12

Oral Delivery of DNA Vector Conjugated with Chitosan and Its Effect on Th1 Polarized Inflammation

Bunsoon Choi[1], Ju Young Choi[1], Do-Hyun Jo[2] and Seonghyang Sohn[1]
[1]*Laboratory of Cell Biology, Institute for Medical Sciences,*
[2]*Department of Molecular Science and Technology, Ajou University, Suwon, Korea*

1. Introduction

Gene therapy can be defined as the transfer of genetic materials to specific cells in order to exert a therapeutic effect. Gene therapy is a promising approach to the treatment of a wide range of diseases by compensating for defective genes or producing beneficial proteins (Zhao et al., 2009; Mansouri et al., 2004). Gene vectors play many important roles in gene therapy. Recently, nonviral vectors have been increasingly proposed as safer alternatives to viral vectors because of their potential advantages such as ease of synthesis, cell/tissue targeting, low immune response, and unrestricted plasmid size (Leong et al., 1998). Among nonviral systems, cationic polymers have attracted a great deal of attention because they can easily form self-assembling polyelectrolyte complexes between plasmid DNA and cationic polymers and mediate transfection via condensing DNA into nanoparticles, protecting DNA from enzymatic degradation, and facilitating cell uptake and endolysosomal escape (Wang et al., 2002). Among cationic polymers, polyethylenimine (PEI) and chitosan are widely used as nonviral vectors for gene delivery. These compounds have the same ability to enter cells by binding to proteoglycans on cell surfaces and undergoing endocytosis (Lungwitz et al., 2005; Köping-Höggard et al., 2001; Mansouri et al., 2006). However, after uptake, they have very different transfection efficiencies. PEI is considered to be the most effective cationic polymer for gene delivery (Densmore et al., 2009). However, PEI is also associated with dose-dependent toxicity, especially at high molecular weights, which probably explains why it has not yet been used in human studies (Kunath et al., 2003). Conversely, chitosan is degraded in the endosome and the material is then released into the cytoplasm. The material is then transported to the nucleus. Therefore, chitosan is generally considered less effective in gene delivery systems than PEI *in vitro* and *in vivo*. However, it is well known as a biocompatible, biodegradable, and relatively non toxic material with high cationic potential (Lee et al.,1998). Therefore, chitosan nanoparticles could be applied to vectors for gene delivery. In addition, chitosan is a widely available orally administered protein that can also be readily formed into nanoparticles able to entrap plasmid DNA and promote gene expression (Bowman & Leong , 2006).

The purpose of this study was to evaluate chitosans of different molecular weights as DNA complexing agents based on their efficiency at transfecting RAW 264.7 cells and their *in vivo*

effects following oral administration to mice. Additionally, chitosan-pcDNA-EGFP-mIL4 nanoparticle complexes administered to mice were evaluated to determine if they can up-regulate the serum level of IL-4, and therefore ameliorate T helper type 1 polarized inflammation in herpes simplex virus induced inflammation.

2. Methods

2.1 Preparation of vector particle

The pCIN-mIL4 vector was constructed by inserting IL-4 cytokine genes into pCI-neo (pCIN; Gibco-Invitrogen, Rockville, MD). The IL-4 gene was amplified by PCR from MFG-muIL-4 plasmids with specific IL-4 primers and then inserted into pCIN to generate pCIN-mIL4 (Lee et al., 1999). Plasmid DNA was purified on Qiagen columns (Qiagen, Chatsworth, CA), after which it was mixed with chitosan or precipitated onto nanoparticles (Gan & Wang, 2007). Next, 2 μL of different molecular weight (3kDa, 10 kDa, 50 kDa) 2% chitosans dissolved in 0.1% acetic acid were mixed with 1 μg of pCIN-mIL4 DNA vector. To produce chitosan-DNA nanoparticles, chitosan was dissolved in 1% acetic acid (chitosan acetate) (0.35%w/v), after which chitosan-DNA nanoparticle complexes were precipitated by centrifugation and confirmed by agarose gel electrophoresis. Briefly, 50 μg of pCIN-mIL4 DNA was dissolved in 5 ml of 20% sodium sulfate, after which an equal volume of chitosan acetic acid and DNA solution was mixed and centrifuged. The supernatant was then discarded and stored at 4 °C.

2.2 Chitosan treatment

Chitosan with different molecular weights (3, 10, and 50 kDa) was dissolved in 0.1 M acetic acid solution and then filtered to remove the insoluble particles. The pH was subsequently adjusted to 8.0 with 1 M NaOH, which resulted in the formation of white precipitates. The precipitated chitosan was subsequently washed thoroughly using deionized water until a neutral pH was attained, at which point the product was vacuum dried at room temperature for 24 h (Gan & Wang, 2007).

2.3 *In vitro* transfection of RAW 264.7 cells with IL-4 DNA

RAW 264.7 cells, which are a murine macrophage cell line, were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Before transfection, cells were harvested, washed twice, and resuspended in 12-well plates at a density of $1x10^5$ cells per well. Cells were then transfected with a total of 1 μg of pCIN-mIL4 vector using 2 μL of oligofectamine (Gibco-Invitrogen, Rockville, MD) and/or chitosan. Finally, cells were cultured for 48 h in medium containing 10% FBS, harvested, and analyzed for IL-4 expression.

2.4 *In vivo* administration of IL-4 DNA

The pCIN-mIL4 or pCIN DNA vector was administered orally once a day for two to three days. Each dose carried 20 μg of DNA into the mice. In normal mice, intestinal tissues and serum were collected day 2 and 5 after the last administration. In herpes simplex virus induced inflammatory Behcet's disease mice, 20 μg of pCIN-EGFP-mIL4 was mixed with chitosan and then orally administered for three consecutive days. At 20 days after last administration, the mice were sacrificed and the intestinal tissues were collected for confocal microscopy. Mice were bred in temperature and light controlled conventional rooms (20-22

°C, 12 h light cycle starting at 8:00 a.m.) during which time they had free access to food and water. During the experimental period, the animals were closely observed. Mice were handled in accordance with protocols approved by our institutional animal care committee.

2.5 RNA isolation & RT-PCR

Total RNA was isolated by acid guanidium thiocyanate - phenol - chloroform extraction (Chomczynski & Sacchi, 1987). Spleen tissues were homogenized in 1 mL of extraction buffer, which was composed of 4 M guanidine solution (Aldrich, Milwaukee, WI, USA), 25 mM sodium citrate pH 7.0, 0.5% sodium N-lauroyl sarcosinate (Fisher, Pittsburgh, PA), and 0.1 M 2-mercaptoethanol (Sigma, St. Louis, MO). A 1/10 volume of chloroform: isoamyl alcohol (49: 1) was added to the samples, which were incubated on ice for five minutes, then centrifuged at 10,000 x g for 15 minutes at 4 °C. RNA contained in the upper aqueous phase was collected, precipitated with an equal volume of isopropanol, and washed twice in 70% EtOH. RNA pellets were dissolved in distilled water, quantified by OD 260/280, and then visualized in an ethidium bromide stained agarose gel. Two micrograms of total RNA were reverse transcribed using a cDNA kit (Gibco BRL, Grand Island, NY, USA), oligo dT primers and AMV reverse transcriptase to generate cDNA for use as a template in PCR amplifications. Two microliters from the reverse transcriptase reaction were then added to PCR reaction mixtures composed of 50 mM KCl pH 8.4, 20 mM Tris-HCl, 2.5 mM $MgCl_2$, 200 μM dNTPs, 2.5 U of Taq polymerase (Gibco BRL), and 1.2 μM primers. The specific primers were as follows:

β-actin (Murray et al., 1990)	(S) 5' - TGGAATCCTGTGGCATCCATGAAAC - 3'
	(A) 5' - TAAAACGCAGCTCAGTAACAGTCCG - 3'
IL-4 (Lee et al., 1986)	(S) 5' - ACGCCATGCACGGAGATGGAT - 3'
	(A) 5' - CAAGCATGGAGTTTTCC - 3'

2.6 Determination of IL-4 levels in mice

The serum levels of IL-4 in mice administered pCIN-mIL4 or chitosan-pCIN-mIL4 nanoparticles were measured by ELISA using commercially available IL-4 ELISA kits (R&D systems Inc., Minneapolis, MN).

2.7 Confocal microscopy

Intestinal tissues isolated from mice administered chitosan-pcDNA-EGFP-mIL4 mixture were sectioned in cryostat for confocal microscopy. Sectioned tissues were observed under a confocal microscope (Zeiss, Germany).

2.8 Animals, introduction of Behcet's disease symptoms and treatment of Behcet's disease mice with chitosan-pcDNA-EGFP-mIL4

Four to five-week-old male ICR mice were used for this experiment. The earlobes of the mice were scratched with a needle and then inoculated with 1.0 x 10^6 plaque forming units/ml of HSV type 1 (F strain). Virus inoculation was conducted twice with a 10-day interval between treatments, after which the mice were observed for 16 weeks. Mice were bred in temperature- and light-controlled conventional rooms (20-22 °C, 12 h light cycle starting at 8:00 a.m.) with free access to food and water. During the experimental period, the animals were closely observed and photographed. Animals were handled in accordance to a protocol approved by our institutional animal care committee.

A revised Japanese classification with minor modifications was used to classify symptomatic mice with Behcet's disease. Briefly, oral, genital and other skin ulcers (including bulla and crust) and eye symptoms were classified as major symptoms, while arthritis, gastrointestinal ulcers and neurological disorders were identified as minor symptoms. Mice with at least one major and one minor symptom were classified as having Behcet's disease. Of the total number of HSV-injected mice, 15% developed Behcet's disease -like symptoms. Treatments that led to the disappearance of symptoms or a decrease in the lesion size of greater than 20% were classified as effective. Scoring of the severity of Behcet's disease was followed by determination of the Behcet's disease activity index, as outlined in the Behcet's disease Activity Forum (www.behcet.ws/pdf/BehcetsDiseaseActivityForm pdf). Among the symptoms in patients, mouth ulceration, genital ulceration, erythema, skin pustules, skin ulceration, joints-arthritis, diarrhea, red eye (right, left), reduced vision (right, left), loss of balance, discoloration, and swelling of the face were selected and analyzed in the Behcet's disease mouse model. The score of each symptom was one, and after the score was computed the total was used to determine the severity of Behcet's disease. Symptomatic mice were photographed on the starting day of drug administration and on day 20 after chitosan-DNA vector administration. Chitosan-pcDNA-EGFP-mIL4 was orally administered to Behcet's disease-like mice for three consecutive days. Placebo was administered to Behcet's –like disease mice in an identical manner.

2.9 Statistical analysis

All data are represented as the mean ± SE. Statistical differences between control and IL-4 vector injected groups were determined using the Student *t* test and Bonferroni correction. Statistical analysis was conducted using MedCalc® version 9.3.0.0.

3. Results

3.1 pCIN-mIL4 vector was mixed with chitosan *in vitro*

1 µg of pCIN-mIL4 DNA vector and 2 µL of 2% of chitosan (3 kDa, 10 kDa, and 50 kDa) were mixed and loaded into 1% agarose gel. The DNA vector was well complexed with chitosan as shown in Figure 1.

Lane 1: DNA marker, Lane 2: pCIN-mIL4, Lane 3: pCIN-mIL4 + 10 kDa chitosan mixture, Lane 4: pCIN-mIL4 + 50 kDa chitosan mixture, Lane 5: pCIN-mIL4 + 3 kDa chitosan mixture

Fig. 1. pCIN-mIL4 and chitosan complex was observed upon agarose gel electrophoresis.

3.2 pCIN-mIL4 DNA vector was transfected into RAW 264.7 cells with or without chitosan

To determine if the Chitosan-pCIN-mIL4 DNA vector mixture could deliver pCIN-mIL4 DNA vector into the cells and if the delivered DNA could be expressed to mRNA, pCIN-mIL4 DNA vector was transfected into RAW 264.7 cells with or without chitosan. The mixture containing 50 kDa chitosan-pCIN-mIL4 strongly increased IL-4 mRNA expression in RAW 264.7 cells when compared to 10 kDa chitosan or 3 kDa chitosan or untreated groups based on analysis by reverse transcriptase PCR (RT-PCR) (Figure 2).

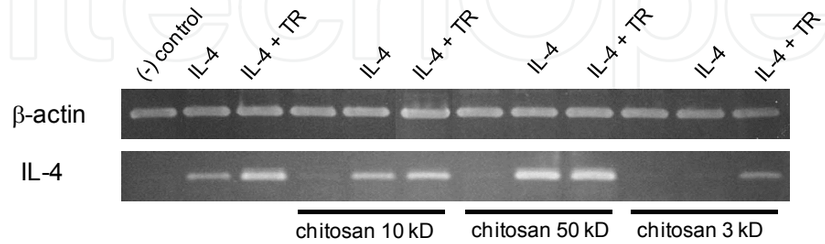

Fig. 2. pCIN-mIL4 DNA vectors mixed with three kinds of chitosan were transfected into RAW 264.7 cells. IL-4 mRNA expression was detected by RT-PCR. Oligofectamin was used as a transfection reagent, and compared to chitosan.

3.3 Chitosan-pCIN-mIL4 DNA vector nanoparticles were transfected to RAW 264.7 cells without oligofectamin

To compare the efficiency of delivery of DNA vector between the chitosan-DNA vector mixture and chitosan-DNA vector nanoparticles, pCIN-mIL4 DNA vector dissolved in sodium sulfate was mixed with 50 kDa chitosan-acetic acid solution, and then transfected to RAW 264.7 cells without oligofectamin. After 48 h, the cells were harvested and analyzed for IL-4 expression by RT-PCR. Chitosan-pCIN-mIL4 DNA nanoparticle transfected cells strongly expressed IL-4 mRNA when compared to the pCIN control vector, chitosan-pCIN nanoparticle, pCIN-mIL4 vector and chitosan transfected groups (Figure 3). The mRNA expression of IL-4 did not differ between chitosan-pCIN-mIL4 mixture and chitosan-pCIN-mIL4 nanoparticle group. There was also no difference between groups treated with 1 μg and 2 μg of pCIN-mIL4 DNA-chitosan nanoparticles. The efficiency of delivery of the DNA vector was similar between the chitosan-DNA vector mixture and chitosan-DNA vector nanoparticles.

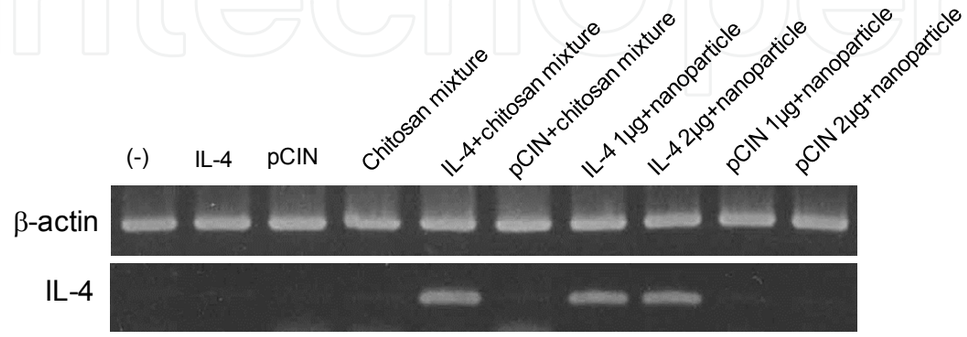

Fig. 3. Comparison of the chitosan-pCIN-mIL4 DNA vector mixture and chitosan-pCIN-mIL4 DNA vector nanoparticles in *in vitro* transfection of IL-4 vector by RT-PCR.

3.4 Chitosan-pCIN-mIL4 DNA vector mixture was orally administered to normal mice

After oral administration of the 50 kDa chitosan-pCIN-mIL4 DNA vector mixture to normal mice, the serum level of IL-4 was detected by ELISA (n=5 per each bar). Two doses of chitosan-pCIN-mIL4 DNA mixtures (5 μg DNA vector + 15 μg chitosan/mouse, 10 μg DNA vector + 30 μg chitosan/mouse) were applied to normal mice for two consecutive days. In addition, DNA vector (5 μg, 10 μg DNA vector/mouse) or chitosan (15 μg, 30 μg chitosan/mouse) was separately administered to mice as controls. The mice were sacrificed on day 5 after the last administration, and the sera were collected and subjected to ELISA. In addition, the intestinal tissues were isolated on day 2 and 5 after treatment and analyzed for the presence of IL-4 mRNA in the 10 μg DNA vector + 30 μg chitosan/mouse treated group. Administration of the 10 μg pCIN-mIL4 DNA vector + 30 μg chitosan mixture led to a significant increase in the serum level of IL-4 (10.25±2.9 pg/ml) when compared to mice that received 10 μg pCIN-mIL4 DNA vector alone (7.0±0.99 pg/ml; p=0.035) (Figure 4A). The

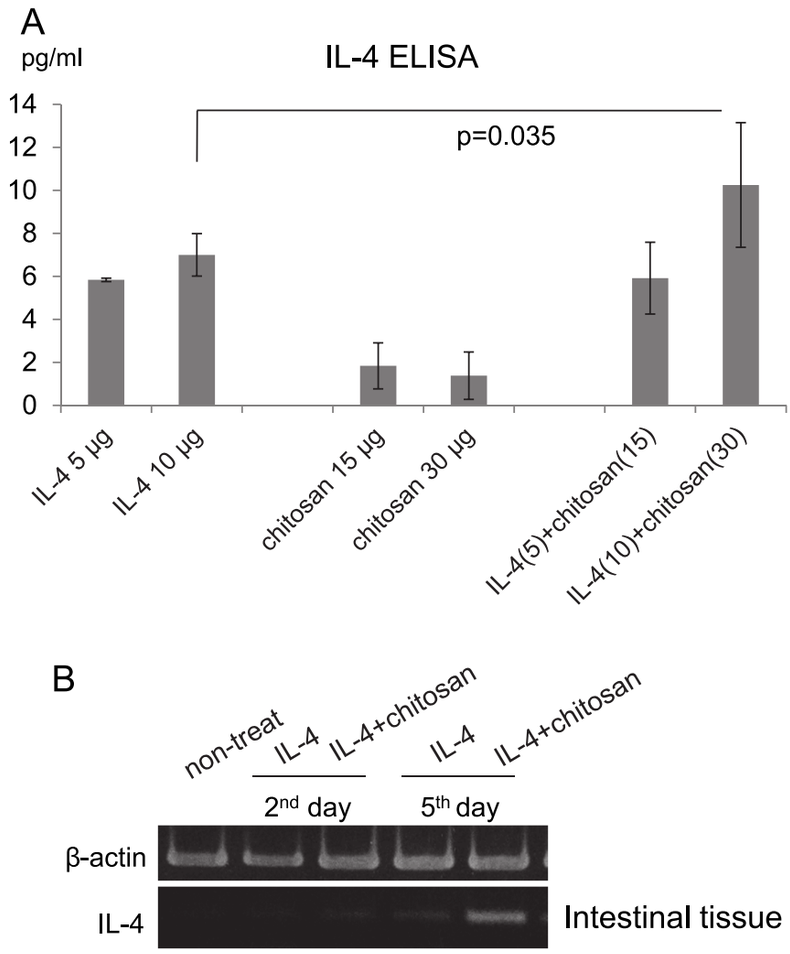

Fig. 4. Oral administration of the chitosan-pCIN-mIL4 DNA vector mixture to normal mice up-regulated the serum level of IL-4 and IL-4 mRNA expression in intestinal tissues.

intestinal tissues isolated on day 2 and 5 after administration showed increased IL-4 mRNA expression when compared to non-treated mice (Figure 4B). Specially, IL-4 mRNA was

strongly expressed on day 5 after administration of the chitosan-pCINmIL4 DNA vector mixture when compared to the pCINmIL4 DNA vector administered group.

3.5 Confocal microscopy in intestinal tissues of normal mice treated with chitosan-pcDNA-EGFP-mIL4 DNA vector mixture

To confirm the aforementioned results microscopically, the pcDNA-EGFP-mIL4 DNA vector was mixed with chitosan, after which 20 μg of the mixture was administered once a day for two consecutive days. At 10 and 20 days after last administration, the intestinal tissues were isolated and cryosectioned for subsequent observation under the confocal microscope. GFP was strongly observed in the intestinal tissue at day 10 after administration and remained day 20 (Figure 5). GFP was not detected in the mice treated with chitosan or pcDNA vector alone, nor was it detected in the mice treated with pcDNA-EGFP-mIL4 DNA vector.

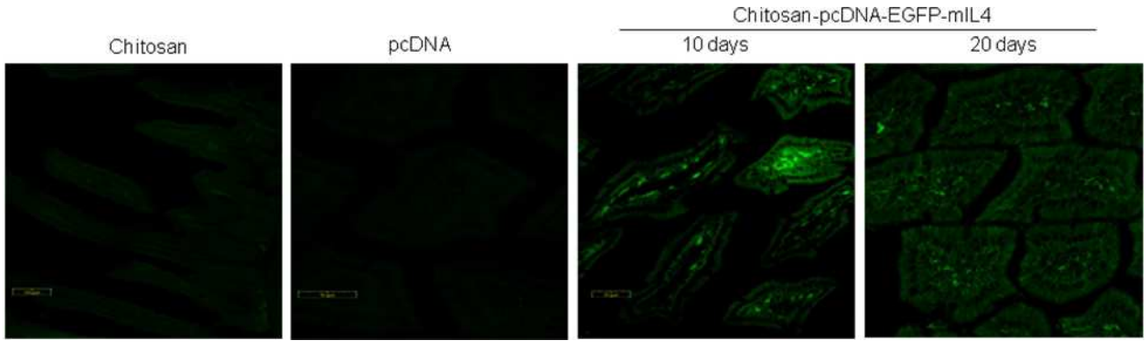

Fig. 5. Oral administration of the chitosan-pcDNA-EGFP-mIL4 DNA vector mixture to normal mice resulted in GFP in the intestinal tissues as determined by confocal microscopy.

3.6 Confocal microscopy in intestinal tissues of chitosan-pcDNA-EGFP-mIL4 DNA vector mixture treated Behcet's Disease (BD)-like mice

To confirm the aforementioned results microscopically and determine if the treatment could improve herpes simplex virus induced inflammatory BD symptoms, 20 μg of pcDNA-EGFP-mIL4 DNA vector was mixed with chitosan, and then administered to BD mice orally once a day for three consecutive days. At day 20 after the last administration, the intestinal tissues were isolated and cryosectioned for subsequent observation under the confocal microscope. GFP was strongly observed in the intestinal tissue (Figure 6A). However, GFP was not detected in the chitosan or pcDNA vector treated mice. Moreover, 20 days after last administration, the change in cutaneous ulceration of BD symptoms was photographed and compared to the symptoms before treatment. The symptoms improved after the treatment (Figure 6B), and the increased IL-4 level likely influenced this improvement.

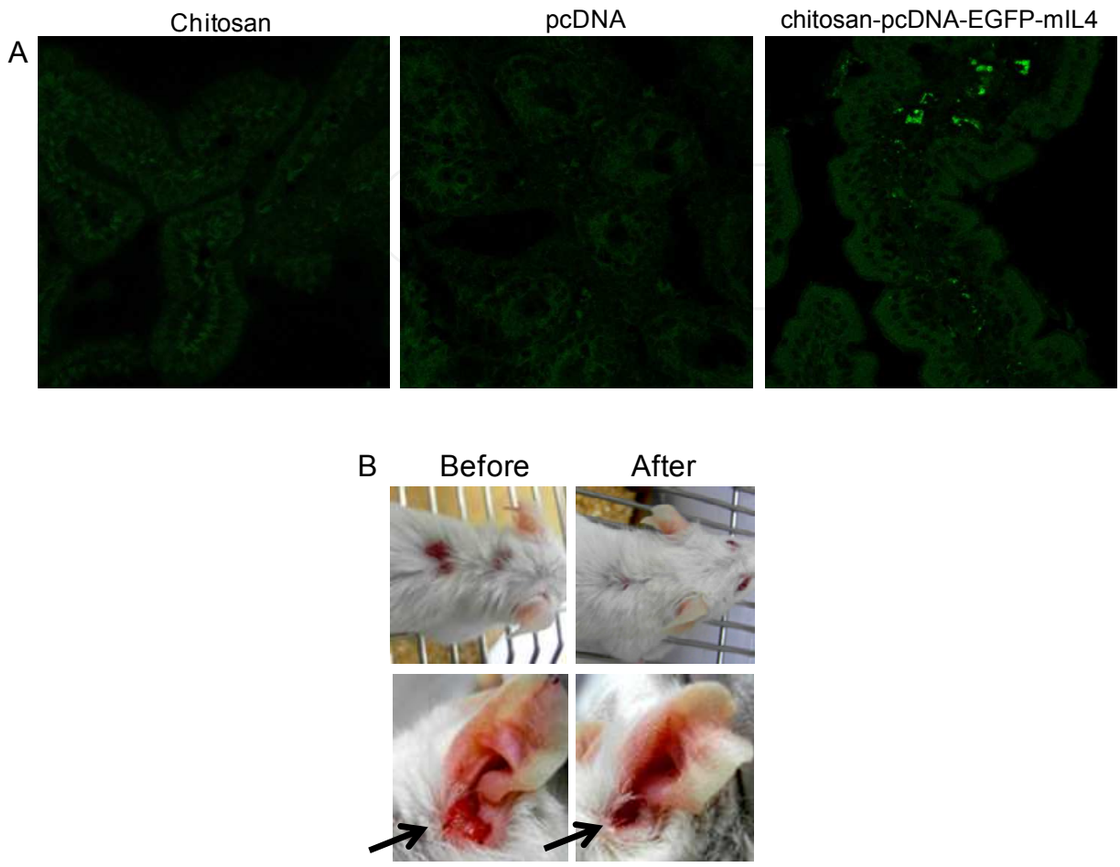

Fig. 6. Oral administration of the chitosan-pcDNA-EGFP-mIL4 DNA vector mixture to Behcet's disease mice resulted in GFP in the intestinal tissues (A) and improved the cutaneous symptoms of BD (B).

4. Discussion

In recent years, gene transfer technology has been shown to increase and evolve (Evans, 2004); however, the development of an effective non-viral gene delivery system has not yet been accomplished (Fernandes et al., 2000). Chitosan can encapsulate DNA vector to form complexes that are absorbed to the cell surface. The complexes are then endocytosed and transported to the nucleus by escaping the endosomal and lysosomal systems (Ishii et al., 2001). There are several indications that the transfection activity of chitosan-DNA vector complexes is dependent on the cell line being transfected (Erbacher et al., 1998). Several factors, including the molecular mass of chitosan, plasmid concentration, stoichiometry of the complex, serum concentration and pH of the transfection medium, can affect the transfection activity and cell uptake (Ishii et al., 2001). In the present study, we used chitosan with three different molecular weights as nanoparticles for *in vitro* transfection

experiments. Among the treatments, the 50 kDa chitosan was the most effective agent for delivery of pCIN-mIL4 vector into the RAW 264.7 cells.

Oral delivery is attractive due to factors such as ease of administration, convenience to the patient, and improved compliance (Tighe et al., 1998). The oral delivery of biomaterials such as peptides, proteins, DNA vectors, and siRNA is the greatest challenge facing the drug delivery system. Chitosan is a natural cationic polysaccharide obtained from deacetylation of chitin that is found in crustacean shells. Chitosan is a biocompatible, non-toxic, biodegradable and mucoadhesive polymer, with the ability to form gels at low pH. In addition, the degradation of chitosan occurs via the microflora available in the colon. These properties could provide a basis for the preparation of controlled release formulations, particularly for colon-specific drug delivery (Tavakol et al., 2009; Hejazi & Amiji, *2003*). Chitosan has been shown to increase the transcellular and paracellular transport of macromolecules across intestinal epithelial monolayers (Angelova & Hunkeler, 2001). Chitosan has more recently been used successfully to deliver a reporter gene encoding chloramphenicol acetyl transferase orally to enterocytes, Peyer's patches and mesenteric lymph nodes (MacLaughlin et al., 1998). Roy et al. confirmed the effectiveness of orally delivered chitosan-DNA nanoparticles for inducing protective immunity in the peanut allergy mouse model (Roy et al., 1999).

In the present study, we constructed a chitosan-pCIN-mIL4 DNA vector mixture. Our results demonstrated that the chitosan-pCIN-mIL4 mixture was able to deliver DNA vector to the cell and that oral administration of chitosan-pCIN-mIL4 mixture increased the IL-4 mRNA level and serum protein level of IL-4 significantly when compared to treatment with pCIN-mIL4 alone. The administration of chitosan pCIN-mIL4 DNA vector to mice *in vivo* more strongly increased the mRNA expression in intestinal tissues and protected against degradation until DNA vector reach tothe intestinal tissue. GFP intensity was still very strong in the intestinal tissues when compared to DNA vector alone.

In summary, our results demonstrate that chitosan is a good candidate for the development of novel gene delivery systems. Treatment with an oral chitosan- pCIN-mIL-4 mixture can lead to expression of IL-4 mRNA and protein in intestinal tissues and increased serum levels of IL-4. Chitosan can encapsulate and protect pDNA, enabling effective transfer of the GFP gene into cells *in vivo*. Thus, chitosan-DNA vector mixture could be a promising method for gene therapy by oral administration.

5. Acknowledgements

This work was supported from the National Research Foundation of Korea (NRF) by the Ministry of Education, Science and Technology (2010-0011130) and the Korean Health Technology R&D Project, Ministry for Health, Welfare & Family Affairs, Republic of Korea. (A100535).

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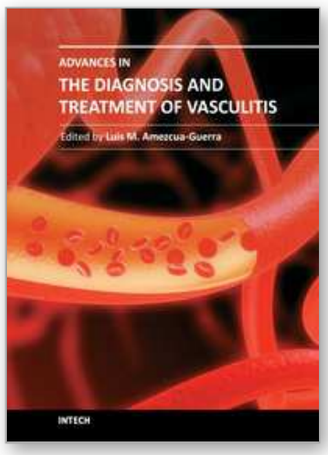

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Edited by Dr. Luis M Amezcua-Guerra

ISBN 978-953-307-786-4
Hard cover, 366 pages
Publisher InTech
Published online 09, November, 2011
Published in print edition November, 2011

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Bunsoon Choi, Ju Young Choi, Do-Hyun Jo and Seonghyang Sohn (2011). Oral Delivery of DNA Vector Conjugated with Chitosan and Its Effect on Th1 Polarized Inflammation, Advances in the Diagnosis and Treatment of Vasculitis, Dr. Luis M Amezcua-Guerra (Ed.), ISBN: 978-953-307-786-4, InTech, Available from: http://www.intechopen.com/books/advances-in-the-diagnosis-and-treatment-of-vasculitis/oral-delivery-of-dna-vector-conjugated-with-chitosan-and-its-effect-on-th1-polarized-inflammation

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InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821

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