Gene expression and antitumor effect following im electroporation delivery of human interferon α2 gene

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KEY WORDS electroporation; gene transfer techniques; gene therapy; interferon-alpha; leukemia

ABSTRACT

AIM: To investigate the gene expression and antitumor effect following im electroporation delivery of human interferon α2 (hIFN-α2) gene. **METHODS:** The pcD_2/hIFN-α2 was injected into the middle of the quadriceps muscle of female BALB/c mice or the leukemia-bearing female BALB/c nude mice, and then electroporation was given to the injection site. Optimal electrical parameters and the efficiency of gene transfer was studied with hIFN-α2 ELISA kit. The HL-60 tumor model in BALB/c nude mice was used to investigate therapeutic effects of im electroporation delivery of pcD_2/hIFN-α2. **RESULTS:** The optimal conditions for the electric pulses were as follows: voltage at 200 V/cm; pulse duration at 40 ms per pulse; number of pulse at 6 pulses and frequency at 1 Hz. Under optimal conditions, the serum hIFN-α2 levels in electroporation group (160 μg/L±31 μg/L) were 45-fold higher than those of nonelectroporation group (3.6 μg/L±1.6 μg/L, $P<0.01$). The growth of leukemia was inhibited more obviously and the survival time of the leukemia-bearing nude mice was prolonged after im electroporation delivery of pcD_2/hIFN-α2 100 μg or 200 μg. **CONCLUSION:** Electroporation was an efficient method for the delivery of plasmid DNA and im electroporation delivery of pcD_2/hIFN-α2 was effective in treating leukemia.

INTRODUCTION

Interferon-alpha was the first cytokine to be used in clinical trials that proved to be useful in the treatment of several cancers including renal cell carcinoma, hairy cell leukemia, malignant melanoma, basal cell carcinoma, and multiple myeloma[1,2]. The most impressive results were observed in hairy cell leukemia, with an overall response rate of 90 %. However, the half-life of interferon recombinant protein is so short that high doses need to be administered repeatedly to obtain an effective concentration. Such administration lead to the repetitive fluctuation between extremely high peak levels to basal levels of IFN-α in the serum and may cause untoward systemic effect[3], and some IFN-α clinical trials have been disappointing[4]. Therefore, different methods have been devised to improve the therapeutic efficacy. One of such methods is direct intratumoral injection of IFN-α genes into tumor cells[5-6]. Although this method is efficient in animal models, it has obvious clinical limitation as it is not practical for metastatic lesions and microscopic residual lesions after surgery.

Skeletal muscle is an attractive tissue for somatic gene delivery because it is large, well vascularized, and easily accessible for im injection and it has good capacity for protein synthesis and very slow turnover, and

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Received 2002-08-12 Accepted 2003-03-06

has the ability to take up plasmid after im adminstration[7-9]. Among the nonviral gene delivery technologies, im injection of plasmids or "naked" DNA into muscle has received much attention since the first report by Wolff and colleagues[7]. One advantage of this technique is that it provides stable epi-chromosomal expression over long periods of time[8,9]. Therapeutic application of this strategy has already shown promise in the preparation of DNA vaccines for use in livestock and humans[10,11]. However, despite optimization and modification, this method is hindered by low level of transfection efficiency. Recently, direct im DNA injection in combination with electrical stimulation (*in vivo* electroporation) has been used as a very effective nonviral physical technique of gene delivery[12-14]. This method also directs long-term and stable transgene expression. In the present study, human IFN-α eukaryotic expressing vector was generated and was chosen to study the influence of different electrical parameters on gene transfer thoroughly. Based on the results, the gene expression efficiency of electro-injection of pcD_2/hIFN-α2 was examined and its therapeutic effect on the human leukemia-bearing nude mice was also investigated.

MATERIALS AND METHODS

Plasmid construction, preparation, and purification Eukaryotic expression vectors pcD_2 was constructed in our laboratory[15]. The 760 bp *Bam*H I- *Xba* I hIFN-α2 cDNA fragment was obtained by PCR of fetal liver DNA with the sense and antisense primers: 5'-CGGATCCCATCTACAATGGCCTTGACCTTTGCTTTACTG-3'and 5'- CTGTAAGGGACTAGTGCCTTAAGAGCTG-3', respectively. The PCR products were cloned into pGEM-Teasy (Promega, USA), then subcloned into pcD_2 vectors at the *Bam*H I- *Xba* I sites to construct plasmid pcD_2/hIFN-α2. All plasmids were manufactured with the Qiagen Giga Endo-Free Prep kit (Valencia, CA, USA), characterized by restriction enzyme digestion, and quantitated by 260/280 nm absorption. All plasmid preparation contained a high percentage of supercoiled DNA (80 %-90 %) and no RNA was detectable by gel electrophoresis.

Experimental animals and tumor cell lines Female BALB/c mice and BALB/c nude mice, 6-8 weeks old, were obtained from the Experimental Animals Center for Peking University Health Science Center (Grade II, Certificate No SCXK11-00-0004). BALB/c nude mice were bred in a laminar flow room operated under sterile conditions. All animals were maintained in standard condition under a 12-h light/dark cycle, provided irradiated food and water. Human HL-60 myeloid leukemia cells lines were kindly provided by Tumor Research Center, Peking University. Cell culture was performed in RPMI-1640 (Gibco, BRL) supplemented with 10 % fetal bovine serum, benzylpenicillin (100 kU/L) and streptomycin 50 mg/L at 37 ºC in a 5 % CO_2 humidified atmosphere.

Electroporation delivery of DNA plasmid Unless otherwise stated, 200 μg pcD_2/hIFN-α2 in 50 μL of 0.9 % saline was injected into quadriceps muscles of anesthetized female BALB/c mice with 26G syringe. Immediately after pcD_2/hIFN-α2 injection, 2 stainless steel needles (0.2 mm in diameter and 20 mm in length; the gap between the two needles was 5 mm) as electrodes were inserted to encompass the pcD_2/hIFN-α2 injection sites and each injection site was submitted to 6 pulses of 40-ms duration at a voltage-to-distance ratio 200 V/cm, delivered at a frequency of 1 Hz, using an electroporator (BTX T820, USA).

RT-PCR analysis Muscle tissue around the electro-injection region was excised for total RNA extraction using total RNA isolation kit (Promega, USA) and was then reverse transcribed. The first strand cDNA was synthesized from 1 μg total RNA in a 20 μL reaction volume following the standard reverse transcription protocol using random hexamers as primers. Resultant cDNA was amplified in 100 μL PCR mixture using primers as mentioned above in plasmid construction. All RT products were also subjected to GAPDH gene amplification (GAPDH was served as an internal standard). The primer set used was 5'-TCCCTCAAGATTGTCAGCAA-3'and 5'-AGATCCACAACGGATACATT-3', amplified a region of 306 bp.

Western blot analysis Muscle extract and serum were used for Western blot analysis to determine hIFN-α2 gene expression. Briefly, 100 μg of total protein from muscle extract and 15 μL of serum were subjected to SDS-PAGE and the separated proteins were transferred to a nitrocellulose membrane. Immunoblotting was performed with a primary anti-hIFN-α polyclonal antibody (PBL Biomedical Laboratories, New Brunswick, NJ, USA) and anti-rabbit secondary antibody conjugated with horsedish peroxidase (HRP). The HRP signal was generated by incubation with a chemiluminescence detection kit (Amersham, Alameda, CA, USA) and exposure on X-ray film.

Measurement of hIFN-α2 levels hIFN-α2 lev-

els in serum samples were detected with an hIFN-α ELISA Kit (ENDOGEN). Serum was obtained from blood samples drawn from the carotid artery of mice.

Treatment of human leukemia-bearing nude mice via electro-injection of hIFN-α2 gene in muscle Female BALB/c nude mice were sc injected 0.2 μL of 1×10^7 HL-60 leukemia cells and sc tumors were formed. When the tumor size was 5 mm×5 mm, the leukemia-bearing nude mice were randomly divided into 4 groups (6 mice each group) to receive different treatments. In group A, 50 μL of 0.9 % saline was injected into quadriceps muscles of anesthetized female BALB/c nude mice with 26G syringe and each injection site was submitted to 6 pulses of 40-ms duration at a voltage-to-distance ratio 200 V/cm, delivered at a frequency of 1 Hz, using an electroporator. In group B, mice were treated with im electroporation delivery of 200 μg pcD_2. In group C, mice were treated with im electroporation delivery of 100 μg pcD_2/hIFN-α2. In group D, mice were treated with im electroporation delivery of 200 μg pcD_2/hIFN-α2. Then, treated leukemia-bearing nude mice were followed on a daily basis for survival. The sc tumor growth was measured in mm, using a caliper, and was recorded as mean diameter [longest surface length (a) and width (b), (a+b)/2][16].

Statistics Results were expressed as mean ±SD. Statistical analysis was carried out using the Student's *t* test. A value of $P<0.05$ was considered statistically significant.

RESULTS

Effect of the electroporative parameters on gene expression The experiment optimized the electrical parameters in order to improve the efficiency of gene transfer. pcD_2/hIFN-α2 200 μg was injected into quadriceps muscles of anesthetized female BALB/c mice with 26G syringe and different electroporative parameters were given. Three days later, blood samples were obtained from the carotid artery of mice, and their serum hIFN-α levels were measured by ELISA. At 6 pulse, 40 ms per pulse and 1 Hz, enhancement of expression was detected from a threshold field strength of 40 V/cm and was optimal at 200 V/cm. Transgene expression was reduced at 240 V/cm, probably because of muscle cells damaged during electroporation at high voltage (Fig 1A). At 200 V/cm, 6 pulse, and 1 HZ, the hIFN-α levels increased with the duration of each pulse up to 40 ms and the reduction of expression was observed at 80 ms (Fig 1B). At 200 V/cm, 40 ms per pulse, and 1 Hz, 6 pulses led to the highest enhancement (Fig 1C). At 200 V/cm, 40 ms per pulse, and 6 pulse, 1 Hz resulted in the highest expression and expression clearly leveled off at 2 Hz (Fig 1D). The optimal condition for electroporation gene transfer was shown to occur at 200 V/cm, 40 ms in duration, 6 pulse, and 1 Hz. These results indicated that there was a balance between gene expression and impairment of muscle. The balance would be damaged if more than optimal parameters were used.

Effect of the electroporation on gene expression To quantitatively evaluate gene delivery efficiency with and without electroporation, serum hIFN-α levels were assessed by ELISA in 3 d after im delivery of pcD_2/hIFN-α2 with or without electroporation. The serum hIFN-α2 levels of electroporation group (160 μg/L±31 μg/L) was 45-fold higher than those of nonelectroporation group (3.6 μg/L±1.6 μg/L, $P<0.01$, Fig 2).

hIFN-α2 gene expression in skeletal muscles RT-PCR analysis of RNA samples extracted from mice muscle 3 d after im electroporation delivery of pcD_2/hIFN-α2 revealed that hIFN-α2 gene was transcribed in the local muscle (Fig 3). The observed hIFN-α2 protein staining in myofibers and serum further indicated that the hIFN-α2 protein was expressed in the electro-injection skeletal muscle and serum (Fig 4).

Therapeutic effects of im electroporation delivery of pcD_2/hIFN-α2 on tumor growth and survival time of HL-60 cell-bearing nude mice BALB/c nude mice were sc injected 0.2 μL of 1×10^7 HL-60 leukemia cells and sc HL-60 cell growth was found. When the tumor size was about 5 mm×5 mm, pcD_2/hIFN-α2 was transferred into these leukemia-bearing nude mice. The growth of leukemia was significantly inhibited in nude mice treated with im electroporation delivery of pcD_2/hIFN-α2 100 μg or 200 μg, whereas it was not inhibited in nude mice treated with im electroporation delivery of 200 μg pcD_2 (Tab 1). The survival time of leukemia-bearing was prolonged more markedly, with a survival rate of 26.2 % and 44.9 % in the group treated with im electroporation delivery of pcD_2/hIFN-α2 100 μg or 200 μg, respectively, whereas the survival rate of leukemia-bearing nude mice treated with im electroporation delivery of 200 μg pcD_2 was 9.3 % (Tab 2). These data clearly demonstrated that although im electroporation delivery of pcD_2/hIFN-α2 was not able to eradicate the tumor, it was able to reduce the rate of

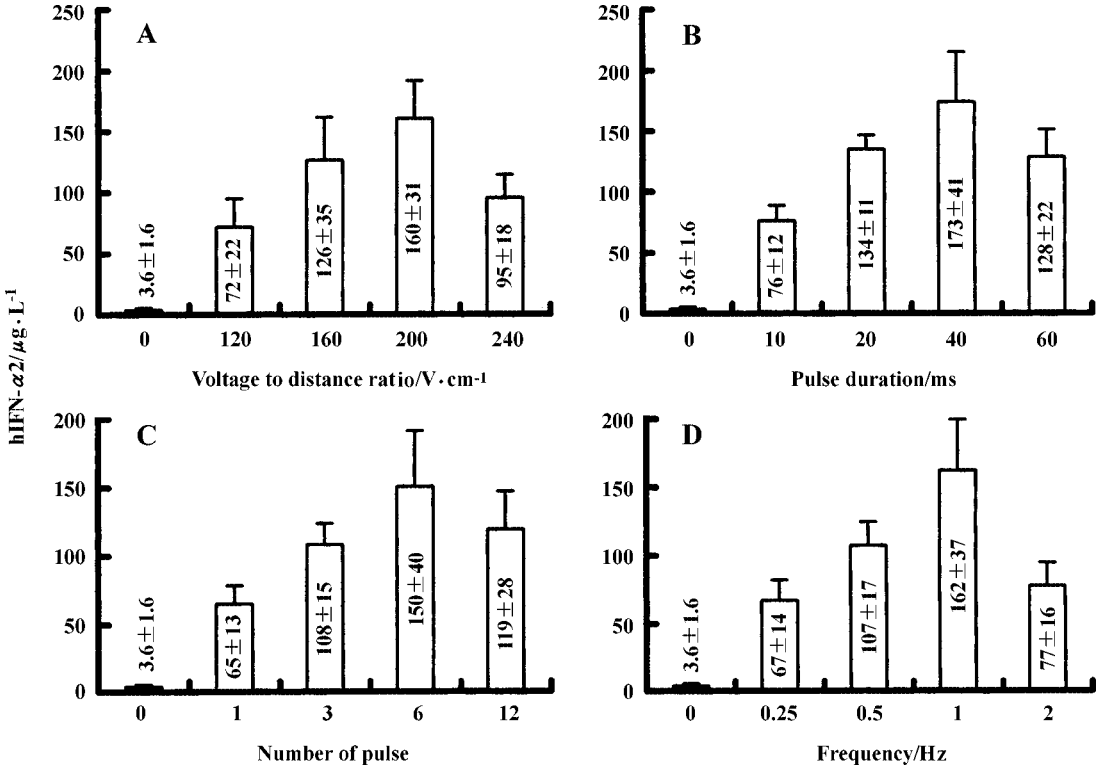

Fig 1. Effect of the electroporative parameters on gene expression. (A) Influence of the applied voltage to distance ratio (40 ms/pulse; 6 pulses; 1 Hz). (B) Influence of pulse duration (200 V/cm; 6 pulses; 1 Hz). (C) Influence of pulse number (200 V/cm; 40 ms/pulse; 1 Hz). (D) Influence of the frequency of pulse delivery (200 V/cm; 40 ms/pulse; 6 pulses). Each bar represents the mean of 5 serum samples.

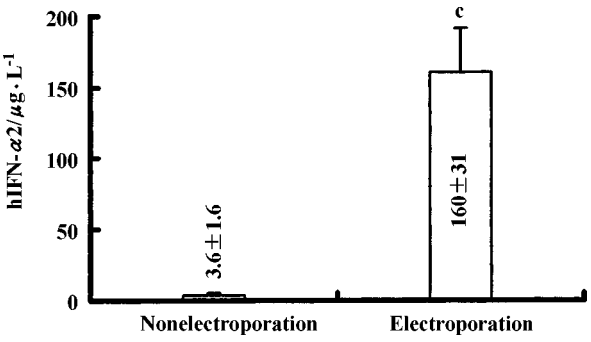

Fig 2. Analysis of gene expression and transfection efficiency with and without electroporation. $^cP<0.01$ *vs* nonelectroporation group. Each bar represents the mean of 5 serum samples.

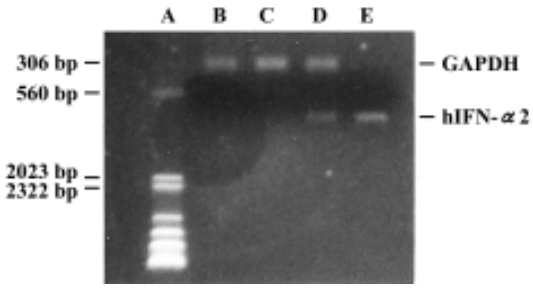

Fig 3. RT-PCR analysis of hIFN-α2 gene expression in muscle after electro-injection of pcD_2/hIFN-α2, control empty plasmid pcD_2 or saline. A: Lambda Hind III marker; B: RNA extracted from electro-injection of saline into muscle; C: RNA extracted from electro-injection of pcD_2 into muscle; D: RNA extracted from electro-injection of pcD_2/hIFN-α2 into muscle; E: the positive control of pcD_2/hIFN-α2. GAPDH was served as an internal standard.

tumor growth significantly.

Time course of pcD_2/hIFN-α2 expression Time course of gene expression by electroporation *in vivo* was determined at optimal electrical parameters. The serum hIFN-α2 levels were already detectable on 1 d (94 μg/L±18 μg/L), reached peak on 3 d (162 μg/L±39 μg/L) and gradually decreased to approximately 35 % of the maximum value by 14 d (57 μg/L±13 μg/L) after im electroporation delivery of pcD_2/hIFN-α2 (Fig 5).

DISCUSSION

The application of electrical parameters to enhance gene transfer *in vivo* is in its infancy. Recently, several independent parametric studies have applied electro-

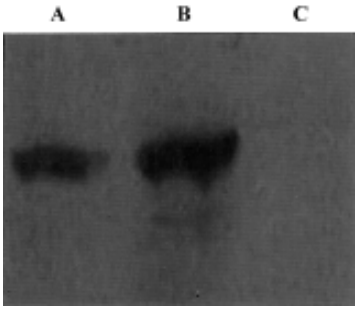

Fig 4. Western blot analysis of hIFN-α2 gene expression in muscle extract and serum obtained on d 3 after electro-injection of pcD_2/hIFN-α2. A: serum samples after electro-injection of pcD_2/hIFN-α2; B: protein extract after electro-injection of pcD_2/hIFN-α2; C: protein extract after electro-injection of pcD_2.

Tab 1. Inhibitory effect of im electroporation delivery of pcD_2/hIFN-α2 on tumor growth of HL-60 cell-bearing nude mice. *n*=5. Mean±SD. [a]*P*>0.05, [b]*P*<0.05, [c]*P*<0.01 *vs* saline. [d]*P*>0.05, [e]*P*<0.05, [f]*P*<0.01 *vs* pcD_2.

Group	Dose /μg	Average diameter of tumor/mm 0 d	7 d	14 d
Saline		5.0±0.20	14.4±1.4	26±3
pcD_2	200	5.1±0.20	14.3±2.0[a]	25±4[a]
pcD_2/hIFN-α2	100	4.9±0.3	12.2±1.9[bd]	20±5[be]
	200	5.1±0.1	10.2±2.1[cf]	15±3[cf]

Tab 2. Effect of im electroporation delivery of pcD_2/hIFN-α2 on survival time of HL-60 cell-bearing nude mice. *n*=5. Mean±SD. [a]*P*>0.05, [b]*P*<0.05, [c]*P*<0.01 *vs* saline. [d]*P*>0.05, [e]*P*<0.05, *vs* pcD_2.

Group	Dose/μg	Survival time/d	Survival rate/%
Saline		19±2.9	
pcD_2	200	21±3[a]	9.3
pcD_2/hIFN-α2	100	24±5[bd]	26.2
	200	28±5[ce]	44.9

poration technology to skeletal muscle *in vivo* and reported high and sustainable levels of reporter gene activity[17,18]. Reporter gene assays simple measure local expression, but by delivering a secreted molecule, we can measure the systemic activity. In this study, we studied the influence of different electrical parameters on gene transfer using human IFN-α2 eukaryotic ex-

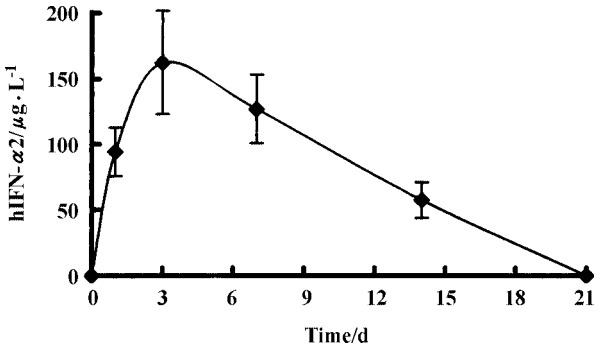

Fig 5. Time-course of serum hIFN-α2 levels after im electroporation delivery of pcD_2/hIFN-α2. Each bar represents the mean of 5 serum samples.

pressing vector. These results are consistent with those of our previous studies [17]. Under optimal conditions, the gene expression efficiency in electroporation group was 45-fold higher than that in nonelectroporation group. These results indicated that im electroporation delivery of naked plasmid DNA was a very powerful method for generating a high and durable level of gene expression.

Although im electroporation delivery of naked plasmid DNA has been used as a very effective nonviral physical technique of gene delivery, there is only a report about the feasibility of delivery of IFN-α2 gene using this method for treating the squamous cell carcinoma (SCCVII)[19]. In the present study, using human HL-60 leukemia-bearing nude mice, we investigate the therapeutic effect of im electroporation delivery of pcD_2/hIFN-α2 on the human HL-60 leukemia. Our result demonstrates that a single im electroporation delivery of pcD_2/hIFN-α2 100 μg or 200 μg could also significantly suppressed the tumor growth and increased the survival time of human HL-60 leukemia-bearing nude mice. These results from our experiment and Li S *et al* indicated that im electroporation delivery of therapeutic gene was an effective method for treating tumor. In addition, a high dose (100 μg or 200 μg) of IFN-α2 DNA plasmid delivered via electroporation reduced the number of administrations for inhibiting tumor growth compared with low dose (10 μg or 40 μg) of IFN-α2 DNA plasmid delivered via electroporation. For example, Im electroporation delivery of 10 μg IFN-alpha once a week for 3 weeks markedly inhibited the squamous cell carcinoma (SCCVII) tumor growth located at a distant site[19]. Such a comparison based on the results from different experimental settings is not conclusive, but a high dose of IFN-α2 DNA plasmid delivered via

electroporation is very appealing for its effectiveness and single administration.

In the course of gene expression by electroporation, we discovered that im electroporation delivery of hIFN-α2 gene 200 μg in mice resulted in relatively stable serum levels over a 2-week period, which was similar to those of Horton *et al*[20]. The result indicated that im electroporation delivery of pcD_2/hIFN-α2 might be less toxic, while efficacy was increased because the appropriate level of hIFN-α2 in serum might maintain for a long time. Short-term (1 h) exposure of tumor cells produced no detectable cytotoxicity, whereas tumor cells continuously exposed to IFN-α2 were significantly inhibited[21].

In summary, these results showed that *in vivo* electroporation was an efficient method for the delivery of plasmid DNA and im electroporation delivery of pcD_2/hIFN-α2 significantly suppressed the growth and increased the survival time of human HL-60 leukemia-bearing nude mice. Therefore, im electroporation delivery of pcD_2/hIFN-α2 had potential usefulness in the treatment of several cancer patients.

ACNOWWLEDGMENT(S) To Prof SU Jin-Yi and MA Da-Long for reading and appraising the English words.

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