examined. All sections are shown at ×400 magnification. Positive cells are stained brown. An arrow in (B) points to a cell representative of positive staining. (A, B) Staining for $CD4^+$ lymphocytes and $CD8^+$ lymphocytes, respectively, from untreated tumors. (C, D) Staining for $CD4^+$ lymphocytes and $CD8^+$ lymphocytes, respectively, from tumors receiving i.t. injection of plasmid DNA encoding IL-12 followed by electroporation. (E, F) Staining for $CD4^+$ lymphocytes and $CD8^+$ lymphocytes, respectively, from tumors following i.m. administration of plasmid DNA encoding IL-12 with electroporation.

suggests that T cells may be a critical factor for obtaining regression of B16.F10 melanoma. An antiangiogenic response may, however, contribute to stabilization of tumor size while an immune response is mounted.

DISCUSSION

This report has demonstrated that IL-12 delivered in the form of plasmid DNA with the aid of electroporation can result in successful regression of B16.F10 tumors. The animals remain disease-free and are resistant to challenge at a distant site. This is the first report to demonstrate nearly a 47% survival rate following gene therapy treatment of established subcutaneous B16.F10 melanoma tumors.

The data reported here support earlier studies suggesting that successful tumor regression is dependent on local rather than systemic expression of IL-12 and IFN-γ [4,9,10]. Both of these cytokines induce numerous factors that may account for their antitumor activity. Interleukin-12 upregulates T and NK cells and stimulates IFN-γ production, which in turn initiates the release of antiangiogenic factors and the possible upregulation of major histocompatibility complex I (MHC I) [1,10]. Direct treatment of the tumor ensures that IL-12 is readily available at that specific site. Therefore, the resulting antitumor effects are directed at the tumor site and not diluted systemically.

Administration of pIRES IL-12 by i.t. injection followed by electroporation resulted in increased IL-12 and IFN-γ expression at the tumor site. Variations in tumor size at the time of treatment may explain the wide range of IL-12 expression observed within the tumors after i.t. *in vivo* electroporation. Larger tumors can more readily accommodate the injected volume, thereby reducing the possibility of decreased levels of plasmid DNA in a small tumor resulting from fluid leakage. A greater number of tumor cells are potentially targeted in larger tumors as opposed to those that are smaller. The surrounding epidermal cells of a smaller tumor may also receive the plasmid. However, these cells have a high turnover rate, and therefore sustained plasmid DNA expression is unlikely.

The highest expression of IFN-γ at the tumor site following i.m. injection and electroporation was measured at 1 pg/mg of tumor on day 17 (Fig. 2B). However, this finding was not constant and was usually observed within very large tumors undergoing ischemic necrosis. It is possible that IFN-γ expression in these tumors resulted from the treatment or was due to infiltrating macrophages attracted to tissue necrosis.

In vivo electroporation following i.m. administration of pIRES IL-12 induced systemic expression of the cytokines IL-12 and IFN-γ. An earlier report from our laboratory showed that i.m. injection of a plasmid encoding IL-12

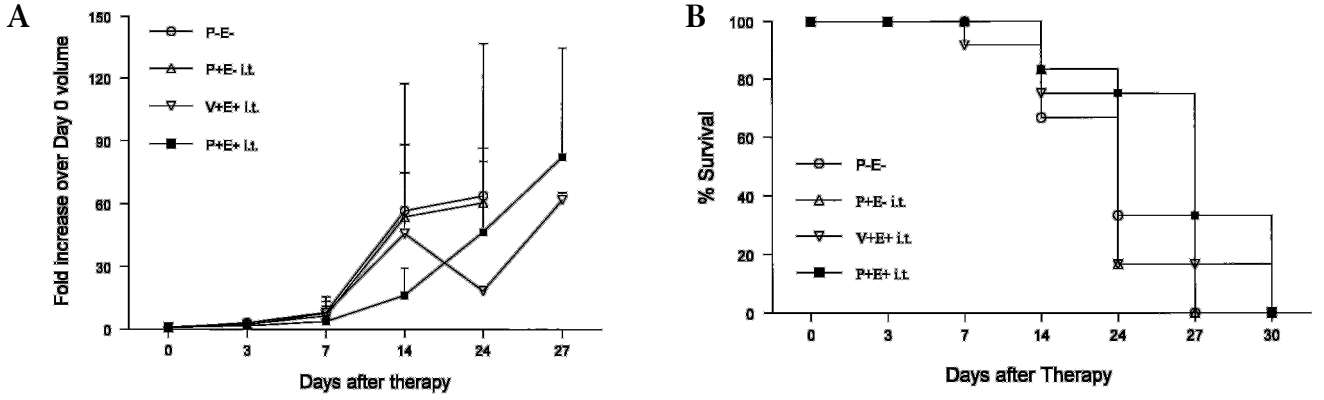

FIG. 5. Administration of IL-12 followed by electroporation does not result in tumor regression in a nude mouse model. (A) Fold increase over day 0 tumor volume following treatment. P, pIRES IL-12; V, control plasmid, pND2Lux; E, electroporation. Mode of delivery: i.t., intratumor. Initial treatment day is day 0; mice were treated again on day 7. The data represent two experiments each, with four mice in each group. Error bars represent standard deviation. Mice were killed when tumor volume exceeded 1000 mm^3. Data are expressed for surviving mice on each day. (B) Percentage survival of mice represented in (A). Mice either succumbed to disease or were killed when tumor volume exceeded 1000 mm^3.

followed by the administration of ms pulses resulted in long-term expression of IL-12 and IFN-γ in the serum of mice. In this report systemic levels of IL-12 and IFN-γ did not peak until day 10 and 14 after the initial i.m. treatment. The untreated mice began to succumb to tumor burden by this time period. In contrast, the i.t. treatment has the advantage of potentially inducing high levels of IFN-γ–dependent antitumor factors directly within the tumor and within 5 days of treatment. These results support previous reports that intratumor expression of cytokines are related to successful tumor regression [4,9,10].

Administration of IL-12 to the tumor with electroporation also induced an infiltration of immune cells, particularly lymphocytes, to the tumor site. The lymphocytes were identified as $CD4^+$ and $CD8^+$ by immunohistochemistry. Very few lymphocytes were observed in untreated animals, animals receiving i.t. injection without electroporation, animals receiving i.t. injection of control plasmid with electroporation, as well as animals receiving i.m. administration of IL-12 and electroporation. The absence of tumor regression and animal survival in the nude mouse model further suggests the critical need for lymphocytes in the treatment of this tumor model.

Another effect of IL-12 is inhibition of angiogenesis. Staining of tumor sections with anti-CD31 antibodies showed the reduction of vascularity following i.t. treatment with IL-12 and electroporation. Again, these effects were not seen in untreated animals or those receiving i.m. treatment. Therefore, an antiangiogenic effect is observed following the same treatment protocol that results in tumor regression.

Although this report investigated numerous effects of IL-12 in relation to tumor regression, other potential influences cannot be ruled out. Schultz *et al.* reported no effects of CpG motif on tumor regression from the IL-12 cDNA in this plasmid but did not determine effects from CpG motifs within the plasmid backbone [8]. Furthermore, IL-12 itself may increase the expression of accessory molecules such as MHC I [10]. These variables and others may also have a function in successful tumor regression.

As mentioned earlier, two other studies have used electroporation for delivery of IL-12 to B16 melanomas but were unsuccessful in obtaining complete regression and disease-free or cured mice [25,26]. In both of these studies, ms pulses were delivered to tumors. In contrast, Heller *et al.* administered μs pulses for delivery of plasmids encoding IL-2 or granulocyte/macrophage colony-stimulating factor (GM-CSF) by *in vivo* electroporation along with electrochemotherapy and demonstrated successful tumor regression [23]. Although different therapeutic molecules were delivered in these studies, this report shows the most substantial therapeutic effect to date by delivering a plasmid encoding IL-12 using μs pulses.

One advantage of electroporation is the ability to tailor the pulse protocols to different tissues. Millisecond pulses are more successful than μs pulses in i.m. delivery for obtaining sustained systemic cytokine expression [18]. Other tissue types, such as skin, are more successfully transfected with μs pulses [14]. The rounder morphology of the B16.F10 tumor cell is more similar to that of a skin cell than the elongated muscle fiber. Therefore, the variances in tumor regression following ms or μs pulse protocols may be the result of not applying the appropriate pulse conditions for the tissue type.

The potential for clinical success makes *in vivo* electroporation a promising treatment modality. Localized gene therapy reduces the risk of adverse side effects associated with systemic recombinant protein therapy. In mice, local delivery of IL-12 by gene gun resulted in similar tumor regression rates as recombinant IL-12 treatment but with fewer adverse side effects [11]. In addition, Lohr *et al.* found that delivery of IL-12 by electroporation did not result in

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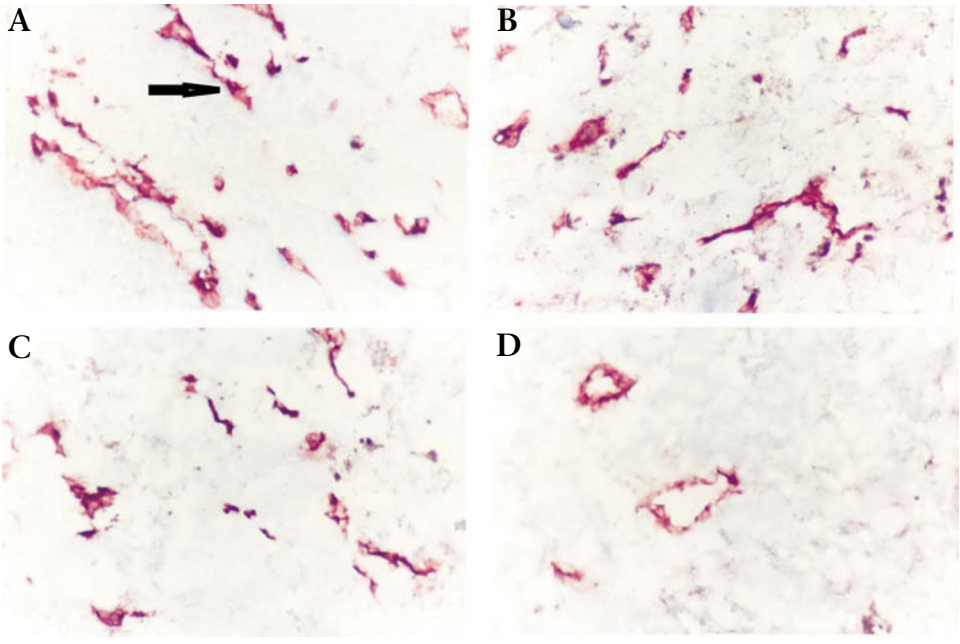

FIG. 6. Immunohistochemical analysis of tumor tissue for the presence of blood vessels. Representative sections rich in vessels are depicted for each treatment. Three sections per tumor were examined. All sections are shown at ×400 magnification. An arrow in (A) points to a representative blood vessel. (A) Presence of blood vessels within tumors on day 0, before treatment. (B) Untreated tumors on day 5. (C) Tumors on day 5 from mice receiving i.m. injection of plasmid DNA encoding IL-12 followed by electroporation. (D) Blood vessels on day 5 from mice receiving i.t. administration of plasmid DNA encoding IL-12 followed by electroporation.

the side effects observed following adenoviral delivery of IL-12 [25]. However, complete regression of established B16.F10 melanoma resulting in disease-free survival was not achieved by either of these treatment modalities. Electroporation is an effective, yet safe, delivery system as shown in electrochemotherapy clinical trials, which used electroporation to target tumors with chemotherapeutic drugs [27]. The delivery conditions used for i.t. treatment in the experiments reported here are similar to those used for electrochemotherapy clinical trials. Those studies revealed these conditions to be tolerable for patients and did not result in damage to normal tissue [27].

In summary, we report a treatment modality that can eradicate established B16.F10 melanoma tumors and result in resistance to renewed tumor growth following challenge. After i.t. delivery of plasmid DNA encoding IL-12 by *in vivo* electroporation, 47% of mice showed complete regression of their tumors and remained disease-free. These mice were challenged with B16.F10 tumor cells, and five of seven remained tumor-free for an additional 100 days, after which they were humanely killed. We also show that i.t. injection of plasmid DNA encoding IL-12 and electroporation is more effective than i.m. delivery for promoting tumor regression and prolonging animal survival. The success of this treatment in this tumor model stems from the local expression of IL-12 and IFN-γ, infiltrating lymphocytes, and inhibition of angiogenesis within the treated tumor.

TABLE 1: Tumor blood vessel counts from C57BL/6 mice in each treatment group

	P^-E^-	P^-E^- i.t.	P^+E^- i.m.	V^+E^+ i.t.	P^+E^+ i.m.	P^+E^+ i.t.
Tumor 1	24	10	27	20	17	6
Tumor 2	32	21	32	28	38	12
Tumor 3	49	28	39	39	38	18

P^-, No plasmid; E^-, no electroporation; P^+, pIRES IL-12; E^+, electroporation; V^+, control plasmid, pND2Lux; i.t., intratumoral; i.m., intramuscular.

MATERIALS AND METHODS

Tumor cells and mice. B16.F10 murine melanoma cells (CRL 6475; American Type Culture Collection, Rockville, MD) were maintained in Dulbecco's minimal Eagle's medium (DMEM) supplemented with 10% FCS and 0.2% gentamicin. Cells were trypsinized and washed in sterile PBS before injection. The left flank of C57BL/6 mice (National Cancer Institute, Bethesda, MD) was shaved and 1×10^6 cells in 50 μl of sterile PBS were injected subcutaneously. When challenged, mice were injected with 5×10^5 B16.F10 cells in the right flank. Tumors were measured using digital calipers, and treatment was begun when tumors reached 3–5 mm in diameter, ~ 7–10 days after injection. Tumor volume (v) was calculated using the formula $v = a^2b\pi/6$, where a = the smallest diameter and b = the perpendicular diameter. Mice were housed in accordance with AALAM guidelines.

Plasmid DNA. pIRES IL-12 was a gift from Karin Moelling (University of Zurich, Zurich, Switzerland). Briefly, pIRES IL-12 contains both subunits joined by an internal ribosomal entry site (IRES) behind a single cytomegalovirus (CMV) promoter [8]. Robert Malone (Gene Delivery Alliance, Inc., Rockville, MD) donated the pND2Lux, which encodes the reporter gene luciferase. Qiagen Mega Kits (Qiagen, Valencia, CA) were used for plasmid preparations. pIRES IL-12 was prepared with an endotoxin-free kit.

Intratumor treatment. Mice were anesthetized using 97% oxygen and 3% isoflurane. Tumors were injected with 50 μl (1 μg/μl) plasmid DNA in sterile saline using a tuberculin syringe with a 25-gauge needle. A circular applicator containing six penetrating electrodes ~ 1 cm in diameter [15,28] was inserted into the tumor. Briefly, six rotating pulses were delivered at 1500 V/cm (99 μs, 1 Hz) using a BTX T820 pulse generator and autoswitcher (BTX, San Diego, CA) [15,28].

Intramuscular treatment. Mice were anesthetized as described earlier. The skin surrounding the gastrocnemius muscle was shaved. Plasmid DNA diluted

in sterile saline (50 μl, 1 μg/μl) was injected into the gastrocnemius muscle using a tuberculin syringe and a 25-gauge needle. An applicator specially designed for the mouse gastrocnemius containing four penetrating electrodes in a rectangular pattern (2 × 5 mm) was inserted into the muscle surrounding the injection site. A total of 12 pulses was delivered segmentally at 100 V/cm (20 ms, 1 Hz) using a BTX T820 pulse generator. A manual switch was used to administer three pulses in each of four directions as follows, with number of electrodes active given in parentheses: across 2 mm distance (4); first diagonal (2); second diagonal (2); and across 5 mm distance (4). The first treatment (day 0) was delivered to the left leg and the second treatment (day 7) was delivered to the right leg.

ELISA. Mice were humanely killed using CO_2 asphyxiation, and then blood and tumors were collected on each day from four mice per treatment group. For detection of cytokines in the serum, blood was collected by cardiac puncture and stored at 4°C overnight. Serum was extracted from blood samples by centrifugation (3 minutes at 5000 rpm) at 4°C, and stored at –20°C until analyzed. To measure cytokine levels within the tumor tissue, the tumors were removed, frozen immediately on dry ice, weighed, and then stored at –80°C. For analysis, the tumors were thawed, and 1 ml of a solution containing PBS and 10% protease inhibitor cocktail (P8340; Sigma, St. Louis, MO) was added. The tissues were kept on ice, homogenized using a PowerGen 700 (Fisher Scientific, Pittsburgh, PA), centrifuged for 3 minutes at 5000 rpm at 4°C, and then supernatants were assayed by ELISA. Both serum and tumor samples were analyzed using murine IFN-γ and IL-12 p70 ELISA kits (R&D Systems, Minneapolis, MN). Serum levels were calculated as pg of cytokine per ml of serum. Cytokine levels in the tumor were calculated as pg of cytokine per mg of tumor.

Histology. Mice were humanely killed by CO_2 asphyxiation. Tumors were excised and placed in 50-ml conical tubes containing 10 ml of 10% formalin. The tissue was stained with H&E after fixation, as follows: after fixation in 10% neutral buffered formalin for 6 hours, representative tissue samples were processed into paraffin blocks using a Miles VIP tissue processor (Miles Inc., Mishawaka, IN). Briefly, tissues were dehydrated in ascending grades of ethanol, cleared in xylene, and infiltrated in paraffin (Tissue Prep 2; Fisher Scientific). Following embedding, tissues were sectioned on a standard rotatory microtome and 4-μm sections were retrieved from a waterbath and mounted on glass slides. Three sections per tumor were examined. Sections were heat-dried and stained with H&E (Richard-Allan Scientific, Kalamazoo, MI) using standard histologic techniques. Using a synthetic mounting medium, coverslips were then placed.

Immunohistochemistry. Immunohistochemical staining was conducted to examine the tumors for the presence of $CD4^+$ lymphocytes, $CD8^+$ lymphocytes, and blood vessels using the following antibodies: rat anti-mouse CD4, rat anti-mouse CD8a (Ly2), and rat anti-mouse CD31 (PECAM-1), respectively (PharMingen, Cambridge, MA). Mice were humanely killed by CO_2 asphyxiation. Tumors were excised with scissors and the skin removed, then immediately frozen in a mixture of dry ice and ethanol, and stored at (80°C. Frozen sections of 5 μm were obtained. For immunohistochemical analysis, rat anti-mouse CD4, rat anti-mouse CD8a (Ly2), or rat anti-mouse CD31 (PECAM-1) was applied to tissue sections at a dilution of 1:50 and incubated for 30 minutes, followed by detection with the Vector Elite Rat IgG–Peroxidase kit at 2× concentration (15 minutes each in biotinylated anti-rat IgG and ABC complex). Immunostaining was carried out on the Dako autostainer. Sections were analyzed at ×400 magnification.

Treatment of nude mice. BALB/c athymic nude mice were obtained from the National Cancer Institute and used at 7 weeks of age. B16.F10 cells were prepared as described earlier. Mice were injected subcutaneously in the left flank with 1×10^6 B16.F10 cells in 50 μl of sterile PBS. Treatment was begun when the tumors reached 3–5 mm in diameter. Mice received intratumor therapy as described earlier.

Statistical methods. Statistical analysis was performed by ANOVA or two-tailed Student's *t*-test.

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REFERENCES

1. Brunda, M., *et al.* (1993). Antitumor and antimetastatic activity of interleukin 12 against murine tumors. *J. Exp. Med.* **178:** 1223–1230.
2. Brunda, M., *et al.* (1995). Role of interferon-γ in mediating the antitumor efficacy of interleukin-12. *J. Immunother.* **17:** 71–77.
3. Harada, M., *et al.* (1998). Role of the endogenous production of interleukin 12 in immunotherapy. *Cancer Res.* **58:** 3073–3077.
4. Yu, W., *et al.* (1997). IL-12-induced tumor regression correlates with *in situ* activity of IFN-γ produced by tumor-infiltrating cells and its secondary induction of anti-tumor pathways. *J. Leukoc. Biol.* **62:** 450–457.
5. Dabrowska, A., Giermasz, A., Marczak, M., Golab, J., and Jakobisiak, M. (2000). Potentiated antitumor effects of interleukin 12 and matrix metalloproteinase inhibitor batimastat against B16.F10 melanoma in mice. *Anticancer Res.* **20:** 391–394.
6. Nanni, P., *et al.* (1998). Interleukin 12 gene therapy of MHC-negative murine melanoma metastases. *Cancer Res.* **58:** 1225–1230.
7. Egilmez, N., *et al.* (2000). *In situ* tumor vaccination with interleukin-12-encapsulated biodegradable microspheres: induction of tumor regression and potent antitumor immunity. *Cancer Res.* **60:** 3832–3837.
8. Schultz, J., Pavlovic, J., Strack, B., Nawrath, M., and Moelling, K. (1999). Long-lasting antimetastatic efficiency of interleukin 12-encoding plasmid DNA. *Hum. Gene Ther.* **10:** 407–417.
9. Toda, M., Martuza, R., Kojima, H., and Rabkin, S. (1998). *In situ* cancer vaccination: an IL-12 defective vector/replication-competent herpes simplex virus combination induces local and systemic antitumor activity. *J. Immunol.* **160:** 4457–4464.
10. Colombo, M., *et al.* (1996). Amount of interleukin 12 available at the tumor site is critical for tumor regression. *Cancer Res.* **56:** 2531–2534.
11. Rakhmilevich, A., *et al.* (1999). Gene gun-mediated IL-12 gene therapy induces antitumor effects in the absence of toxicity: a direct comparison with systemic IL-12 protein therapy. *J. Immunother.* **22:** 135–144.
12. Marshall, E. (1995). Cancer trial of interleukin-12 halted. *Science* **268:** 1555.
13. Titomirov, A., Sukharev, S., and Kistanova, E. (1991). *In vivo* electroporation and stable transformation of skin cells of newborn mice by plasmid DNA. *Biochim. Biophys. Acta* **1088:** 131–134.
14. Heller, R., *et al.* (2001). Intradermal delivery of interleukin-12 plasmid DNA by *in vivo* electroporation. *DNA Cell Biol.* **20:** 21–26.
15. Heller, R., Jaroszeski, M., and Gilbert, R. (1996). *In vivo* gene electroinjection and expression in rat liver. *FEBS Lett.* **389:** 225–228.
16. Aihara, H., and Miyazaki, J. (1998). Gene transfer into muscle by electroporation *in vivo*. *Nat. Biotechnol.* **16:** 867–870.
17. Mir, L., *et al.* (1999). High-efficiency gene transfer into skeletal muscle mediated by electric pulses. *Proc. Natl. Acad. Sci. USA* **96:** 4262–4267.
18. Lucas, M., and Heller, R. (2001). Immunomodulation by electrically enhanced delivery of plasmid DNA encoding IL-12 to murine skeletal muscle. *Mol. Ther.* **3:** 47–53, doi:10.1006/mthe.2000.0233.
19. Heller, L., Jaroszeski, M., Coppola, D., Pottinger, C., and Heller, R. (2000). Electrically mediated plasmid DNA delivery to hepatocellular carcinomas *in vivo*. *Gene Ther.* **7:** 826–829.
20. Wells, J., Li, L., Sen, A., Jahreis, G., and Hui, S. (2000). Electroporation-enhanced gene delivery in mammary tumors. *Gene Ther.* **7:** 541–547.
21. Goto, T., *et al.* (2000). Highly efficient electro-gene therapy of solid tumor by using an expression plasmid for the herpes simplex virus thymidine kinase gene. *Proc. Natl. Acad. Sci. USA* **97:** 354–359.
22. Rols, M., *et al.* (1998). *In vivo* electrically mediated protein and gene transfer in murine melanoma. *Nat. Biotechnol.* **16:** 168–171.
23. Heller, L., Pottinger, C., Jaroszeski, M., Gilbert, R., and Heller, R. (2000). *In vivo* electroporation of plasmids encoding GM-CSF or interleukin-2 into existing B16 melanomas combined with electrochemotherapy induces long-term antitumor immunity. *Melanoma Res.* **10:** 577–583.
24. Niu, G., *et al.* (1999). Gene therapy with dominant-negative Stat3 suppresses growth of the murine melanoma B16 tumor *in vivo*. *Cancer Res.* **59:** 5059–5063.
25. Lohr, F., *et al.* (2001). Effective tumor therapy with plasmid-encoded cytokines combined with *in vivo* electroporation. *Cancer Res.* **61:** 3281–3284.
26. Kishida, T., *et al.* (2001). *In vivo* electroporation-mediated transfer of interleukin-12 and interleukin-18 genes induces significant antitumor effects against melanoma in mice. *Gene Ther.* **8:** 1234–1240.
27. Heller, R., Gilbert, R., and Jaroszeski, M. (1999). Clinical applications of electrochemotherapy. *Adv. Drug Deliv. Rev.* **35:** 119–129.
28. Gilbert, R., Jaroszeski, M., and Heller, R. (1997). Novel electrode designs for electrochemotherapy. *Biochim. Biophys. Acta.* **1334:** 9–14.