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Interleukin-10 expression after intramuscular DNA electrotransfer: kinetic studies

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Abstract

Transfected muscle can be used as a secreting tissue for therapeutic proteins. Skeletal muscle transfection is increased by suitable electric pulse application (electrotransfer). We and others had shown that electrotransfer of interleukin-10 encoding plasmid is an effective strategy in animal models of chronic diseases such as myocarditis, atherosclerosis, or rheumatoid arthritis. In the present work, we have studied murine interleukin-10 production and secretion after i.m. electrotransfer. In immunocompetent mice, serum and muscle mIL-10 levels were enhanced by electrotransfer. Serum mIL-10 concentration reached rapidly a peak level 2 days after electrotransfer. It then decreased to background at day 14. Muscle mIL-10 mRNA and protein remained more stable, being detectable up to 84 days after electrotransfer. A boost reinjection led to similar high level of circulating mIL-10. The fast decrease of serum mIL-10 was not observed in SCID mice.

Keywords: Interleukin-10; Electrotransfer; Kinetics study; Skeletal muscle; Electroporation gene therapy

Previous studies have shown that a simple plasmid DNA injection can efficiently transfect muscle tissue [1]. The transgenic protein expression by muscular fibers is significantly detectable for at least 19 months after injection [2]. Moreover, intramuscular injection of plasmid coding for secreted proteins leads to sustained and detectable concentration of the transgenic protein in blood [3–5]. The therapeutic potential of muscular transfection concerns (i) the treatment of muscle disorders, such as Duchenne's dystrophy, (ii) DNA vaccination, and (iii) systemic secretion of therapeutic proteins such as erythropoietin, cytokines, etc. However, expression level obtained by i.m. 'naked' DNA injection is too low for certain therapeutic applications, although not all of them. We and others have recently developed the use of electric pulse delivery to amplify gene transfer into skeletal muscle. Comparatively to DNA injection alone, the expression level is enhanced 30- to 100-fold by electrotransfer [6–8]. The advantage of using electrotransferred skeletal muscle as a secretory tissue of therapeutic proteins had been illustrated in many instances [3,9–20].

In this work, we have studied muscle electrotransfer with a plasmid coding for murine interleukin-10, an anti-inflammatory cytokine. The IL-10 cytokine is secreted by NK cells, monocytes, B cells derived from the peripheral blood, and T helper 2 cells. This Th2 cytokine has been shown to display major effects on the immune system: IL-10 inhibits secretion of pro-inflammatory cytokines such as TNFα and IL-1 [21], IL-2 [22], or IFNγ [23], and inhibits the T helper 1 cell (Th1) function and differentiation [24,25]. In addition, IL-10 induces proliferation and differentiation of B lymphocytes [26,27] and stimulates mast cells [28]. Interleukin-10 has been shown to be involved in the pathobiology of inflammatory disorders such as (i) LPS-induced endotoxemia, in which endogenous interleukin-10 is rapidly released in mice serum [29], (ii) atherosclerosis, for which IL-10 is detected in early and advanced human atherosclerotic plaques and is associated with low levels of inflammatory markers [30–32], and (iii) rheumatoid arthritis, where IL-10 is expressed in joints of patients [33]. Several studies performed in various experimental inflammatory diseases have demonstrated the beneficial effect of a therapeutic infusion of recombinant IL-10 [34–39]. Moreover we and others had previously shown that interleukin-10 encoding plasmid

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electrotransfer to skeletal muscle exerts some therapeutical effects in different inflammatory disease models such as atherosclerosis, reactive angiogenesis, rheumatoid arthritis, and myocarditis [40–45]. However, the time course of transgenic IL-10 in electrotransferred muscle and in blood is not known. Such a knowledge is essential for further therapeutic applications. In the present study, we have thoroughly followed the kinetics of mIL-10 mRNA and protein production in muscle and of mIL-10 secretion after muscle electrotransfer with a mIL-10 encoding plasmid. Unexpected results were obtained as compared to previously described long-lasting expression of the electrotransferred gene.

Materials and methods

Plasmids and DNA preparation. The mIL-10 pCOR plasmid (pXL 3458) was constructed as previously described [46]. It contains the cytomegalovirus (CMV) promoter (nucleotide −522/+72) inserted upstream of the coding sequence of the murine interleukin-10 (mIL-10). The simian virus 40 (SV40) late polyA signal was placed downstream of the mIL-10 cDNA. The plasmid pXL 3296 (empty pCOR) is the similar construct devoid of coding sequence. The pXL 3010, which is not a pCOR plasmid, contains the human-secreted alkaline phosphatase gene downstream of the CMV promoter and upstream of the SV40 late poly(A) signal. Plasmids were prepared using standard procedures [47,48].

DNA injection and delivery of electric pulses. Six-week-old C57-Bl/6 mice or SCID mice (IFFA Credo, L'Isle d'Arbesle, France) were anesthetized using ketamine and xylazine (113.75 and 3.5 mg/kg, respectively) and both legs were shaved. Plasmid DNA (15 μg in 30 μl 0.9% NaCl) was injected in the tibial cranial muscle, longitudinally, by the means of a Hamilton syringe. About 20 s after plasmid DNA injection, 8 square-wave electric pulses of 200 V/cm, 20 ms each, at 2 Hz were applied through two stainless steel plate electrodes, placed 3.7–4.2 mm apart at each side of the leg as previously described [6,7]. Electrical contact with the shaved leg skin was ensured by means of conductive gel. Electric pulses were generated by an ECM 830 BTX electropulsator (Genetronics, San Diego, CA, USA). A minimum of eight tibial cranial treated muscles was included in each experimental group.

Animal experiments were conducted following the recommendations of the NIH and Aventis local Ethic Committee on Animal Care and Experimentation.

Measurement of mIL-10 and hSeAP concentration. Peripheral blood samples were collected from retro-orbital sinus or from intracardiac puncture at different time-points. Concentrations of circulating mIL-10 protein were determined using a commercial enzyme-linked immunosorbent assay (ELISA) kit specific for murine IL-10 (Biosource International, Camarillo, CA, USA) according to manufacturer's instructions. Muscles were collected and homogenized in PBS and mIL-10 concentration was measured on supernatant after centrifugation of the homogenate using the same ELISA kit as above. For serum hSeAP, the PhosphaLight kit (Tropix, MA, USA) was used.

Analysis of mIL-10 mRNA. Muscles were collected and frozen at −80 °C before RNA extraction. Three mice were included in each experimental group and 3 muscles from different mice were homogenized in 1 ml Trizol LS Reagent (Life Technologies, Invitrogen, Groningen, The Netherlands) using an Ultra-Thurax Diax600 Heidolf (Bioblock Scientific, Illkirch, France). Total RNA was extracted according to manufacturer's instructions. DNA was removed from RNA solution, using 2 U DNase I (DNA-free kit, Ambion, Austin, TX, USA). The degradation of DNA was checked by polymerase chain reaction (PCR) on 1 μl of total RNA using murine skeletal muscle actin primers (see below).

Five micrograms of total RNA was reverse-transcribed using a SuperscriptII RNase H^- Reverse Transcriptase (Life Technologies) with a primer $(dT)_{20}$. To verify cDNA integrity, we amplified the murine skeletal muscle actin cDNA using the sense primer 5′-TCTTGT GTGTGACAACGGCTC-3′ (positions 26–47 on cDNA) and the antisense primer 5′-CAAACATGATTTGAGTCATCTTCTC-3′ (positions to 379–355 on cDNA). Reaction conditions for 40 cycles were as follows: denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C, and extension for 30 s at 72 °C. An extension final step was performed for 15 min at 72 °C. A band at 353 bp was expected for actin cDNA. The presence of mIL-10 cDNA was tested by PCR with the sense primer 5′-ACT TCCCAGTCGGCCAGAG-3′ (positions 95–113 on cDNA) and the antisense primer 5′-AAATCGATGACAGCGCCTCA-3′ (positions 387–268 on cDNA). Reaction conditions were the same as those described for actin with an annealing temperature of 60 °C. We expected a band at 293 bp for mIL-10 cDNA.

Statistical analysis. Variance analysis on log values of the measured parameters and a protected least significance test of Fisher for comparison between treatments has been used. Results are presented on linear scale for clarity.

Results

Kinetics of interleukin-10 concentration in blood and muscles of immunocompetent mice

C57-Bl/6 female mice were injected and electrotransferred or not, with 3 or 15 μg mIL-10 encoding plasmid in each tibial cranial muscle. Mouse interleukin-10 (mIL-10) was detected at day 7 in both muscle and serum, and the level increased with the amount of injected DNA and by using electrotransfer (data not shown). Electrotransfer of mIL-10 encoding plasmid resulted in a high mIL-10 serum concentration, which was immunodetected as early as at day 2 after injection, and which rapidly decreased to background at day 14 (Fig. 1A). The endogenous level of interleukin-10 was undetectable either in blood or in muscles in a control group electrotransferred with a non-coding plasmid. As a control, when mIL-10 was incubated with serum (one volume of mIL-10 solution added to one volume of mice serum), its concentration, as dosed by ELISA, was significantly reduced by 20–30%. Fig. 1B shows the time course of mIL-10 protein in muscles. As in serum, a peak was rapidly reached 4 days after injection. However, the transgenic protein was detectable for at least 84 days, a time much longer than in serum.

Kinetics of murine interleukin-10 mRNA in the muscle of immunocompetent mice

Muscles of control mice electrotransferred with empty plasmid did not express mIL-10 (Fig. 2A, left). In muscles simply injected with pCMV mIL-10, mRNA appeared clearly four days after injection but was slightly detectable at days 8 and 14 (Fig. 2A, right). On the other hand, when muscles were electrotransferred with pCMV mIL-10, mIL-10 mRNA was high at days 2–8, and was detected for at least 84 days (Fig. 2B).

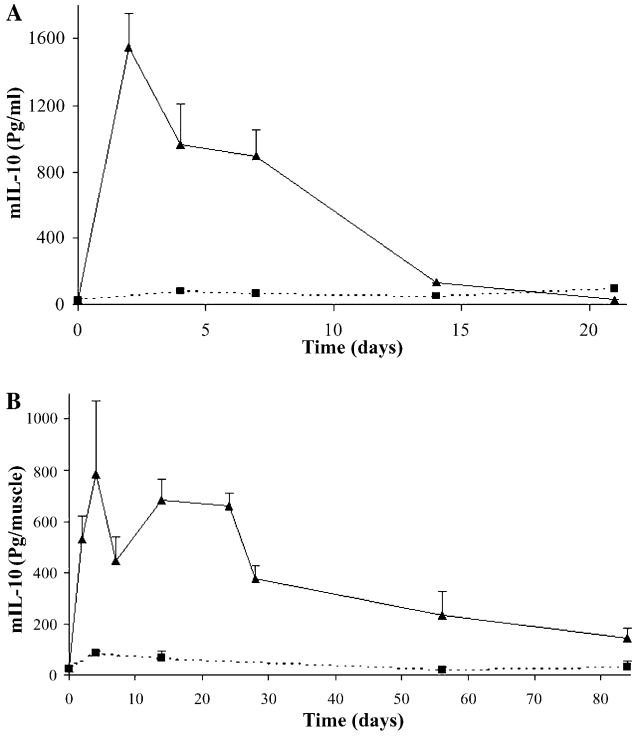

Fig. 1. Time course of mIL-10 concentration in blood and muscle tissue. C57-Bl/6 female mice were injected in both tibial cranial muscles with 15 μg pCMV mIL-10 (triangle symbols, plain lines) or with 15 μg of empty plasmid (square symbols, dotted lines), and electrotransfer was realized. Values are means + SEM ($n = 8$) of mIL-10 concentration in blood serum (A) or muscle (B). For panel B, mIL-10 concentration values in the serum were 642.8 ± 45.8 pg/ml at day 2 and 139.3 ± 29.4 pg/ml at day 14 for mIL-10 electrotransferred group and 11.0 ± 4.9 pg/ml at day 14 for empty plasmid electrotransferred group.

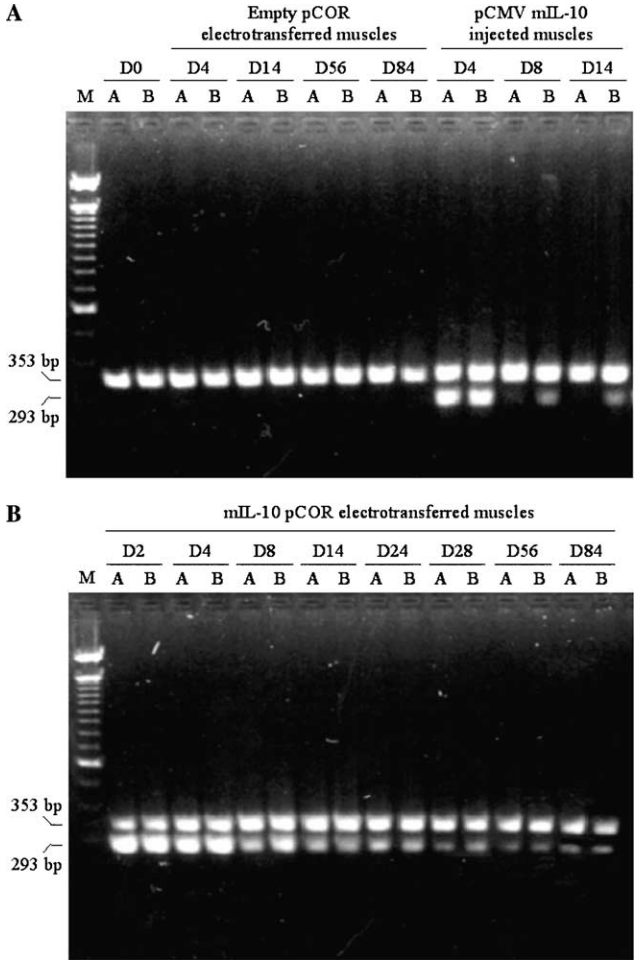

Fig. 2. mIL-10 mRNA in muscles. C57-Bl/6 female mice were injected with 15 μg pCMV mIL-10 or with 15 μg of empty plasmid. Three mice were included in each group and measurement was realized in two groups (A and B). Primers for housekeeping muscle actin and for mIL-10 were used in the same amplification. Actin mRNA amplification allowed us to verify RNA integrity and was revealed by a band at 353 bp, and mIL-10 mRNA amplification fragment appeared at 293 bp. (A) No mIL-10 mRNA amplified fragment appeared in empty plasmid electrotransferred muscles. When muscles were injected with pCMV mIL-10 in the absence of electric pulse delivery, a band at 293 bp appeared clearly at day 4 and was slightly detectable at days 8 and 14. (B) After injection and electrotransfer of 15 μg pCMV mIL-10 the fragment at 293 bp appeared until day 84 and decreased slowly with time.

mIL-10 mRNA level seems almost the same four days after injection of pCMV mIL-10 with or without electrotransfer. However, mRNAs were amplified by a non-quantitative RT-PCR method. Thus, no comparison between two samples can be made.

Reinduction of circulating mIL-10 after a second electrotransfer of pCMV mIL-10

The previous experiment suggested that electrotransfer resulted in sustained production of mIL-10 mRNA and protein in transfected muscle. In order to determine if the rapid decrease in blood circulating mIL-10 could be due to neutralization by a serum factor, we performed a reinduction experiment. Mice were primed with pCMV mIL-10 or empty plasmid electrotransfer at day 0 in one leg. They were subsequently boosted with pCMV mIL-10 electrotransfer at day 14 in the other leg. Thus, a possible effect of the first electrotransfer on the muscle was not taken into account. Two days after the second electrotransfer, mIL-10 level in blood was comparable in the 2 groups of mice (Fig. 3). The blood level of immunodetected mIL-10 was not affected by a previous exposure to circulating transgenic mIL-10. It has to be noted that 2 days after the first electrotransfer, mIL-10 concentration in empty plasmid electrotransferred mice was increased. This could result from electrotransfer-induced inflammation, which has been described by others [49]. Indeed, enhanced production of IL-10 is occurring in inflammatory processes [50].

Kinetics of interleukin-10 and hSeAP serum concentrations in SCID mice

In order to study the secretion capacity of transfected muscular fibers, we co-injected the pCMV mIL-10 with

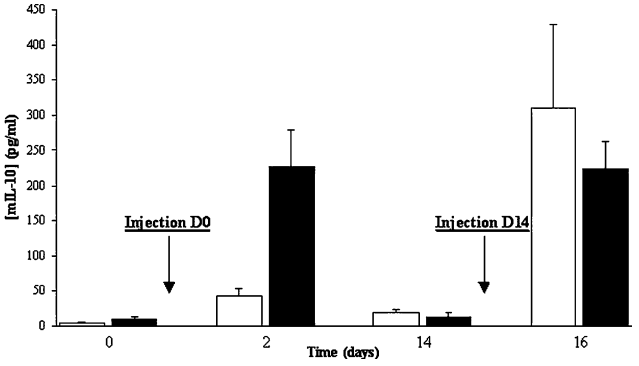

Fig. 3. Effect of the first mIL-10 electrotransfer on mIL-10 secretion after the second electrotransfer. At day 0, we injected and electrotransferred 15 μg pCMV mIL-10 (solid bars) or empty plasmid (white bars) in only one leg of mice. At day 14, we electrotransferred 15 μg mIL-10 encoding plasmid to every mouse in the other leg. Blood samples were collected at days 0 (before injection), 2, 14 (before injection), and 16. Values are means + SEM ($n = 8$) of mIL-10 concentration in serum. Statistics: there is no significant difference in blood mIL-10 concentration between day 2 and 16 for the group that received two pCMV mIL-10 injections. Moreover, there is no significant difference in blood concentration at day 16 between the group that received two pCMV mIL-10 injection and the group that received empty plasmid then pCMV mIL-10.

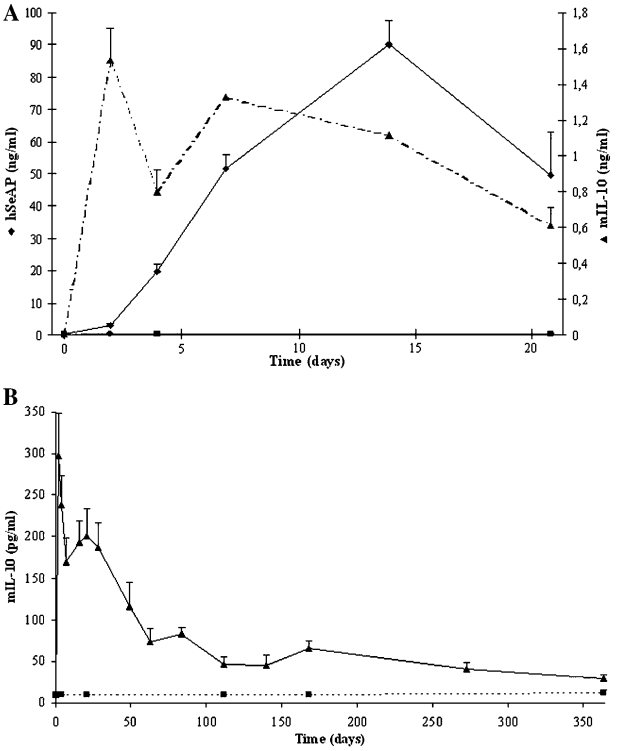

Fig. 4. Time course of mIL-10 in the serum of SCID mice. (A) SCID female mice were injected with a solution containing 15 μg hSeAP encoding plasmid and 15 μg pCMV mIL-10 (triangle) or 15 μg of empty plasmid (square), in a volume of 30 μl. Injection and electrotransfer were realized under sterile conditions. For each individual point, concentrations of hSeAP (plain lines) and of mIL-10 (dotted lines) in the serum are means + SEM ($n = 10$). (B) SCID female mice were injected with 15 μg pCMV mIL-10 (plain line) or with 15 μg of empty plasmid (dotted line) and electrotransferred. In this experiment, 30 mice were included in the mIL-10 group and 10 in the empty group. Concentrations in the serum are means + SEM.

plasmid pXL 3010, which codes for human secreted alkaline phosphatase. It was necessary to perform the study with immunodeficient SCID mice to avoid immune response against the human protein. Serum murine IL-10 and hSeAP time course is represented in Fig. 4A. In this experiment, the kinetics of SeAP concentration in the serum was comparable to that expected [11]. Thus, the presence of mIL-10 encoding plasmid did not modify the secretion capacity of fibers. The kinetics of murine interleukin-10 concentration in SCID serum was clearly different from that in immunocompetent C57-Bl/6 mice serum, as mIL-10 in serum was significantly detectable on day 21 after electrotransfer (Fig. 4A). In another subsequent experiment, despite a lower maximum level, blood mIL-10 remained significantly higher than in control animals up to one year after electrotransfer (Fig. 4B).

Discussion

After injection and electrotransfer of mIL-10 encoding plasmid, mouse interleukin-10 concentration in serum of immunocompetent mice reached a peak 2 days after injection and rapidly decreased to background at day 14. This result was unexpected, because electrotransfer with other plasmid coding for secreted proteins leads to stable secretion for at least 3–12 months [11,17].

We have demonstrated that this decrease did not result from an impairment of protein production, as mRNA and protein were detectable in muscles for at least 84 days. An immune reaction against the transgenic protein is unlikely, since the plasmid used codes the murine form of IL-10. Moreover, a second electrotransfer of pCMV mIL-10 induced a high circulating mIL-10 level of the same value as the one obtained in naive mice after the first electrotransfer. Impairment of muscular fibers secretion capacity cannot be retained as well. Indeed, after muscle electrotransfer of SCID mice muscle with a mix of pCMV mIL-10 and pCMV hSeAP, the hSeAP concentration in serum was increasing for the first two weeks, as expected.

It has to be noted that rapid mIL-10 concentration decrease in the serum of electrotransferred immunocompetent mice has been observed by others for IL-10 [43,51] and other cytokines such as IL-5 [9], IL-12 [20,52], or IL-18 [16]. Thus, this time course profile might be characteristic of cytokines.

A surprising fact was that the mIL-10 level decrease in SCID mice was much slower than in immunocompetent C57-Bl/6 mice. Despite the fact that mIL-10 is a small protein (20 kDa) which can be more rapidly eliminated by renal filtration than hSeAP (57–58 kDa)

[53], and despite the fact that mIL-10 concentration in serum reached rapidly a peak of about 1–2 ng/ml at day 2 after injection while hSeAP reached a peak value within two weeks of about 100 ng/ml, we can note that the two kinetics had the same profile. Therefore, this indicates that the rapid decrease of free mIL-10 in serum of immunocompetent mice cannot be explained by a rapid catabolism and/or renal filtration. Indeed, these factors should be comparable in immunocompetent and SCID mice.

The SCID mice lack mature T and B cells [54]. Since B cells present IL-10 receptor, a greater part of the IL-10 secreted in the blood by electrotransferred muscle might probably remain free in SCID mice plasma because of a lower interaction with IL-10 receptors.

In conclusion, further studies will be necessary to explain the kinetics of transgenic mIL-10 in muscle and in serum after electrotransfer of muscle with plasmid DNA encoding mIL-10. Particularly, the real cytokine level, in free or associated form, in the different compartments will be one essential point to clarify. However, in the context of cytokine gene therapy, several advantageous characteristics have been observed in this work. First, the fast increase of circulating IL-10 to peak value, compatible with therapeutic level, will allow a rapid treatment in case of acute crisis of disease. Second, the rapid decrease of serum mIL-10 might represent an advantage if it is representative of a return to normal cytokine level after the gene therapy. And third, the possibility to repeat DNA injection and electrotransfer is an interesting therapeutic approach since it allows to obtain a similar level of cytokine than the first treatment.

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