J. Biochem. **133**, 423–427 (2003)
DOI: 10.1093/jb/mvg055

Sustained Expression of Fc-Fusion Cytokine Following *In Vivo* Electroporation and Mouse Strain Differences in Expression Levels

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Received December 22, 2002; accepted January 16, 2003

We previously demonstrated that cytokine expression following intramuscular gene transfer of a naked plasmid is increased 2 logs by *in vivo* electroporation, but the relatively low expression levels of the encoded protein is still a limitation for successful gene therapy and gene function studies. We recently reported that the serum viral IL-10 levels achieved by electroporation-mediated intramuscular delivery of pCAGGS-vIL10, a viral IL-10-expressing plasmid, can be further enhanced by modifying the plasmid into an immunoglobulin fusion protein expression plasmid, pCAGGS-vIL10/Fc. Here we examined the applicability of this approach to the expression of an endogenous cytokine, IL-10, in two different inbred mouse strains. We obtained sustained high serum levels of IL-10 in C3H/HeJ mice (C3H), but the level and duration of the gene expression was mouse-strain dependent. Although the serum IL-10 level was also increased by using the IL-10/Fc gene plasmid in C57BL/6 mice (B6), IL-10/Fc and a luciferase reporter showed significantly lower levels in B6 than in C3H mice, and the persistence of pCAGGS-IL10/Fc expression ranged from several days in B6 mice to more than one month in C3H mice. These results suggest that the electroporation-mediated intramuscular delivery of the immunoglobulin fusion protein expression plasmid is simple and very efficient, but mouse strain differences in transgene expression should be taken into consideration in its use.

Key words: electroporation, gene delivery, IL10/Fc fusion cytokines, mouse strain differences, naked plasmid DNA.

Abbreviations: IL-10, interleukin-10; CAG promoter, cytomegalovirus immediate-early enhancer-chicken β-actin hybrid promoter; ELISA, enzyme-linked immunosorbent assay.

Research on cytokine gene therapy has demonstrated its potential for the treatment of several autoimmune diseases, infectious diseases, and cancer (*1*–*3*). Gene transfer technology would circumvent some of the problems associated with the systemic delivery of various recombinant cytokines, such as rapid degradation, short half-life (*4*, *5*), systemic adverse effects (*6*, *7*), and the high cost of manufacture.

Among the techniques for *in vivo* gene transfer, the delivery of genes as naked plasmid DNA, a non-viral vector, is an attractive alternative that may overcome problems associated with gene delivery by viral vectors, which can provoke mutagenesis and carcinogenesis or induce immune responses. Moreover, there are fewer size constraints on inserts for naked plasmid DNAs than for current viral vectors.

Muscle has a large mass and is a candidate tissue for producing recombinant proteins. The direct injection of naked plasmid DNA into muscle is simple, inexpensive, and safe, but the transfection is limited to the injection site, and the expression levels are not satisfactory. Several methods have been introduced to enhance the *in vivo* efficiency of plasmid delivery and expression, including *in vivo* electroporation (*8*, *9*). Recently, we developed a plasmid vector that expresses secretory proteins as immunoglobulin fusion proteins (*10*). Here we examined its applicability to *in vivo* electroporation-mediated gene transfer and the expression of interleukin-10 (IL-10) in two strains of inbred mice. We also investigated mouse strain differences in transgene expression using intramuscular injection of a luciferase-expressing plasmid with or without *in vivo* electroporation. The results indicate that the electroporation-mediated transfer of the modified plasmid is superior to that of the unmodified plasmid in terms of the serum level of the encoded protein and its duration, and that these parameters are also affected considerably by the mouse strain used.

MATERIALS AND METHODS

Animals—Two commonly used inbred mouse strains, C57BL/6 and C3H/HeJ, were purchased from CLEA Japan (Osaka). C57BL/6 mice were used at 9 weeks and C3H/HeJ at 7 weeks of age, to match the body weight of the two strains throughout the study. The mice were maintained under specific pathogen-free conditions in the animal facility at the Osaka University Medical School.

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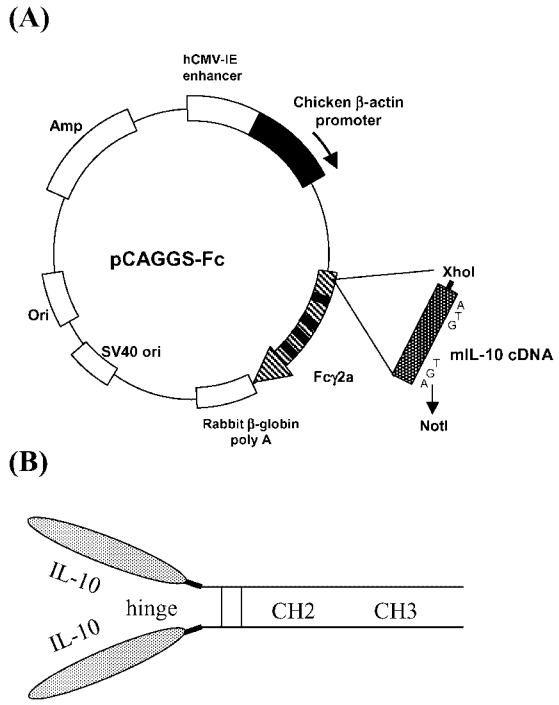

Fig. 1. **Plasmid structure.** (A) The structure of the fusion protein IL-10/Fc expression plasmid. Plasmid pCAGGS-Fc was constructed by inserting the Fc fragment (into which *Xho*I–*Not*I sites were introduced just before the hinge sequence to use as a cloning site for the cDNA to be fused with Fcγ2a) between the CAG promoter and a 3′-flanking sequence of the rabbit β-globin gene of the pCAGGS expression vector. The fragment encoding the IL-10 cDNA had an *Xho*I site just before the start codon and a *Not*I site replacing the stop codon, and was introduced at the *Xho*I–*Not*I site of pCAGGS-Fc to create the IL-10/Fc expression vector (pCAGGS-IL10/Fc). (B) The postulated structure of the homodimeric IL-10/Fc fusion protein.

Plasmid Construction—Plasmid pCAGGS-IL10 was constructed by inserting a truncated form of the mouse IL-10 cDNA into the unique *Xho*I site between the CAG (cytomegalovirus immediate-early enhancer-chicken β-actin hybrid) promoter and a 3′-flanking sequence of the rabbit β-globin gene of the pCAGGS expression vector (*11*, *12*). The murine Fcγ2a cDNA was generated by RT-PCR from the total RNA extracted from an IgG2a-secreting hybridoma cell line (a gift from Dr. M. Miyasaka, Osaka University, Osaka). *Xho*I and *Not*I restriction sites were introduced upstream of the Fcγ2a sequence. Plasmid pCAGGS-Fc was constructed by inserting the Fc fragment into the pCAGGS expression vector between the CAG promoter and a 3′-flanking sequence of the rabbit β-globin gene (*10*). The IL-10 fragment was amplified from pCAGGS-IL10 using a 5′ primer that included an *Xho*I restriction site located just before the start codon (ATG) and a 3′ primer that included a *Not*I site that replaced the IL-10 stop codon (TGA). The resulting IL-10 fragment was introduced into the *Xho*I–*Not*I site of pCAGGS-Fc to produce pCAGGS-IL10/Fc (Fig. 1A). Plasmid pCAGGS-luc, containing firefly luciferase cDNA driven by the CAG promoter, was constructed by inserting the 1,689-bp *Hin*dIII–*Xba*I luciferase gene fragment (from the pGL3-Basic vector, Promega, Madison, WI), after it was filled in with klenow, into the blunted *Eco*RI site of pCAGGS. All fragments were confirmed using a standard sequencing technique. Plasmids were amplified in *E. coli DH10B* cells, extracted by the alkaline lysis method, and purified by two cycles of ethidium bromide–CsCl equilibrium density gradient ultracentrifugation. After further purification by isopropanol precipitation, phenol and phenol/chloroform extraction, and ethanol precipitation, the plasmids were dissolved in TE buffer. The quantity and quality of the purified plasmid DNA were assessed by optical density at 260 and 280 nm and agarose gel electrophoresis.

Intramuscular DNA Injection and Electroporation—Mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body weight). Plasmid DNA (pCAGGS-luc, pCAGGS-IL10, or pCAGGS-IL10/Fc) was injected into the bilateral tibialis anterior muscles of two strains of mice (50 μg each side; total, 100 μg/mouse; at 1.5 μg/μl in PBS) using an insulin syringe with a 27-gauge needle, and electric pulses were delivered as described previously (*8*). Briefly, a pair of electrode needles with a 5-mm gap 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 T820M; BTX, San Diego, CA). Three pulses of 100 V followed by three more pulses of the opposite polarity were administered to each injection site at a rate of one pulse/s, with each pulse being 50 ms in duration.

Luciferase Activity Assay—One day after intramuscular injection with pCAGGS-luc alone or pCAGGS-luc followed by electroporation, the mice were sacrificed. The bilateral tibialis anterior muscles were dissected, frozen in liquid nitrogen, and immediately homogenized in passive lysis buffer (Promega, Madison, WI). After centrifugation at 15,000 ×*g* for 15 min at 4°C, 30 μl of the supernatant was added to 100 μl of luciferase assay reagent (Promega, Madison, WI). Relative light units were determined for 30 s using a luminometer (Luminoskan TL Plus).

Enzyme-Linked Immunosorbent Assay (ELISA)—Serum samples obtained from the two strains of mice subjected to pCAGGS-IL10 or pCAGGS-IL10/Fc were assayed for IL-10 using an ELISA kit (Endogen, Woburn, MA) according to the manufacturer's instructions. Note that the IL-10/Fc protein concentration determined by this ELISA indicates the concentration of the IL-10 equivalent recognized by the monoclonal antibody used in this ELISA system.

Transfection of Plasmid DNA—BMT-10 cells, an SV40 T antigen-producing monkey cell line (*13*), are a suitable host for the expression vectors based on the chicken β-actin promoter (*12*). Cells were cultured in 24-well plates and transfected with 4 μg/well of pCAGGS-IL10 and pCAGGS-IL10/Fc using Lipofectamine™ Reagent (Invitrogen, Corp., Carlsbad, CA) according to the manufacturer's protocol. After 72 h of incubation at 37°C in a CO_2 incubator, the media were collected and the levels of IL-10 released from the cells were determined.

Western Blot Analysis—The translation product of pCAGGS-IL10 or pCAGGS-IL10/Fc was examined by Western blot analysis of the culture medium from BMT-10 cells transfected with pCAGGS-IL10 or pCAGGS-IL10/Fc and serum samples obtained four days after the

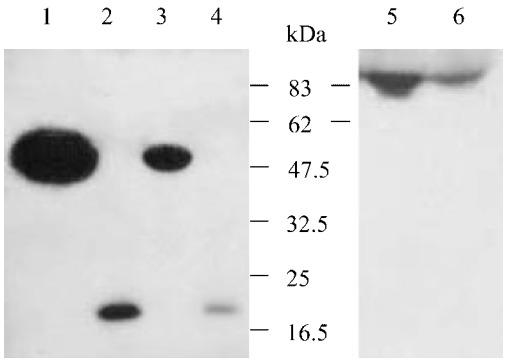

Fig. 2. **Western blot analysis of IL-10/Fc expression.** Left panel: serum samples from mice 4 days after electroporation-mediated intramuscular injection of pCAGGS-IL10/Fc (lane 1), pCAGGS-IL10 (lane 2), and culture medium 72 h after BMT-10 cells were transfected with pCAGGS-IL10/Fc (lane 3) or pCAGGS-IL10 (lane 4) were subjected to SDS-PAGE under reducing conditions. Right panel: samples of serum (lane 5) and culture medium (lane 6) from pCAGGS-IL10/Fc transfer were also run under non-reducing conditions.

mice received an electroporation-mediated intramuscular injection of pCAGGS-IL10 or pCAGGS-IL10/Fc. The samples (culture media or sera) were mixed with a one-third volume of SDS-PAGE loading buffer with or without 5% 2-mercaptoethanol, heated at 95°C for 5 min, and fractionated in 12% polyacrylamide gels. Proteins were electrotransferred onto an IPVH membrane (Nihon Millipore Ltd., Tokyo). The membrane was blocked overnight at 4°C in buffer containing 20 mM Tris-HCl, pH 7.6, 137 mM NaCl, 0.1% Tween-20, and 5% skim milk, and incubated for 2 h at room temperature with a 1:1,000 diluted monoclonal antibody against mouse IL-10 (Endogen, Woburn, MA) in blocking buffer. The membrane was washed and then incubated for 1 h at room temperature with 1:2,000 diluted HRP-goat anti-rat antibody (Zymed, San Francisco, CA) and visualized with an ECL detection system (Amersham).

Statistics—Results are expressed as the mean ± SD. Statistical analysis was performed by one-way ANOVA and Student's *t*-test. A value of $p < 0.05$ was considered significant.

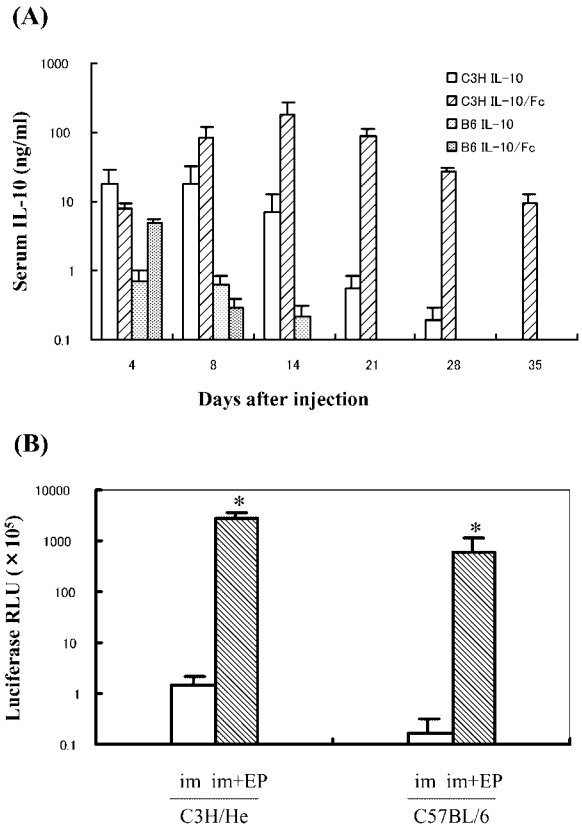

Fig. 3. **Expression of transgenes in two strains of mice.** (A) Time-course of the serum concentration of IL-10 after the *in vivo* electroporation-mediated gene transfer of either pCAGGS-IL10 or pCAGGS-IL10/Fc in two strains of mice. Mice received injections of 100 μg of pCAGGS-IL10 (C3H, $n = 5$; B6, $n = 4$) or pCAGGS-IL10/Fc (C3H, $n = 5$; B6, $n = 4$) in the bilateral tibialis anterior muscle, and electric pulses were delivered. Blood was collected at various time points after DNA injection. Serum IL-10 levels were determined by ELISA. The values represent the mean ± SD. (B) Luciferase expression in the tibialis anterior muscles of two strains of mice, C3H and B6, after an intra-muscular injection of 50 μg pCAGGS-luc (blank bar. C3H, $n = 3$; B6, $n = 3$) or 50 μg pCAGGS-luc followed by electroporation (striped bar. C3H, $n = 3$; B6, $n = 3$). The luciferase activity was determined 24 h after injection. Data are expressed as the mean ± SD. * $p < 0.001$, im + EP *versus* im in both C3H and B6. im denotes intramuscular injection; im + EP denotes electroporation-mediated intramuscular injection.

RESULTS

Detection of Transferred Gene Expression by Western Blot Analysis—As shown in Fig. 2, immunoblotting of the culture medium from BMT-10 cells transfected with pCAGGS-IL10 for 72 h and serum from the mice injected 4 days previously with pCAGGS-IL10 revealed the successful expression of mIL-10 with the expected molecular size of 22-kDa (Fig. 2, lanes 2 and 4). Culture medium from BMT-10 cells transfected with pCAGGS-IL10/Fc and serum from mice injected with pCAGGS-IL10/Fc showed a single band at 48-kDa under reducing conditions, suggesting that IL-10 was linked to the 26-kDa Fcγ2a portion (Fig. 2, lanes 1 and 3). Under non-reducing conditions, the band migrated at a molecular size of 96 kDa (Fig. 2, lanes 5 and 6), suggesting that the fusion protein was produced as a homodimer, as shown in Fig. 1B. These results demonstrate the successful expression of the delivered genes encoding IL-10 and the IL-10/Fc fusion protein, both *in vitro* and *in vivo*.

Serum IL-10 Levels after IL-10 and IL-10/Fc Gene Transfer by In Vivo Electroporation in Two Strains of Mice, C3H/HeJ and C57BL/6—To compare serum levels of IL-10 and IL-10/Fc after electroporation-mediated gene delivery in two strains of mice, serum samples were collected at various time points after the injection of 100 μg of plasmid per mouse. As shown in Fig. 3A, in C3H mice, the serum levels of IL-10 reached their peak (18.4 ± 13.4 ng/ml) on day 8 in the pCAGGS-IL10 group, then gradually decreased. However, the serum IL-10 levels reached their peak (182.9 ± 93.5 ng/ml) on day 14 in the pCAGGS-IL10/Fc group, then gradually decreased thereafter, but remained very high on days 28 (26.8 ± 3.5 ng/ml) and 35 (9.5 ± 2.9 ng/ml). The peak levels of serum IL-

10 in the pCAGGS-IL10/Fc group were almost 10-fold higher than in the pCAGGS-IL10 group.

In contrast, in B6 mice, the serum levels of IL-10 reached their peak (0.7 ± 0.3 ng/ml) on day 4 in the pCAGGS-IL10 group, which is lower than that in the C3H mice, then gradually decreased. The serum levels of IL-10 reached their peak (4.9 ± 0.6 ng/ml) on day 4 in the pCAGGS-IL10/Fc group, and the peak was about 7-fold higher than in the pCAGGS-IL10 group. Although the IL-10 levels were also increased by changing the pCAGGS-IL10 plasmid to an immunoglobulin fusion protein expression plasmid, pCAGGS-IL-10/Fc, the IL-10 levels in the B6 mice were significantly lower than in the C3H mice, and the IL-10 levels increased rapidly to 0.3 ± 0.1 ng/ml by day 8. Thereafter, the difference in the serum IL-10 level between the two strains was more than 100-fold.

These results show that serum cytokine levels achieved by electroporation-mediated intramuscular cytokine gene delivery can be further enhanced by using an Fc-fusion cytokine expression plasmid. *In vivo* electroporation with pCAGGS-IL10/Fc resulted in significantly higher serum levels of IL-10 than electroporation with the plasmid expressing native IL-10 in both strains of mice, but the serum level of IL-10 and its duration were very different between these two strains.

Expression of the Luciferase Gene in C3H/HeJ and C57BL/6 Mouse Strains—To examine strain differences in the expression of another protein, luciferase expression with or without electroporation was compared in the tibialis anterior muscles of the C3H and B6 mouse strains 24 h after gene delivery of the plasmid vector pCAGGS-luc, which expresses luciferase under the CAG promoter. As shown in Fig. 3B, electroporation increases expression by an order of 3 logs in both C3H and B6 mice. However, muscles harvested from the C3H mice showed significantly higher (about 10-fold) luciferase activity than the muscles from B6 mice. This result shows that the level of protein expression induced by intramuscular DNA injection differs greatly between the C3H and B6 mouse strains.

DISCUSSION

Ever since Wolff *et al*. reported that gene expression in skeletal muscle can be achieved by a simple intramuscular injection of naked plasmid DNA (*14*), many physical and chemical approaches have been utilized to improve the efficiency of gene transfer by plasmid DNA. The uptake of plasmid DNA by muscle cells is relatively inefficient (less than 1% of the injected dose), and is limited to cells adjacent to the track of the injection (*15*). The reproducibility and efficiency of gene expression in muscle can be improved by preinjecting a large volume of hypertonic sucrose (*16*) or molecules inducing muscle regeneration, such as bupivacaine (*17*, *18*). We previously demonstrated that *in vivo* electroporation increases the number of muscle cells that take up plasmid DNA and probably increases the copy number of plasmid introduced into each muscle cell. Electric pulses generally increase gene expression up to 100-fold compared with the injection of naked plasmid DNA into muscle without electroporation (*8*). This was further confirmed in our present study, where we show that luciferase activity is increased by electroporation by an order of 3 logs in both C3H and B6 mice. Indeed, in larger mammals, including primates, gene transfers following the i.m. administration of naked DNA are not as efficient as in mice, but can be greatly improved by electroporation (*19*). Moreover, under our experimental conditions, there was no obvious systemic toxicity observed, except for a very small degree of muscle damage caused by the electroporation. The body weight gain of mice injected with the plasmid was similar to that observed for control mice without injection (data not shown).

In the present study, we constructed an expression plasmid encoding a fusion protein, IL-10/Fc. Fusion cytokines (*20*) with Fc segments secreted as homodimers offer advantages over native proteins, such as a prolonged circulating half-life, a characteristic of Ig, and a higher avidity for the ligand. Importantly, it has been confirmed that Fc-fusion proteins possess the biologic functions of the native cytokine moiety (*10*, *20–22*). In addition, to enhance systemic fusion protein expression, we coupled the intramuscular injections with local electroporation. The results demonstrate that electroporation enhances plasmid gene expression in the muscle of two mouse strains, C3H and B6, and that the serum levels of the encoded protein resulting from electroporation-mediated intramuscular gene delivery are further improved by using an immunoglobulin fusion protein expression plasmid in both strains of mice. However, these two strains of mice exhibited different serum IL-10 levels. The expression level of the delivered gene (the IL-10/Fc or luciferase reporter gene) was significantly higher in C3H mice than in B6 mice, and a marked variability in the persistence of gene expression in the two strains was observed (from more than one month in C3H mice to several days in B6 mice). We currently cannot explain exactly why the expressed IL-10 levels in circulation are so different in these two strains of mice, but the different luciferase activities in the muscle (Fig. 3) suggested that the protein production in these two strains of mice is very different. The different strains may have different abilities to utilize the CAG promoter of the plasmid construct or, alternatively, may have different responses to the presence of immunostimulatory sequences in the vector DNA that could cause cytokine-mediated promoter attenuation (*23*). In any case, it is important to consider strain differences in *in vivo* gene transfer experiments.

In summary, the present study demonstrates successful long-term high expression levels of an IL-10/Fc fusion protein in C3H mice using a simple intramuscular electroporation-mediated gene delivery method. This approach can be used in gene therapy studies and to investigate the *in vivo* biological functions of cytokines as well as other secretory proteins. This study also suggests that strain differences in transgene expression should be seriously considered in *in vivo* gene transfer experiments.

This work was supported by a grant from the Research for the Future Program of the Japan Society for the Promotion of Science (JSPS-RFTF97I00201). This work was also supported by

a grant from the Ministry of Education, Culture, Sports, and Scince and Technology of Japan.

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