Inhibition of Established Subcutaneous and Metastatic Murine Tumors by Intramuscular Electroporation of the Interleukin-12 Gene

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Key Words
In vivo electroporation • Interleukin-12 • Cytokine gene therapy • Antitumor effect • 38C13 B-cell lymphoma • CT-26 colon adenocarcinoma • B16F1 melanoma

Abstract
In vivo electroporation (EP) of the murine interleukin-12 (IL-12) gene in an expression plasmid (pIL-12) was evaluated for antitumor activity. EP transfer of pIL-12 into mouse quadriceps muscles elicited significant levels of serum IL-12 and interferon-γ. Intramuscular EP of pIL-12 resulted in complete regression or substantial inhibition of 38C13 B-cell lymphoma, whereas pIL-12 delivered by gene gun or intramuscular injection without EP showed little therapeutic effect. Impressive antitumor activity by intramuscular EP was also demonstrated in animals with advanced malignant disease. At day 14 after 38C13 tumor inoculation, all animals were found to carry large tumors and to have metastases; without treatment, most died within a week. A single intramuscular EP of pIL-12 resulted in regression of 50% of large subcutaneous tumors and significantly prolonged the lifespan of these animals. Moreover, animals that were previously cured of 38C13 tumors by in vivo EP treatment significantly suppressed tumor growth when challenged 60 days later. In vivo EP of the IL-12 gene was also effective in suppressing subcutaneous and lung metastatic tumors of CT-26 colon adenocarcinoma and B16F1 melanoma cells. Together, these results show that intramuscular electrotransfer of the IL-12 gene may represent a simple and effective strategy for cancer treatment.

Introduction
Many cytokines, either administered systemically or expressed as transgenes by tumor cells, have been intensively investigated as potential anticancer agents. Among the cytokines evaluated, interleukin-12 (IL-12) has been shown to confer potent antitumor activities. IL-12 is a heterodimeric cytokine produced primarily by activated antigen-presenting cells and which mediates a broad range of effects on both innate and acquired immunity [16]. It has been well documented that IL-12 can augment the cytotoxic activities of natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), facilitate type I T helper (Th) cell development, and regulate production of many cytokines, particularly interferon-γ (IFN-γ) production from NK and T cells [8, 18]. IL-12 also possesses IFN-γ and IFN-inducible protein 10-dependent antiangiogenic activity [13]. These diverse biological functions make IL-12 a potent therapeutic agent for malignant diseases [44, 49]. Administration of recombinant IL-12 locally or systemically has been reported to induce potent antitumor
activity in a variety of murine tumor models, causing regression of established tumors [5, 34, 53] and inhibiting formation of both experimental metastases [5, 34] and spontaneous metastases [3]. However, in these studies, repeated delivery of recombinant IL-12 on a daily basis was required to achieve the maximal therapeutic activity, which was also usually associated with dose-dependent toxicity [11, 17]. Alternatively, recombinant viruses, including retroviruses [55], pox viruses [29], and adenoviruses [4, 9], have been used to deliver IL-12 systemically or by local injection of high titers of a virus into the tumor mass. Modification of fibroblasts [45, 56], tumor cells [46], or dendritic cells [37] by viral or nonviral vectors has also been used to deliver IL-12. These alternative approaches for IL-12 delivery have various limitations, such as the induction of host antivector cellular immunity in the adenovirus system [22], potential integrational mutagenesis in the retroviral system [31], and a relatively low transfection efficiency of nonviral plasmid DNA, even when delivered in complexes with cationic liposomes [24].

Electroporation (EP) has been widely used to introduce exogenous molecules, including DNA, into cultured cells [35, 54]. This system provides much higher transfection efficiencies compared with other nonviral transfer systems. EP has also been used to transfer chemotherapeutic agents into tumors in vivo, a process known as electrochemotherapy. The combination of a local injection of an anticancer drug, such as bleomycin, and in vivo EP has been shown to be an effective anticancer treatment in a variety of animal models for different types of cancers [14, 21, 33]. Moreover, electrochemotherapy for human malignant tumors has achieved significant (33–96%) complete response rates in several clinical trials [21]. Recently, in vivo EP was shown to be effective in introducing reporter genes into a variety of organs and tissues, including mouse muscles [1], mouse skin [48], mouse myeloma [43], chicken embryos [32], rat liver [20], rat brain [36], and rat corneal endothelium [39]. This approach has also been successfully used in animal models for the production of functional proteins, such as erythropoietin [28] and interleukin-5 [1], from transfected muscle tissues. These studies clearly demonstrate that gene transfer into muscles by in vivo EP is more efficient for producing sustained serum levels of therapeutic proteins than is a simple intramuscular DNA injection.

In the present study, we investigated whether in vivo EP can be applied in cytokine gene therapy for treating malignant diseases. A plasmid vector (pIL-12) encoding the p35 and p40 subunits of murine IL-12 was electrotransferred into muscle tissues to treat a variety of subcutaneous or metastatic murine tumors, including 38C13 B-cell lymphomas, CT-26 colon adenocarcinomas, and B16F1 melanomas. We also compared the antitumor activities of in vivo EP with other nonviral gene delivery methods (intramuscular and gene gun delivery).

**Materials and Methods**

**Mice**

Female C3H/HeN mice, 10 weeks old, were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). Female BALB/c and C57BL/6 mice, 8 to 10 weeks old, were obtained from the Laboratory Animal Facility, Institute of Biomedical Sciences, Academia Sinica (Taipei, Taiwan). Animal care was provided in accordance with the guidelines approved by the Animal Committee of the Institute of Biomedical Sciences, Academia Sinica, Taiwan.

**Cell Lines**

38C13 murine B-cell lymphoma is a carcinogen (7,12-dimethylbenz(a)anthracene)-induced tumor originally produced in a T-cell-depleted C3H/cB mouse [2]. CT-26 is a colon adenocarcinoma cell line derived from BALB/c mice treated with N-nitroso-N-methylurethane [12]. B16F1 is a metastasizing subline of the B16 melanoma that arose spontaneously and is syngeneic with C57BL/6 mice [47]. Cell lines were maintained in RPMI-1640, 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Sigma Chemical, St. Louis, Mo., USA) at 37°C with 5% CO2 in a humidified incubator. 38C13 cells were grown in the above medium supplemented with 50 μM 2-mercaptoethanol.

**Plasmids and DNA Preparation**

The pIL-12 plasmid that produces biologically active murine IL-12 was described previously [10, 25]. It contains the p35 and p40 coding sequences of murine IL-12 under the control of discrete cytomegalovirus promoters. Plasmid pcDNA3 containing the cytomegalovirus early promoter/enhancer sequence was used as a control plasmid in this study. Plasmid DNA was purified from transformed Escherichia coli strain DH5α using a Qiagen Plasmid Giga Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions and was stored at −70°C as pellets. The DNA was reconstituted in sterile saline at a concentration of 1 mg/ml for experimental use.

**Intramuscular DNA Injection and Electroporation**

In vivo EP was performed as previously described [1] with modifications. Briefly, mice were anesthetized with acepromazine maleate (Fermenta Animal Health Co., Kansas, Mo., USA). Fifty micrograms of plasmid DNA was injected into the bilateral quadriceps muscles using a disposable insulin syringe with a 27-gauge needle. A total of 100 μg of plasmid DNA in various combinations of pIL-12 and pcDNA3 was injected into each mouse. Immediately after injection, a pair of electrode needles was inserted into the muscle to a depth of 5 mm to encompass the DNA injection sites, and electric pulses were delivered using an electric pulse generator (Electro Square Porator ECM 830; BTX, San Diego, Calif., USA). The shape of the pulse was...