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Gene therapy of experimental autoimmune thyroiditis mice by *in vivo* administration of plasmid DNA coding for human interleukin-10[1]

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KEY WORDS Interleukin-10; gene therapy; thyroiditis; autoimmune diseases

ABSTRACT

AIM: To investigate the effect of interleukin-10 (IL-10) gene on experimental autoimmune thyroiditis mice. **METHODS:** Mice were immunized to induce autoimmune thyroiditis with porcine thyroglobulin (pTg), and thyroids of mice were injected with IL-10 DNA. On d 28 after immunization with pTg, mRNA expression of IL-10 in thyroid glands was detected and thyroid specimens were histopathological studied. **RESULTS:** The mRNA expression of IL-10 was detected in thyroid glands on d 7 and 14 after injection of IL-10 plasmid DNA or on COS-7 cells 48 h after IL-10 plasmid DNA transfection. In addition, hIL-10 levels in culture media significantly increased 48 h and 72 h after IL-10 plasmid DNA transfection. Infiltration index of lymphocytes (1.1±0.4) in thyroids of IL-10-treated mice was significantly lower than that of pcDNA3-null-treated mice (2.2±0.5) ($P<0.01$). Compared with pcDNA3-null control mice, IL-10-treated mice had lower levels of serum IFN-γ ($P<0.01$). **CONCLUSION:** The direct injection of DNA expression vectors encoding IL-10 into thyroid significantly inhibited development of lymphocytic infiltration of thyroid of autoimmune thyroiditis mice, and alleviated the progression of this disease.

INTRODUCTION

Although true prevalence of autoimmune thyroiditis (Hashimoto's thyroiditis) is uncertain, it is common and may be increasing in frequency. The mean incidence in women is 3.5 cases per 1000 people per year and in men is 0.8 cases per 1000 people per year[1]. Hashimoto's thyroiditis (HT) is a chronic autoimmune disease characterized by a progressive destruction of thyroid epithelial cells and reduced production of thyroid hormones. Thyroid gland presents a marked lymphocyte infiltration, while a diffuse fibrosis tends to replace the parenchyma[2-4]. Up to now, its pathogenesis is not completely known, but many studies have suggested that cell-mediated autoimmune mechanisms are pathogenetically involved[5]. Indeed a T-cell clone specifically cytotoxic for autologous thyroid cells in a patient with Hashimoto's disease is reminiscent of animal models of cytotoxic T-cells associated with experimental autoimmune thyroiditis (EAT)[6]. Previous

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studies showed that treatment of EAT with cytokines such as IL-10 *in vivo* could protect from thyroid cell destroyed[7]. However, because the half-lives of these cytokines were very short, these recombinant cytokines were administered for large amounts and repeatedly[8]. More recently, *Fas* ligand (*Fas*L) gene therapy could prevent thyroid infiltrated by lymphocyte[9]. The experiment was performed using *Fas*L plasmid DNA direct injection to thyroid.

The purpose of this study was to investigate the effect of IL-10 gene on EAT in mice using poly-*L*-lysine-IL-10 DNA-liposome complexes directly injected into thyroid.

MATERIALS AND METHODS

Animals C57BL/6J female mice aged 8-12 weeks, weighing approximately 30 g (Shanghai Experimental Animal Center, Chinese Academy of Sciences, Grade II, Certificate No SYXK-SHANGHAI 2002-0023) were kept under specific pathogen-free conditions in our animal facility.

Expression vector construction The human IL-10 (hIL-10) 530 bp cDNA fragment was produced by reverse transcription-polymerase chain reaction (RT-PCR) from concanvalin (ConA)-stimulated human peripheral blood lymphocytes and conformed by sequencing analysis. The primers (sense primer: 5'-CGGAATTCCACCATGCACAGCTCAGC-3' and antisense: 5'-CGTCTAGAGATGTCTCAGTTTCGTATC-3') were used by PCR. The amplified hIL-10 cDNA fragment containing entire coding sequences was cloned into the pcDNA3 vector under control of CMV enhancer/promotor. The restriction enzymes *Eco*R I and *Xba* I were performed for digestion of aim fragment and pcDNA3 plasmid, and 508 bp fragment of hIL-10 was cloned into pcDNA3 vector[10].

Plasmid DNA preparation Large-scale plasmid DNA preparations were produced by alkaline lysis method using a Qiagen kit (Qiagen, CA). Plasmids were delivered in a complexes with poly-*L*-lysin (PLL) (Sigma, USA) and lipofectamine (Invitrogen, Life Technologies). For the direct gene transfer of PLL-DNA-liposome complexes, 2.5 μg plasmid DNA was first mixed with 720 ng PLL in a volume of 15 μL Opti-MEM (Invitrogen, Life Technologies). After a 15-min incubation at room temperature to allow DNA-PLL condensation, the mixture was added to a 10 μL solution of 25 μL lipofectamine in Opti-MEM[9]. Thirty minutes after incubation, the preparation was applied to the mice.

***In vitro* transfection** COS-7 cells were cultured on 24-well plates at a concentration of 2×10^4 cells per well for 24 h. Cultures were initiated in DMEM containing 10 % fetal calf serum, supplemented with 100 kU/L benzylpenicillin at 37 ºC for 24 h with 5 % CO_2. Then the growth media were removed and the cells were washed with PBS. After adding transfection media (including 3 μg IL-10 plasmid and 18 μL lipofectamine per well), COS-7 cells were incubated for 48 h and 72 h, after which the culture media were collected and stored at -70 ºC for hIL-10 expression assay by ELISA .

Determination of IL-10 levels in culture medium The concentrations (ng/L) of hIL-10 in collected culture media were measured using ELISA (Senxiong Biotech Co, Shanghai).

Immunization of animals and gene therapy Porcine thyroglobulin (pTg) (Sigma, USA) was emulsified in complete Freund's adjuvant (CFA) for immunization on d 0 and in incomplete Freund's adjuvant (IFA) for challenge on d 14. CFA suspension contained 1 g/L of mycobacterium tuberculosis. CFA and 100 μg of pTg was injected sc[7]. On d 21 after immunization, the mice were divided into IL-10-treated group (n=10) and control group (n=12). Our experimental method was previously described[9]. Briefly, mice were anesthetized ip with pentobarbital. Using sterile procedures, a lateral neck dissection was performed to visualize the thyroid gland and dissect it free of surrounding fascia and muscle while maintaining the capsule intact and blood supply constant. The connective shaft surrounding the thyroid gland was applied using a 50 μL syringe. This procedure allowed a direct contact between the PLL-IL-10 DNA-liposome complexes and the thyroid gland without any effraction (equivalent amounts of 2.5 μg pcDNA3-null plasmid DNA were injected to thyroid of control mice). After 1-2 min of contact, the wound was closed in layers. Mice were killed on d 7 and 14 after injection of plasmid DNA.

RT-PCR On d 7 and 14 after injection of plasmid DNA to thyroid gland, total RNA was extracted from thyroid by TRIzol. In addition, total RNA was extracted from COS-7 cells incubated with IL-10 DNA transfection media for 48 h. Total RNA was treated with DNase I, then 0.5 μg RNA was used in a first-strand cDNA synthesis using oligo-dt primer, and PCR was performed from 1:20 of the cDNA reaction. The PCR reaction

generated a 508-bp fragment of human IL-10 by previously described primers and a 591-bp fragment of murine β-actin (sense primer 5'-AACGAGCGGTTCCGATGCCCTGAG-3' and anti-sense primer: TGTCGCCTTCACCGTTCCAGTT-3'). The RT-PCR products were detected by 1.5 % agarose gel on electrophoresis.

Histopathological studies of thyroid specimens The histological grade of EAT was assessed by three doctors using blind evaluation of thyroid specimens. The mononuclear cell infiltration of the thyroid gland was graded as previously described[11]. Grade 1: interstitial accumulation of inflammatory cells between two or more follicles; Grade 2: one and two foci of inflammatory cells reaching at least the size of one follicle; Grade 3: 10 % to 40 % of the thyroid gland replaced by inflammatory cells; Grade 4: >40 % of the thyroid gland replaced by inflammatory cells.

Serum IFN-γ concentrations Eight hours after mice were injected ip with ConA 20 mg/kg[12], blood samples were obtained from IL-10-treated and pcDNA3-null-treated mice on d 28 after immunization with pTg. Sera were appropriately diluted and assayed for the concentration of IFN-γ by ELISA according to the antibody manufacturer recommendation (Senxiong Biotech Co, Shanghai). Plates were read at 490 nm, and results were expressed as concentrations (ng/L) of IFN-γ.

Statistical analysis Statistical analysis was performed using the software SPSS 9.0 and the Student's *t*-test. P<0.05 was considered significant.

RESULTS

Expression of IL-10 in mouse thyroid after gene delivery *in vivo* or on COS-7 cells after gene transfection *in vitro* IL-10 expression was detected by RT-PCR in thyroid of mice on d 7 and 14 after transferring expression vectors. IL-10 mRNA expressed in thyroid on d 7 and 14 after injection of PLL-IL-10 DNA-liposome complexes (Fig 1), but IL-10 mRNA was not detected in thyroid injected with pcDNA3-null plasmid DNA, while control β-actin mRNA was detected in both. However, IL-10 mRNA was not detected in thyroid on d 28 after injection of PLL-IL-10 DNA-liposome complexes (data not shown). In addition, we next determined by RT-PCR the expression of IL-10 mRNA on COS-7 cells 48 h after DNA transfection. Therefore, IL-10 mRNA was detected only on COS-7 cells transferred with IL-10 plasmid DNA, but not in those transferred with pcDNA3-null (Fig 1).

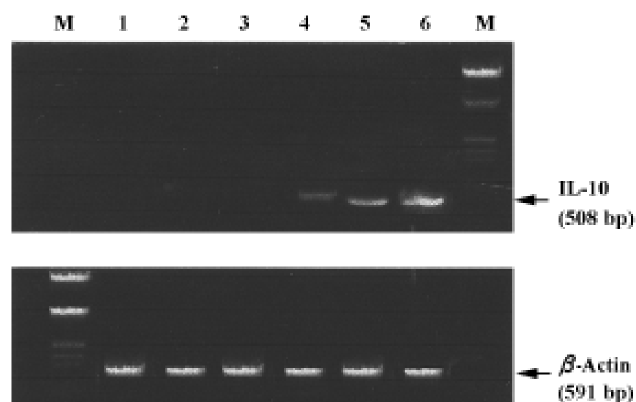

Fig 1. IL-10 mRNA expression by RT-PCR. Lane 1, 2: represent thyroid from control mice on d 14 and 7 after injection of pcDNA3-null DNA (*n*=2), respectively. Lane 4, 5: represent thyroid from IL-10-treated mice on d 14 and 7 after injection of PLL-IL-10 DNA-liposome complexes (*n* =2), respectively. Lane 3, 6: represent IL-10 mRNA expression on COS-7 cells 48 h after pcDNA3-null DNA and IL-10 plasmid DNA transfection, respectively. COS-7 cells (2×10^4 cells per well) transferred IL-10 plasmid DNA (3 μg) after adding lipofectamine (*n*=3) or equivalent amounts of pcDNA3-null plasmid DNA (control, *n*=3) were collected. The amplification of β-actin served as an internal standard. M: marker.

Increased IL-10 levels in culture medium At 48 and 72 h after IL-10 plasmid DNA was transferred into COS-7, hIL-10 concentrations at the collected culture media also markedly increased using ELISA measurement (Fig 2), they were (627±95) ng/L and (1168±153) ng/L, respectively. In contrast, after pcDNA3-null transfection, hIL-10 concentrations at culture media were not detected at the same times.

Inhibition of lymphocytic infiltration of the thyroid by transfer of IL-10 gene To determine whether IL-10 expression could alter the course of ongoing EAT, because the first sign of EAT appears about three weeks postpriming, mice were treated with PLL-IL-10 DNA-liposome complexes or pcDNA3-null on d 21 after primary immunization. Mice were killed one week after DNA injection (d 28 after immunization). The mononuclear cell infiltration of the thyroid gland usually reaches its peak on d 28 after immunization. The histopathological studies of thyroid specimens showed that thyroids of pcDNA3-null-treated mice had markedly lymphocytic infiltration (Fig 3). Compared with pcDNA3-null control mice, the thyroids of IL-10-treated mice had lower lymphocytic infiltration index (P<0.01, Tab 1).

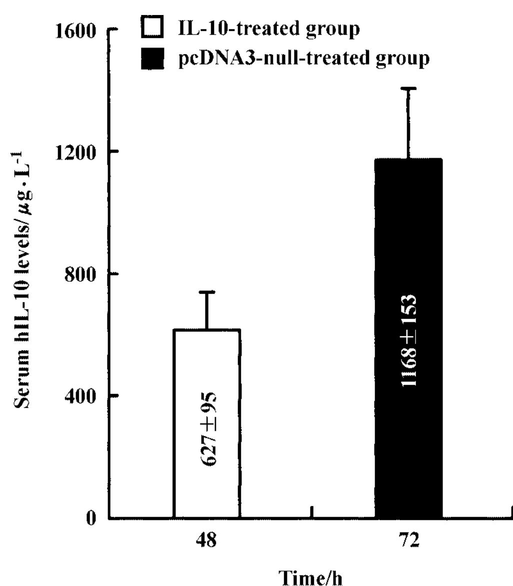

Fig 2. ***In vitro*** **DNA transfection efficiencies of IL-10 plasmid DNA on COS-7 (3 μg DNA per well, 2×10^4 cells per well). After 48-h and 72-h culture, the culture medium was collected and the hIL-10 level was measured by ELISA. *n*=3. Mean±SD.**

Tab 1. Effect of IL-10 gene transfer on EAT. Mean± SD. ^{c}*P*<0.01 *vs* pcDNA3-null-treated mice.

Group	*n*	Lymphocytic infiltration index in thyroid d 28
IL-10-treated mice	8	1.1 ±0.4^c
pcDNA3-null-treated mice	10	2.2 ±0.5

EAT: experimental autoimmune thyroiditis

Reduced serum IFN-γ levels To investigate whether IL-10 gene therapy could affect the expression of IFN-γ, serum IFN-γ levels were detected using ELISA. The results showed that serum IFN-γ levels of IL-10-treated mice were significantly lower than that of pcDNA3-null-treated mice (*P*<0.01, Fig 4).

DISCUSSION

In this study, PLL-IL-10 plasmid DNA and liposome complexes were directly injected to thyroid of EAT in mice. We observed that IL-10 mRNA expression in thyroid appeared for as long as 14 d. Moreover, it needed small amounts of plasmid DNA with liposome. The higher IL-10 mRNA expression on COS-7 cells

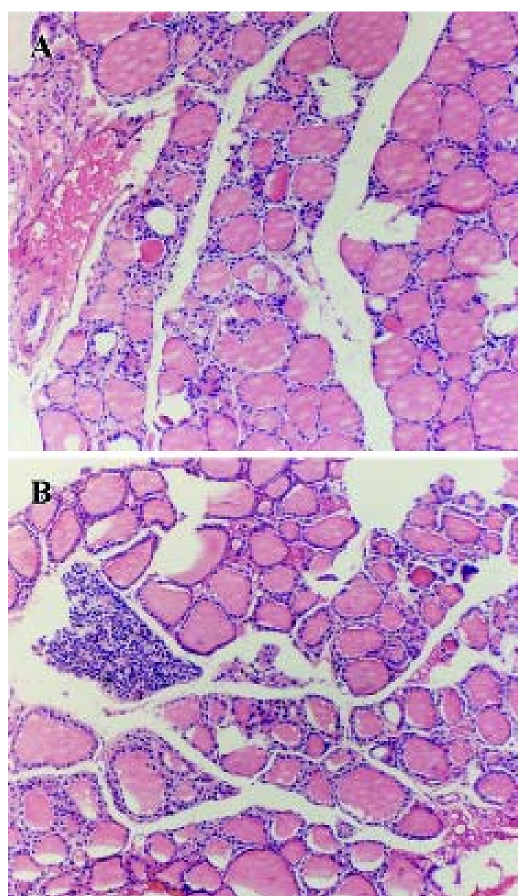

Fig 3. Histological aspect of thyroids on d 28 after immunization with pTg in IL-10 (A) or pcDNA3-null-treated mice (B).

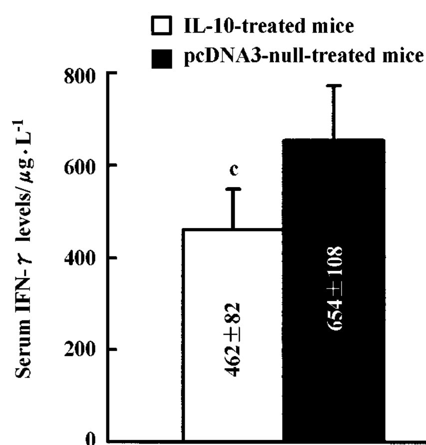

Fig 4. Serum IFN-γ levels were measured by ELISA. *n*=8 (IL-10-treated group), *n*=10 (Control group). Mean ±SD. ^{c}*P*<0.01 *vs* pcDNA3-null-treated mice.

and elevated IL-10 concentrations in culture media were observed using *in vitro* transfection of IL-10 plasmid DNA. Previous studies had also the similar results[13]. In addition, we found that IL-10 plasmid DNA im in-

jection could significantly suppress delayed-type hypersensitivity (DTH) reaction in mice[10]. As a result, this cloned hIL-10 gene had biologic functions.

EAT is a classic model of autoimmune thyroiditis with pTg immunization. The characteristic of this model is the same as human Hashimoto's thyroiditis. The first signs of EAT appearing on d 21 after immunization were characterized by lymphocyte infiltration of thyroid[9]. According to expression of IL-10 plasmid DNA *in vivo*, the injection time of IL-10 plasmid DNA was on d 21 after primary immunization in this study. As a result, IL-10 gene therapy could significantly inhibit lymphocytic infiltration of thyroid, and protected from destruction of thyroid follicular cells. IL-10 could suppress activation of T-lymphocytes, and decrease the number of infiltrated $CD4^+$ and $CD8^+$ lymphocytes in the thyroids. Previous study showed that treatment of recombinant human IL-10 *in vivo* could reduce development of the EAT, and the effect of IL-10 could probably be explained by an increase of the physiologic apoptosis susceptibility of activated T-lymphocytes, rather than by a direct apoptosis induction by the cytokine itself[7]. The apoptosis of lymphocytes appeared on d 3 after IL-10 injection (d 21 after immunization by pTg). This phenomenon was not observed on d 28 after immunization in the present study. In addition, we do not know if the apoptosis of lymphocytes appeared on d 21 after immunization in our study, because we did not choose that experimental time point.

In the present study, serum IFN-γ levels in IL-10-treated mice were significantly lower than that of pcDNA3-null-treated mice. IL-10, as Th2 cell cytokine, could significantly suppress cytokine release from Th1 cells, particularly IFN-γ. IFN-γ plays an essential role in pathogenesis of autoimmune thyoiditis[13,14]. The previous studies showed that EAT was induced in susceptible mice injected intrathyroidally with IFN-γ, in contrast, the progression of EAT was marked alleviation when these mice were injected a monoclonal antibody to IFN-γ[15]. Therefore, IFN-γ secretion was reduced in IL-10-treated mice, it could significantly decrease the number of thyroglobulin-specific $CD8^+$ cells. Furthermore, activation of macrophages induced by IFN-γ to secrete proinflammatory and cytotoxic molecules including IL-1 and TNF-α were suppressed. These effects could prevent against lymphocyte infiltration of thyroid and damage of thyroid follicular cells. Therefore, the development of EAT was markedly reduced.

In the present study, we used liposome and PLL to increase transfection effectivity of IL-10 plasmid DNA. The liposomal transfection reagent lipofectin is a 3:1(w/w) mixture of the cationic lipid DOSPA and the neutral lipid DOPE[16]. More recently, several cationic lipid-based gene delivery systems have been tested for gene transfer both *in vivo* and *in vitro*. Furthermore, cationic lipid-based systems have been proven useful in many *in vivo* applications in animal model and in human clinical trials[16-18]. In addition, at relatively low concentrations of lipid/plasmid complexes, little or no toxic effect has been reported in mice, rabbits, pigs, and nonhuman primates after systemic administration of the complexes[19,20]. So, PLL-plasmid DNA-liposome complexes were used for gene therapy of EAT. In addition, because thyroid gland of mouse is a very small organ, it is very difficult that large amounts of plasmid DNA are injected to thyroid gland. The method, which is direct injection of thyroid gland, is also an acceptable strategy for human Hashimoto's thyroiditis.

In conclusion, we injected the mixture of PLL-IL-10 plasmid DNA-liposome to thyroid gland of EAT in mice, and determined that IL-10 gene transfer thyroid could significantly reduce lymphocytic infiltration of thyroid. The results suggested that IL-10 expression restricted to thyroid might have a curative effect on ongoing EAT in the mice. Furthermore, the mixture of PLL-plasmid DNA-liposome had relatively long time of expression *in vivo* and little or no toxic effect to human. As a result, this method is potentially applicable to the treatment of human Hashimoto's thyroiditis. Further studies in this field could contribute to the therapy of Hashimoto's thyroiditis.

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