Effective Tumor Therapy with Plasmid-encoded Cytokines Combined with in Vivo Electroporation

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Abstract

Plasmids may have unique advantages as a gene delivery system. However, a major obstacle is the low in vivo transduction efficiency. In this study, an electroporation-based gene transduction approach was taken to study the effect of interleukin (IL)-2 or IL-12 gene transduction on the growth of experimental murine tumors. Significant intratumoral gene transduction was achieved by electroporation of tumors that had been injected with naked plasmids encoding reporter genes and cytokine genes (IL-2 and IL-12) under the control of a constitutive cytomegalovirus promoter. In addition, significant tumor growth delay could be achieved in a murine melanoma line B16.F10 with the cytokine genes. Most importantly, systemic transgene levels were negligible when compared with intratumoral adenovirus-mediated IL-12 gene delivery, which leads to significantly higher systemic cytokine levels. Therefore, naked plasmids and in vivo electroporation-mediated cancer gene therapy may be therapeutically efficacious while maintaining low systemic toxicity.

Introduction

Recombinant viral vectors account for the majority of gene delivery approaches used in current cancer gene therapy studies. Examples include adenovirus, herpes virus, and retrovirus vectors. The main advantage of viral vectors is their high efficiency of gene transduction. However, there are also many potential problems, such as efficiency of production and safety. For example, adenovirus is one of the most commonly used vector systems for gene therapy. Most of the adenovirus vectors used are rendered replication defective. However, it has been reported that new unwanted variants can develop as a result of recombination during the production process. In addition, adenoviruses can elicit strong immune responses that will diminish efficacy in later administrations and could represent a hazard for adverse reactions. At higher doses, biosafety is also a big concern. For example, even with strictly local infection, there may be systemic toxicity because of virus leaking into the systemic circulation, then infecting the liver with high efficiency (1, 2).

Plasmid DNA, on the other hand, is a relatively safe alternative to viral vectors. The toxicity is generally very low, and large-scale production is relatively easy. However, a major obstacle that has prevented the widespread application of plasmid DNA is its relative inefficiency in gene transduction. Therefore, most applications for plasmid DNA have been limited to vaccine studies with a few exceptions (3–5).

Methods that can significantly enhance the efficiency of plasmid DNA transduction efficiency will greatly extend the utility of this promising mode of gene transfer. Electroporation has long been used to effectively transport molecules, including DNA, into living cells in vitro (6). More recent reports demonstrated that it can safely be used in vivo, e.g., by enhancing the local efficacy of chemotherapeutic agents (7, 8). When used in conjunction with DNA plasmids, it greatly enhances the local transfection efficiency over plasmid injection alone (9).

In this study, we applied this new approach to antitumor immunogene therapy. We report the effectiveness and possible advantages of local gene therapy with plasmid encoded mIL-27 and mIL-12 in combination with in vivo electroporation in a murine melanoma tumor model with particular attention to intratumoral and systemic transgene levels.

Materials and Methods

Tumor Models/Cell Culture. B16.F10 (American Type Culture Collection, Manassas, Virginia) is a metastasizing subline of the B16 melanoma that arose spontaneously and is syngeneic with C57BL/6 mice. In vitro it is maintained in DMEM. Transplantation into C57BL/6 mice at 105 cells/animal establishes tumors in 100% of mice. 293 cells (American Type Culture Collection, Manassas, Virginia) were used for adenovirus propagation and were maintained in DMEM as well. All cell culture media were supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and 1 g each of penicillin/streptomycin (Life Technologies, Inc., Grand Island, NY) per 100 ml.

Plasmids. The pEGFP-N1 plasmid was obtained from Clontech Corp. (Palo Alto, CA). It encodes the EGFP protein under the control of a CMV promoter. Plasmid pNGVL-mIL2 and pNGVL-mIL12 were obtained from the NGVL at the University of Michigan (Ann Arbor, MI). They encode mIL-2 and mIL-12 genes under the control of the CMV promoters, respectively. Plasmid pNGVL-β-gal was also obtained from the NGVL. It encodes a nuclear targeted β-gal gene under the control of the CMV promoter.

Adenovirus Vector. The adenoviral vector Ad5IL12 (kindly provided by Dr. Frank L. Graham, McMaster University, Hamilton, Ontario, Canada) used in this study was described previously and is based on an Ad5 recombinant system (10). In short, both mIL-12 subunit cDNAs were inserted in the E1 region and placed under control of the murine CMV promoter. Efficient expression of both IL-12 subunits was achieved by placing an internal ribosome entry site in-between. Viruses were propagated in 293 cells and purified by CsCl banding according to a standard protocol (11).

In Vivo Electroporation and Adenovirus Infection. Animal care and experimental procedures were in accord with institutional guidelines. All animals were anesthetized before virus or plasmid injection/electroporation with Ketamine/Xylazine at 1.80 mg/mouse. After tumors grew to sizes of 5–7 mm in diameter (or 65–179 mm3 in volume), they were injected intratumorally either with Ad5IL12 (3 × 107 particles in 50 μl of PBS) or with 50 μg

[The text continues with further details on methods and results, not transcribed here.]
of either EGFP-, β-gal-, mIL-2- or mIL12 plasmid in 50 μl PBS. Animals intended for combination treatment underwent subsequent electroporation within 1–5 min. Electroporation pulses were delivered with 2 × 2-cm stainless steel plates attached to a caliper electrode (Genetronics, Inc., San Diego, CA). Electrode Gel (Signa Gel; Parker Laboratories, Inc., Fairfield, NJ) was applied to the electrodes to reduce the interfacial resistance and maintain good electrical contact between electrode and skin. The caliper electrodes were clamped on the tumor in an approximate dorsal-ventral orientation to avoid placing any bone within the electric field. The distance between electrodes was ~6 mm. Square wave electric pulses were generated with an Electro Square Porator T820 (Genetronics, Inc.). Three pulses (100V/50 ms) were delivered, followed by three more pulses at the opposite polarity. These electroporation parameters were selected based on previous reports (12–14) and our own preliminary experiments.

Detection of Reporter Gene Expression. About 48 h after injection of either EGFP or β-gal plasmid injection with or without consecutive electroporation, animals were sacrificed, and tumors were harvested. Animals were anesthetized and sacrificed by cervical dislocation. Tumors injected with β-gal plasmid were fixed in 4% paraformaldehyde/PBS for 48 h at 4°C. Tumors were sectioned at 250 μm. Sections were rinsed three times for 30 min each in rinse buffer (100 mM sodium phosphate, 2 mM MgCl₂, 0.01% deoxycholic acid, and 0.02% NP40) at room temperature. Sections were stained for 24 h at room temperature. Staining solution was rinse buffer, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. After 24 h, staining solution was removed, and sections were stored in 70% ethanol before mounting (in 70% ethanol) for microscopy.

Measurement of IL-12 Levels. mIL-12 levels in serum samples and tumor extracts were detected with an mIL-12 ELISA kit (R&D Systems, Minneapolis, MN) that detects the heterodimer of p35 and p40 (p75) with a detection level of 8 pg/ml. Serum was obtained from blood samples drawn from the tail vein of animals/data point. For detection of intratumoral mIL-12, untreated control tumors and tumors at different time points after injection or plasmid injection/electroporation were harvested (two to four animals/data point). Tumors were homogenized in PBS (with Complete protease inhibitor; Boehringer Mannheim) and spun down, and supernatant was collected for measurement.

Tumor Growth Delay Studies. About 10⁵ B16.F10 cells in 50 μl of PBS were transplanted in the right hind limbs of C57BL/6 mice. Treatment was initiated when the tumors had reached a mean diameter of 5–7 mm (corresponding to a tumor volume of 65–179 mm³). Each treatment group consisted of nine animals and was treated with a single intratumoral injection of 50 μg of plasmid encoding EGFP, mIL-2, or mIL12 with or without subsequent electroporation, respectively. The injection was carried out in 50 μl of PBS. Tumor volume was determined by measuring the largest (L) and the smallest (S) diameters of the tumor and calculated as V = π/6(L × S²). Growth curves are plotted as the mean relative treatment group tumor volume ± SE. Relative tumor growth rate were calculated for the first 6 days after treatment.

Histological Examination of the Tumors. Tumors from different treatment groups were excised at the end of experiments (15 days after initial treatment). They were then deep frozen in liquid nitrogen with Tissue-Tek OCT compound (Sakura, Torrance, CA) as embedding medium, sectioned, and mounted. They were then stained with H&E and evaluated at ×400.

Results

Intratumoral Expression of Reporter Genes in Vivo. Electroporation of the plasmids were carried out according to conditions established by previous reports (12–14) and our own experience. B16.F10 tumors grown to 5–7 mm in diameter (or 65–179 mm³) in volume were injected with the reporter plasmids. Expression of reporter genes (EGFP and β-gal) after plasmid injection and electroporation in tumor tissue was assessed in fresh tissue sections (at 250 μm) by light microscopy (transmission and fluorescence imaging; Fig. 1). For both reporter systems, very few cells were positive when only naked DNA without consecutive electroporation was injected [Fig. 1a (EGFP) and 1c (β-gal)]. The combination with electroporation resulted in consistently efficient transduction of a higher number of cells with both reporter genes [Fig. 1b (EGFP) and 1d (β-gal)]. In EGFP experiments, plasmid injection with electroporation allowed 3–8% (as evaluated by fluorescence-activated flow cytometry) of all of the cells in the tumor mass to be transduced with the EGFP gene in comparison with <0.1% in tumors injected with EGFP plasmid alone.

Intratumoral and Systemic Expression of Therapeutic Genes in Vivo. To quantitatively evaluate local and systemic transgene expression as a consequence of electroporation and control gene transfer approaches, mIL-12 (as a heterodimer of p35 and p40 subunits) levels were assessed in tumor and serum of untreated and treated animals. The mIL12 levels in the serum and tumors were below the detection threshold (8 pg/ml) in untreated or electroporation-alone control animals. Fig. 2 is a summary of the peak mIL-12 levels in treated animals. With the mIL-12 plasmid injection alone, the cytokine level in the tumor reached from below the level of detection (day 2) to a peak of 0.3 ng/g (day 5), whereas the level in the serum reached from below the level of detection (day 2) to a peak of 0.4 ng/ml (day 5). In those tumors that were injected with the control GFP-encoding plasmid, the tumor and serum levels of the cytokine were similar to those with mIL-12 alone, indicating that the low level of cytokine expression observed with the mIL-12 plasmid injection alone were perhaps the result of DNA injection itself rather than any specific gene expression. When mIL-12 plasmid injection was combined with electroporation, the cytokine level reached 2.6 ng/g on day 2 and 5.4 ng/g on day 5 in the tumor, whereas the level in the serum reached from below the level of detection (day 2) to 0.35 ng/ml, similar to that achieved with plasmid injection alone. In comparison, in animals that were intratumorally injected with a therapeutically effective dose [according to previous studies in our laboratory (15)] of 3 × 10⁸ plaque-forming units of AdIL12, an adenovirus encoding the mIL-12 gene under the control of the CMV promoter, the mIL-12 levels were
between 7.2 (day 2) and 5.7 ng/g (day 5) in the tumor, whereas the peak value (at day 2) in the serum reached 20 ng/ml. For both modalities, tumor mIL-12 levels returned to baseline at 9 days after treatment. Therefore, significant tumor levels of IL-12 can be achieved in vivo by combining mIL-12 plasmid injection and electroporation when compared with AdIL12 injection. Serum levels, however, were greatly elevated after local AdIL12 injection (reaching a maximum of 20 ng/ml), while they were close to nonspecific DNA control after the combination of plasmid injection and electroporation (maximum of 0.4 ng/ml; Fig. 2). In addition, apparent toxicity was observed in animals injected with AdIL12, consistent with our earlier observations (15). The toxicities include weight loss, apathy, and splenomegaly (15).

Significant Tumor Growth Delay after Electro-Gene Therapy. Experiments were then conducted to examine the antitumor efficacy of the combined electroporation/plasmid DNA transfer approach. Fig. 3A shows the mean relative volumes (± SE) for B16.F10 tumors in C57BL/6 mice treated with injections of control plasmid (EGFP), mIL-2 or mIL-12 with or without in vivo electroporation. Fig. 3B shows the relative growth rate of different groups in the first 6 days after treatment. Electroporation in conjunction with control plasmid (pEGFP-N1) did not result in significant growth delay over control plasmid alone or nontreated controls. In addition, injections of naked mIL-2 or mIL-12 plasmid alone did not result in any significant growth delay over control plasmid. The combination of electroporation with either mIL-2 or mIL-12 plasmid resulted in a significant growth delay of approximately 5–15 days when compared with both control plasmid plus electroporation (P < 0.01) and the respective naked cytokine plasmids (P < 0.05), with mIL-12 plus electroporation being the most effective. These experiments were conducted three times to ensure the reproducibility of the experiments. In all three experiments, a similar pattern of tumor growth delays was observed. Additional experiments (with the IL-12 plasmid) indicate that it is possible to carry out a second plasmid injection and electroporation to extend the duration of tumor suppression (data not shown). As to the mechanisms of the antitumor effect of IL-12, our past study (15) indicates that stimulation of T cells, natural killer cells, and the antiangiogenic properties of IL-12 all played important roles in suppressing tumor growth in the B16.F10 model.

Discussion

IL-2 and IL-12 have shown promising antitumor effects in preclinical studies by improving immune responses against tumors, although their mechanism of action has not completely been elucidated as yet (2, 10, 16–21). Exploitation of these antitumor properties in humans, however, has thus far been hampered by significant systemic toxicity (19, 22–24). Confining the expression of those genes to the tumor by using intratumoral injection of viral vectors had some success (10, 20). Even with this approach, however, dose-limiting systemic toxicity has nevertheless been observed by several investigators in animal models (1, 2, 15). Our observations of elevated serum levels of IL-12 in animals treated with intratumoral injection of adenovirus encoding IL-12 and associated toxicities, most likely because of adenovirus leaking into the systemic circulation and infecting parenchymatous organs (24), fall in line with these reports.

Increasing the gene transduction efficiency of naked plasmid DNA should alleviate some of the problems associated with virus-mediated gene transfer. Recently, successful in vivo transfer of IL genes into muscle (9) and of marker and therapeutic suicide genes into normal tissues and tumors (9, 25–28) have been reported. In this report, we describe the efficient use of IL-based immunotherapy and electroporation in a murine melanoma tumor. Our results support the idea that the combination of injecting plasmids encoding secretable therapeutic genes (such as IL-12) with electroporation leads to significant local antitumor effects with reduced systemic cytokine levels when compared with gene therapy based on an adenoviral vector. Even with the simple electroporation protocol used in these experiments, satisfactory local gene expression and a distinct therapeutic effect could be observed. Systemic transgene levels, however, were greatly reduced with the plasmid/electroporation combination when compared with the adenovirus approach. The observed lower systemic cytokine levels
should reduce the systemic toxicity of DNA-based cytokine therapy. Another advantage of this approach is its inherent low immunogenicity, greatly facilitating multiple, repeated applications, which may be necessary in many circumstances.

The transfection efficiency in vivo depends on a multitude of parameters, such as the amount of plasmid, time between plasmid injection and electroporation, temperature during electroporation, and above all electrode geometry and pulse parameters (field strength, pulse length, pulse sequence, and others; Refs. 26, 28, and 29). Much progress has been made improving the electroporation paradigm, and it is very likely that additional improvements will be achieved. Electro-gene therapy, therefore, may emerge as a viable alternative to virus vector-based approaches, especially in tumors that are not easily infected with the current vectors and where secretable therapeutic genes are used.

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