Prevention of Autoimmune Insulitis by Delivery of Interleukin-4 Plasmid Using a Soluble and Biodegradable Polymeric Carrier

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Purpose. We delivered interleukin-4 (IL-4) plasmid (pCAGGS-IL-4) using the biodegradable polymer, poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA), to prevent autoimmune insulitis in NOD mice.

Methods. The pCAGGS-IL-4/PAGA complex was transfected to 293T cells. The expression level of IL-4 was measured by ELISA. The pCAGGS IL-4/PAGA complex was injected once to NOD mice intravenously at the age of 4 weeks. RT-PCR was performed to evaluate the level of the IL-4 mRNA in the liver. At 6 weeks after the injection, the grade of insulitis of the mice was evaluated by double blind methods.

Results. *In vitro* transfecton assays showed that PAGA enhanced the expression of IL-4 in 293T cells. RT-PCR of the liver showed that IL-4 was expressed highest in the complex injected group. In the plasmid/PAGA complex injected group, the prevalence of severe insulitis in NOD mice was markedly improved, suggesting that PAGA enhanced the delivery of IL-4 plasmid.

Conclusion. The pCAGGS-IL-4/PAGA complex is an effective system to prevent autoimmune insulitis in NOD mice and applicable for the prevention of autoimmune diabetes.

KEY WORDS: autoimmune insulitis; diabetes; gene delivery; Interleukin-4; poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA).

INTRODUCTION

Numerous studies have established that autoimmune type 1 diabetes results from the activation of Th type 1 (Th1) lymphocytes (1–6). Th1 and Th type 2 (Th2) cells are mutually inhibitory and the increased production of the Th2 cytokines suppresses Th1 differentiation (7,8). Therefore, the gene therapy systems for the prevention of type 1 diabetes have been developed by employing Th2 cytokines. It was previously reported that the systemic delivery of interleukin-10 (IL-10) plasmid into NOD mice prevented autoimmune insulitis effectively (9,10). Nitta et al. administrated naked IL-10 plasmid to NOD mice intramuscularly (9). In addition, we administrated IL-10 plasmid/polymer complex into NOD mice intravenously to improve the cellular uptake and plasmid stability. The results showed that DNA/polymer complexes were more effective than naked DNA for the prevention of insulitis (10).

Interleukin-4 (IL-4) has also been used in gene therapy trials to prevent autoimmune disease. Previous reports showed that development of transgenic NOD mice expressing IL-4 in their islets prevented insulitis or diabetes completely (11,12). Recently, somatic gene therapy with the IL-4 gene was also attempted in NOD mice. The delivery of the IL-4 gene using adenovirus improved autoimmune insulitis by 10% compared with a non-injection control group at the age of 15 and 30 weeks (13). Although viral vectors are currently the most effective way for gene transfer, they have disadvantages, compared with nonviral carriers. Viral vectors introduce serious concerns about endogenous virus recombinations, oncogenic effects, and immunologic reactions (14,15). The recent death of a young patient with liver ornithine transcarbamylase deficiency following intrahepatic adenoviral injection raises many questions about the safety of viral vectors (16). This safety problem of viral vector gene therapy necessitates nonviral gene therapy.

Nonviral carriers have some advantages over viral carriers. They can accommodate large-size DNA, be modified with appropriate ligands for specific cell targeting, and be administered repeatedly. Nonviral delivery systems generally consist of cationic liposomes, linear cationic polymers and their conjugations (17). However, nonviral gene delivery has its limitations such as toxicity and low transfection efficiency. Therefore, biodegradable cationic polymers have been introduced as a gene delivery system that can improve the transfection efficiency and cytotoxicity (18). Recently, a biodegradable polymeric carrier, poly[α-(4-aminobutyl)-L-glycolic acid] (PAGA), was synthesized to increase biocompatibility and transfection efficiency (19). PAGA was designed with a hydrolytically labile ester bond. Therefore, PAGA was degraded rapidly to monomer (L-oxylysine) or small oligomer under physiological conditions and did not show any detectable cytotoxicity (19). Also, PAGA showed the improved transfection efficiency compared with its analog, poly-L-lysine (19). In this study, we developed the IL-4 plasmid delivery system using PAGA. *In vitro* transfection assays demonstrated that PAGA enhanced IL-4 expression in 293T cells. After intravenous administration, the expression of IL-4 in the liver was confirmed by RT-PCR. After 6 weeks, the insulitis of each group was evaluated. From the results, it has been shown that pCAGGS-IL-4/PAGA is effective in the prevention of autoimmune insulitis in NOD mice.

MATERIALS AND METHODS

Synthesis of PAGA

PAGA was synthesized as described previously (19,20). Briefly, 10 g of sodium nitrite solution was added dropwise to 20 g of CBZ-L-lysine in a mixture of 200 ml of 2 N H_2SO_4 and 200 ml of acetonitrile with constant stirring on ice. Stirring was continued for an additional 7 h on ice and 12 h at room temperature. The resulting solution was then extracted with ether and precipitated with petroleum ether and then recrystalized in dichloromathan/hexane. CBZ-L-lysine was polymerized by melting condensation at 150°C under vacuum for 5 days. The polymer was cooled and dissolved in chloroform followed by precipitation with methanol. Two grams of the dried polymer was dissolved in 28 ml of DMF containing 2 g of activated palladium on carbon (10% Pd-C). Ninety milliliters of 85% formic acid was added slowly to the polymer

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solution, and 15 h after stirring at room temperature the solution was filtered to remove Pd-C. Forty milliliters of 2 N HCl was added and the volume was reduced under vacuum. PAGA was finally precipitated with a large excess of acetone, dried under vacuum, and stored at –70°C until further use. PAGA was characterized in terms of molecular weight and biodegradability using MALDI-TOF-mass spectrometry and in terms of purity using NMR spectroscopy.

Preparation of pCAGGS-IL-4

Mouse IL-4 expression pCAGGS plasmid was amplified in E. coli DH5α (Gibco-BRL, Gaithersburg, Maryland), and purified by Qiagen Plasmids Maxi Kits (Qiagen, Valencia, California). The purity and identity of the plasmid were confirmed by absorbance measurements at 260 nm and 280 nm and by agarose gel electrophoresis following restriction enzyme digestion.

Preparation of pCAGGS-IL-4/PAGA Complex

The pCAGGS-IL-4/PAGA complex was prepared by self assembly. PAGA (1.23 mg) was dissolved in 2 ml of phosphate-buffered saline (PBS, pH 7.3). One hundred microliters of the PAGA solution was slowly dropped into 100 μl of the plasmid solution (0.5 mg/ml) and left at room temperaure for 30 min for complex formation. The complex formation of PAGA/DNA was routinely monitored by 1.0% agarose gel electrophoresis.

In vitro Transfection Assay

293T cells were maintained in DMEM medium supplemented with 10% FBS in a 5% CO_2 incubator. For the transfection assays, the cells were seeded at a density of 4.5×10^4 cells per well in 24-well micorassay plates at 24 h before transfection. The pCAGGS-IL-4/PAGA complex was prepared at a charge ratio of 1:3 (-/+). The growth media was removed and the cells were washed twice with fresh media. After the addition of transfection media, 293T cells were incubated for 24 h in a CO_2 incubator. The media were collected and stored at –70°C until use for the IL-4 expression assay. The amount of IL-4 was measured by using an enzyme-linked immunosorbent assay (ELISA, optEIA mouse IL-4 set, Pharmingen, San Diego, CA) as described in the manufacturer's manual.

Animals and the Injection of pCAGGS IL4/ PAGA Complex

Four-week-old female NOD mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and were kept under specific pathogen-free conditions. Two hundred microliters of plasmid/PAGA complex at a charge ratio of 1:3 (-/+) was injected into the tail vein of NOD mice at a dose of 50 μg of DNA per mouse. NOD mice were sacrificed at scheduled times by cervical dislocation following anesthetization with methoxyflurane (Schering-Plough Animal Health, Union, NJ) inhalation. The pancreas and liver were harvested and stored at –70°C until use.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from NOD mice liver at scheduled times by acid-guanidium thiocyanate-phenol-chloroform extract as described previously (21), using RNAwiz (Ambion, Austin, Texas). The prepared RNA was treated with RNase free DNase I for 30 min to eliminate contaminating DNA. The concentration of RNA was measured by the absorbance at 260 nm. The integrity of RNA was confirmed by formaldhyde-formamide denatured agarose gel electrophoresis. Briefly, reverse transcription was performed with 2 μg of total RNA at 37°C for 1 h using AMV reverse transcriptase (Promega, Madison, WI). Specific oligonucleotide primers were forward primer 5′-GCTGGTTATTGTGCTGTCTC-3′, backward primer 5′-GTTCAAAATGCCGATGATCTCT-3′. For PCR, the following reaction cycle was used: 40 cycles at 94°C for 1 min, 60°C for 1 min and 72°C for 1 min 30 s followed by an extension of 10 min at 72°C. PCR products were analyzed by electrophoresis on 1.5% agarose gels.

Measurement of Insulitis in NOD Mice

For hematoxylin-eosin staining, the pancreas was fixed in 20% formalin containing 75 mM phosphate buffer at pH 7.0, and embedded in paraffin. Five-micron thick sections were cut and stained with hematoxylin-eosin. More than 20 islets from each pancreas were examined using double blind methods from three animal groups (n = 3 in each group). To characterize the progression of insulitis, each islet was assigned by using the following insulitits grading system (22). Grade 3 and 4 were considered as severe insulitis (grade 0, normal islet; 1, mononuclear cell infiltration, largely in the periphery, in less than 25% of the islet; 2, 25% to 50% of islet showing mononuclear infiltration; 3, over 50% of islet showing mononuclear infiltration; and 4, small retracted islet with few mononuclear cells).

RESULTS AND DISCUSSION

Preparation of the pCAGGS-IL-4/PAGA Complex and *in Vitro* Transfection Assay

The expression of IL-4 from pCAGGS-IL-4 was driven by a β-actin promoter and CMV enhancer (Fig. 1). The identity and quality of the prepared pCAGGS-IL-4 was confirmed by agarose gel electrophoresis following restriction enzyme digestion. In this study, PAGA was used as a gene carrier. The characteristics of PAGA were reported in the previous reports (10,19,20). PAGA has excellent characteristics over other gene carriers. PAGA does not have any detectable cytotoxicity (19). PAGA is degraded rapidly under physiological conditions and produces a non-toxic monomer (L-oxylysine) (19). PAGA can condense plasmid DNA into a small size complex. The complex formation with PAGA was confirmed by a gel retardation assay. The complex was completely retarded at a higher charge ratio than 1:3 (-/+). In addition, PAGA can protect DNA from nuclease as described previously (10). Its transfection efficiency is higher than that of its analog, poly-L-lysine (19). The effective average diameter of the complex was measured by dynamic light scattering at this charge ratio. The average diameter of the complex was 179 nm when the complex was prepared at the DNA concentration of 0.25 mg/ml. At higher concentrations, the complex had a tendency to have higher particle size (data not shown). Therefore, in the following experiments, the complex was prepared at a plasmid concentration of 0.25 mg

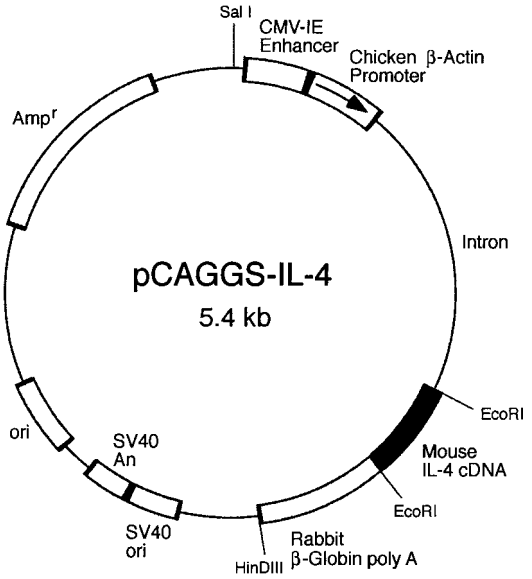

Fig. 1. Diagram of pCAGGS-IL-4 structure

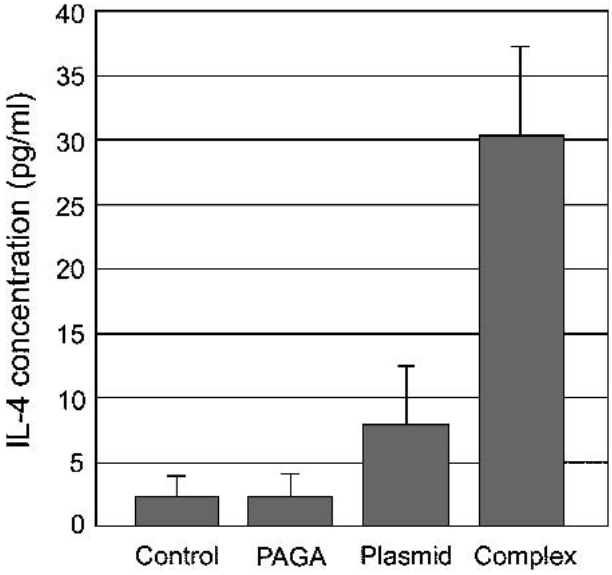

Fig. 2. *In vitro* transfection efficiency of pCAGGS-IL-4/PAGA complex in 293T cells After the transfection of pCAGGS-IL-4/PAGA complex into 293T cells, the media were collected and the IL-4 level was measured by ELISA. Values denote mean and standad deviation. Control, no transfection; PAGA, transfection with PAGA only; Plasmid, transfection with naked plasmid; Complex, transfection with plasmid/PAGA complex.

/ml. In general, low transfection efficiency and cytotoxicity have been major barriers for nonviral gene delivery. However, low transfection efficiency may be overcome by repeated administration. Since PAGA is biodegradable and non-toxic, it may be fit for repeated administration to maintain sustained gene expression. All these characteristics of PAGA make it an excellent gene carrier for IL-4 plasmid.

The pCAGGS-IL-4/PAGA complex was transfected to 293T cells to confirm an enhanced transfection effect of PAGA. Naked DNA was also transfected to 293T cells. The DNA quantity transfected was fixed at 0.5 μg/well in 24 well microassay plates. The results showed that the level of IL-4 expressed in the complex transfected cells was much higher than in the naked DNA transfected cells (Fig. 2). This result suggests that PAGA enhanced the transfection of pCAGGS-IL-4 into 293T cells at a charge ratio of 1:3 (-/+).

The Expression of IL-4 in NOD Mouse Liver

The complex was injected to 4-week-old NOD mice intravenously. Two hundred microliters of the plasmid/PAGA complex or plasmid was injected into the tail vein of NOD mice at a dose of 50 μg of plasmid per mouse. As a control group, same dose of PAGA was injected into NOD mice. At 5 days after the injection, the mice were sacrificed and the liver was harvested. RNA was prepared from the liver. The expression of IL-4 in the liver was evaluated by RT-PCR. To exclude the possibility that the endogeneous IL-4 mRNA or plasmid DNA is amplified, the primers were designed to encompass the intron sequence of the pCAGGS-IL-4 plasmid. After amplification, the expression of IL-4 was detected in the naked DNA injected or the plasmid/PAGA complex injected group (Fig. 3, lanes 2 and 3). The expression level of IL-4 in the complex injected group was higher than that in the naked DNA injected group. However, in the control group, the IL-4 mRNA was not detected (Fig. 3, lane 1).

Insulitis after the Intravenous Injection of pCAGGS-IL-4/PAGA Complex

The prevalence of severe insulitis of NOD mice was evaluated at 6 weeks after the injection of the plasmid/PAGA complex. The mice were sacrificed and the pancreas was harvested. The insulitis of each group (n = 3) was evaluated by using double blind methods. The prevalence of severe insulitis in NOD mice at 10 weeks of age was markedly improved in the complex injected group, compared with the naked DNA injected or the control group (Fig. 4). The results suggest that the PAGA/DNA complex is more effective than naked DNA for the prevention of autoimmune insulitis in NOD mice.

In this report, we developed an IL-4 gene delivery system using pCAGGS-IL-4 and the nonviral carrier, PAGA. As described above, delivery of IL-4 plasmid using PAGA is effective *in vitro* and *in vivo*. To prevent autoimmune diabetes completely, the safety and efficiency of gene delivery system must be improved. The non-toxicity and the effective prevention of autoimmune insulitis suggest that the pCAGGS-IL-4/PAGA system is one of the safe gene therapy systems for the prevention of autoimmune diabetes.

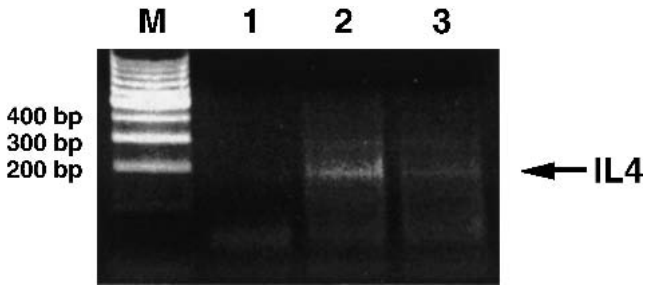

Fig. 3. RT-PCR. Total RNA was isolated from NOD mice liver at 5 days after injection. The IL-4 mRNA was reverse-transcribed and amplified by PCR. Lane 1, PAGA injected group; lane 2, plasmid/PAGA complex injected group; lane 3, naked plasmid injected group. Lane M indicates DNA size marker.

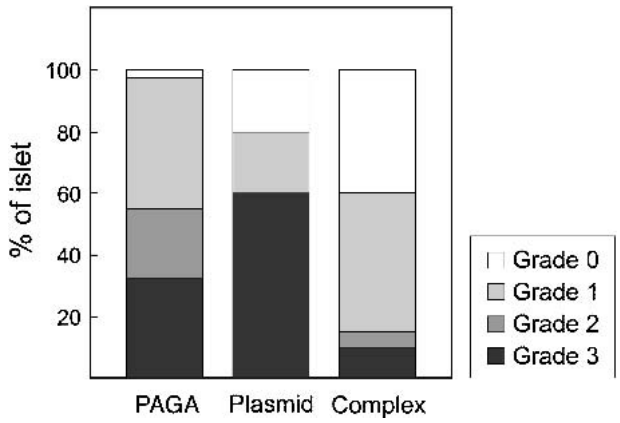

Fig. 4. Insulitis in NOD mice after injection. Insulitis was evaluated by hematoxylin-eosin staining of islets from each pancreas. More than 20 islets from each pancreas were examined using double blind methods from three animal groups (n = 3 in each group). PAGA, PAGA injected group; Plasmid, naked plasmid injected group; Complex, plasmid/PAGA comoplex injected group.

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