



The immunologic effect of TGF-beta1 chitosan nanoparticle plasmids on ovalbumin-induced allergic BALB/c mice

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Abstract

Studies suggested that an increased transforming growth factor-beta (TGF-beta) level in the intestinal tract helps to alleviate symptoms of food allergy. An efficient method for *in vivo* delivery of TGF-beta is yet to be developed. In this report, we investigated some DNA-based therapeutic strategies. Our results demonstrate that a sustainable increase of TGF-beta protein in mouse intestinal tissue can be induced after oral administration by gavage of a TGF-beta expressing DNA vector that was packed in chitosan nanoparticles. Concomitantly, a significant amelioration of ovalbumin-induced food allergy symptoms was observed. Thus, our study supports this therapy being perhaps a better alternative to the previously reported protein-based strategies.

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Keywords: Chitosan; Food allergy; Plasmid; TGF-beta; Treatment

Introduction

Food allergy represents a considerable public health problem, especially in children, because of the potential severity of allergic hypersensitivity. It is defined as a

'mucosal disease' in the gut that is mainly associated with aberrant Th2-type immune responses and oral tolerance breakdown to food antigens.

Recent studies show that transforming growth factor-beta (TGF-beta) is likely the most important multi-functional cytokine in influencing mucosal T-cell responses which regulate cell growth, differentiation, and survival (Li et al., 2007; von Boehmer, 2007). TGF-beta plays a pivotal role in the induction and maintenance of oral tolerance by facilitating the IgA secretion and dependent conversion of naive T cells into regulatory T (T reg) cells, and also directs T reg cell homing to the gut (von Boehmer, 2007). To date, it has been demonstrated that the T reg cells in the intestinal mucosa have down-regulatory properties for Th1 cells and therefore play an important role in the oral

Abbreviation: Ct, threshold cycle; OVA, ovalbumin; OVA-sIgA, OVA-specific IgA; OVA-sIgE, OVA-specific IgE; RT-PCR, reverse transcription polymerase chain reaction; TGF-beta, transforming growth factor-beta.

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tolerance induction and the IgE response suppression. Epidemiological evidence also supports that TGF- β 1 is a likely decisive factor in diminishing the risk of allergic diseases during infancy partly because oral tolerance to food antigens could be enhanced by TGF- β 1 in early neonatal life (Kalliomaki et al., 1999; Oddy et al., 2003; Okamoto et al., 2005). It was reported that the risk of wheezing at 1 year of age was significantly decreased with increasing TGF- β 1 dose received during breast-feeding (Oddy et al., 2003). More recent studies illustrated that orally administered TGF- β 1 protein had the capacity to modulate food allergy by reducing the total IgE levels and inhibiting the anaphylactic reaction (Kalliomaki et al., 1999; Oddy et al., 2003; Okamoto et al., 2005; Ando et al., 2007). It provides a potential strategy to ameliorate food allergy by increasing the TGF- β 1 level (Okamoto et al., 2005; Bellinghausen et al., 2006; Ando et al., 2007; Wan and Flavell, 2007). This approach, albeit in some cases successful, often requires frequent oral intakes of high dosage of TGF- β 1 (Okamoto et al., 2005). Moreover, because of the fairly speedy clearance of the cytokine *in vivo*, the effective TGF- β 1 protein concentration may decrease relatively quickly and thus varies drastically between administrations (Mowat, 1999; Okamoto et al., 2005; Howell and McAnulty, 2006). The latter, however, limits the efficiency of TGF- β 1 protein-based therapy.

Here, we explored the oral administration by gavage of TGF- β 1 expressing DNA as an alternative method, postulating that by having the cells express TGF- β 1 following *in vivo* transfection, the protein level would remain stable for a relatively long period of time and thereby decrease the demand for frequent drug intake. To improve the delivery of DNA, we packaged the DNA with nanoparticles of chitosan, a naturally occurring polysaccharide. Chitosan is a widely available, mucoadhesive polymer that is able to increase cellular permeability and improve the bioavailability of orally administered protein medication (Roy et al., 1999). Studies showed that DNA condensed and entrapped within chitosan nanoparticles could be better protected from degradation. Moreover, chitosan, which is non-immunogenic, can readily adhere to the intestinal walls and be endocytosed by cells in the gastrointestinal tract, making it an especially attractive vehicle to treat mucosal immunological diseases. We compared different approaches to delivering TGF- β 1 DNA. The observations strongly support the legitimacy of chitosan-mediated DNA-based TGF- β 1 treatments in ameliorating food allergy.

Materials and methods

Plasmid constructs

Plasmid pcDNA3.1⁽⁺⁾/TGF- β 1 was kindly provided by Dr. Shan Jiang from Shanghai Jiao Tong

University, Shanghai, China. Eukaryotic expression plasmid pBudCE4.1 was purchased from Invitrogen, Inc. (Carlsbad, CA). To construct the TGF- β 1 expression vectors, a *Hind*III and *Bam*HI fragment from pcDNA3.1⁽⁺⁾/TGF- β 1, which contains the full-length mouse TGF- β 1 cDNA, was subcloned into pBudCE4.1. The latter vector contains two promoters which facilitated the construction of a co-expression plasmid containing TGF- β 1 and another gene that is currently being studied in another project (data not shown). The constructs were sequenced and then further confirmed against published sequences in the GenBank database. All plasmid DNA was prepared using a QIAGEN kit following the instructions provided by the manufacturer.

Cell culture and transient transfection

COS-7 cells (American Type Culture Collection) were grown in Dulbecco's modified Eagle's medium (DMEM; Invitrogen), supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin, at 37 °C in 5% CO₂ (Zhao et al., 2002). Transfection of COS-7 cells with pBudCE4.1/TGF- β 1 or control pBudCE4.1 plasmid was performed with lipofectamine (Invitrogen) as described previously (Zhao et al., 2002). Cells and cell supernatant were collected 48 h after transfection.

Reverse transcriptase-PCR (RT-PCR) analyses

TRIzol reagent (Invitrogen) was used to prepare RNA from COS-7 cells which were transfected with either pBudCE4.1/TGF- β 1 or control pBudCE4.1 plasmid (vector control). RNA was then further purified using DNase and RNA Secure (Ambion, Austin, TX) to remove contaminating DNA and to inactivate RNases. The RNA purity and quality were assessed by inspection of the 18S and 28S rRNA bands using agarose gel electrophoresis. The Stratascript II reverse transcription kit (Invitrogen) was used with oligo dT primers to reverse-transcribe 5 μ g of total RNA into cDNA. RT-PCR analysis was performed as described (Nakayama et al., 1992). The products (0.2 μ g each) were assayed by PCR with the TGF- β 1 primers listed in Table 1. The 10 μ l PCR mixture includes 50 mM KCl, 1.5 mM MgCl₂, 0.001% [w/v] gelatin, 10 mM Tris-HCl, (pH 8.3), 200 μ M dNTPs, 150 μ M of each primer, and 0.5 units of TaqDNA polymerase (Applied Biosystems, Foster City, CA). The PCR conditions are summarized in Table 2. The resulting PCR products were analyzed using 6% acrylamide gels.

Table 1. Primers for real time quantitative RT-PCR

Amplified gene	Primers	
	Sequence	Length of amplified fragments (bp)
β -Actin		
Upstream	5' GTCCCGGCCAGCCAGGTCCAG 3'	210
Downstream	5' CCTAAGGCCAACCGTGAAAAGATG 3'	
TGF- β 1		
Upstream	5' GCC CTG GAT ACC AAC TAT TGC 3'	285
Downstream	5' GCA GGA GCG CAC AAT CAT GTT 3'	
IFN- γ		
Upstream	5' GGC TGT TTC TGG CTG TTA CTG C 3'	233
Downstream	3' CGC TTA TGT TGT TGC TGA TGG 3'	
IL-4		
Upstream	5' TAG TTG TCA TCC TGC TCT T 3'	359
Downstream	5' GTC TTT CAG TGA TGT GGA C 3'	

Western blot analyses

Culture supernatants from transfected COS-7 cells were collected and proteins were extracted as described (Okamoto et al., 2004). After separation by SDS-PAGE, proteins were electrotransferred to a polyvinylidene difluoride membrane (Hybond P). The membrane was blocked with phosphate-buffered saline (PBS), containing 0.1% Tween 20 and 5% dried milk (buffer C), and then incubated with polyclonal anti-TGF- β 1 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) in buffer C at room temperature for 1 h, followed by incubation with the horseradish peroxidase (HRP)-labeled secondary antibody (1:2000; KPL, Gaithersburg, MD) in buffer C at room temperature for 1 h. Finally, the TGF- β 1 band was visualized using enhanced chemiluminescence.

Preparation of chitosan–DNA nanoparticles

Chitosan (deacetylation degree: 90%, molecular weight: 305 kDa, Golden-Shell Biochemical Co., Ltd., Zhejiang, China) was dissolved in acetic acid, adjusted to pH 5.7 with NaOH, diluted with sterile ddH₂O to a final concentration of 0.02% (w/v), sterile-filtered (0.122 μ m micropore diam.), and stored at 4 °C overnight. An equal volume of chitosan solution was added to the plasmid DNA solution (200 μ g/ml) containing 45 mmol/L Na₂SO₄, and mixed, and then incubated in a 55 °C water bath for 20 min. The mixtures were vortexed (2500 rpm) for 30 s, and placed at room temperature for 30 min before *in vivo* administration. The DNA encapsulation rate and mean diameter of the nanoparticles examined under a phase contrast microscope (Nikon,

Tokyo, Japan) were higher than 95% and 200 nm, respectively.

Animals and groups

Six-week-old female BALB/c mice fed on ovalbumin (OVA)-free diet (provided by the Shanghai Animal Center of the Chinese Academy of Sciences) were sensitized intraperitoneally and challenged orally with OVA, as described previously (Knippels et al., 1998; Li and Li, 2006). All mice were randomly allocated into six groups (20 mice each), including five experimental groups and one no intervention control group (negative control, NC). Mice in the five OVA-allergic groups received one of the following treatments, respectively: oral chitosan/pBudCE4.1/TGF- β 1 plasmid (eTGfO, encapsulated TGF- β , oral), intramuscular pBudCE4.1/TGF- β 1 plasmid (nTGfM, naked TGF- β , im), oral naked pBudCE4.1/TGF- β 1 plasmid (nTGfO, naked TGF- β , oral), oral TGF- β 1 protein (prTGfO, Recombinant Human Latent TGF- β 1 Protain, oral) (R&D, Minneapolis, MN), and oral encapsulated empty plasmid pBudCE4.1 (eVo, encapsulated empty vector, oral) as positive control for OVA-allergic mice. Sensitization and challenge were performed as following: 10 μ g of OVA (Sigma-Aldrich, St. Louis, MO) and 1 mg of Al(OH)₃ in 0.5 ml of sterile normal saline was injected intraperitoneally on the 1st day. On the 15th day, a second intraperitoneal injection of 0.5 ml of sterile normal saline solution containing 10 μ g of OVA was given as the enhanced sensitization. On the 30th and 45th days, the gut was challenged repeatedly with 0.5 ml of OVA (1 mg/ml) in normal saline orally. On the 15th, 30th, and 45th days, mice

Table 2. Conditions of real-time quantitative amplification by LightCycler PCR

Gene	Pre-denaturation			Denaturation			Annealing			Extension			Number of cycles
	Temperature (°C)	Duration (s)	Variation rate of temperature (°C/s)	Temperature (°C)	Duration (s)	Variation rate of temperature (°C/s)	Temperature (°C)	Duration (s)	Variation rate of temperature (°C/s)	Temperature (°C)	Duration (s)	Variation rate of temperature (°C/s)	
β	94	120	20	94	30	20	60	30	20	72	12	2	40
TGF- β	94	120	20	94	30	20	61	30	20	72	20	2	40
β													
IFN- γ	94	120	20	94	30	20	63	30	20	72	20	2	40
IL-4	94	120	20	94	30	20	59	30	20	72	20	2	40

were simultaneously treated by TGF- β 1 plasmid or protein, respectively, after enhanced sensation and challenge. The volume for the treatment was 0.5 ml per mouse; the DNA contents was 50 μ g per mouse and the protein amount was 5 μ g per mouse as described (Okamoto et al., 2004). On the 45th day, 1 h after the last treatment with TGF- β 1, 50% of the mice (10 mice per group) were sacrificed by cervical dislocation, and the Peyer's Patch and lavage fluids in the small intestine, and peripheral serum samples were collected and immediately frozen and stored. On the 59th day, the remaining mice (10 mice per group) were sacrificed 1 h after the repeated OVA challenge (1 mg/ml) orally without subsequent TGF- β 1 administration. The samples were collected and stored as described above. All experimental protocols were approved by the Shanghai Jiaotong University, Animal Subjects Committee of Shanghai Jiaotong University.

Immunohistochemistry

The jejunum was harvested and immediately immersed in 10% PBS (pH 7.4) buffered formalin for 3 h at room temperature. After dehydration through a gradient series of ethanol solutions, the tissue samples were embedded in low-melting paraffin. To inactivate endogenous HRP, sections (3 μ m in thickness) were incubated for 40 min in methanol and 3% hydrogen peroxide solution, then washed in PBS and incubated overnight at 4 °C with polyclonal antibodies against TGF- β 1 (1:200 in PBS; Santa Cruz Biotechnology). The sections were washed with PBS for 5 min, and then incubated with HRP-labeled secondary antibody (1:2000; KPL). After 10 min wash with PBS, the sections were incubated for 1–3 min with 3,3'-diaminobenzidine-tetrahydrochloride (DAB; 1 drop of DAB + 1 ml of H₂O₂; Sigma-Aldrich). To verify the detection specificity, the primary antibody was omitted, and the sections were incubated with the secondary antibody alone. Finally, the sections were counterstained with Mayer's haematoxylin and examined under an Olympus BX51 photomicroscope (Olympus Optical Co., Tokyo, Japan).

Assessment of TGF- β 1, IFN- γ , and IL-4 mRNA expression in Peyer's Patch of the small intestine

Quantitative real-time RT-PCR was performed using a Roche LightCycler RNA Master SYBR Green I kit (for one-step RT-PCR) and a Roche LightCycler Instrument (Roche, Shanghai, P.R. China) (Li et al., 2005). The internal control was beta-actin. Primer sequences and amplification conditions are listed in

Tables 1 and 2. The threshold cycle (Ct) value was calculated from the fluorescence signal ratio using a second derivative method (SDM), provided by the LightCycler software, version 3.5. A negative control was set by replacing sample template with DNA-free sterile distilled water. Every sample was assayed twice. Quantitative real-time RT-PCR was used to measure the expression of TGF-beta1, IFN-gamma, IL-4 mRNAs in Peyer's Patch in small intestine. Therefore, Peyer's Patches were collected and the tissues were used for cDNA preparation and quantitative RT-PCR. RNA solution was first diluted to 400 ng/μl, then 2-fold serially to 200, 100, 50, and 25 ng/μl, respectively. The diluted samples were then used as templates for PCR amplification. Each product dissolution curve showed a single peak, indicating that a single fragment was amplified with no dimerized primers. Agarose (2%) gel electrophoresis confirmed that the product was of the correct size. Slope, coefficient (r), intercept, amplification efficiency ($E = 10^{(-1/\text{slope})}$), and standard curve of mRNA amplification were obtained by LightCycler software: $Y/\text{or } Ct_{\text{target gene}} = \text{slope} \times X_{\text{target gene}} + a_{\text{target gene}}$, $Y/\text{or } Ct_{\text{internal control gene}} = \text{slope}_{\text{internal control gene}} \times X_{\text{internal control gene}} + b_{\text{internal control gene}}$. $a_{\text{target gene}}$ or $b_{\text{internal control gene}}$ is referred to intercept. Y was the Ct value of the amplified gene; $\log X$ was the mRNA copy number of the amplified gene. mRNA value of target gene ($\text{mRNA}_{\text{target gene}}$) was normalized to the value for beta-actin mRNA ($\text{mRNA}_{\text{internal control gene}}$) (formula (1) and (2)). The log ratio (i.e. ratio of $\text{mRNA}_{\text{target gene}}/\text{mRNA}_{\text{internal control gene}}$) was used to compare samples from different groups (Table 3).

$$\text{mRNA}_{\text{target gene}} = 10^{\left(\frac{Ct_{\text{target gene}} - a_{\text{target gene}}}{\text{slope}_{\text{target gene}}} \right)} \quad (1)$$

$$\begin{aligned} &\text{mRNA}_{\text{internal control gene}} \\ &= 10^{\left(\frac{Ct_{\text{internal control gene}} - b_{\text{internal control gene}}}{\text{slope}_{\text{internal control gene}}} \right)} \end{aligned} \quad (2)$$

Assays of total IgA and OVA-specific IgA (OVA-sIgA) in intestinal lavage fluids

Total IgA and OVA-sIgA in intestinal lavage fluids were assayed using ELISA (Li and Li, 2006). All mice were starved for 12 h and then sacrificed. Under general anesthesia a blunt-ended tube (10 cm long $\times$ 2 mm in diameter) was inserted into the anus and advanced 5–6 cm into the small intestine. Sterile 0.01 M PBS (2.0 ml) was injected and the wash from the small intestine (i.e., the spontaneous flow of this fluid from the anus over 1 min) was collected and stored at -20°C . The total IgA was assayed using rat-anti-mouse IgA monoclonal antibody (BD Pharmingen, San Diego, CA) as the primary coating antibody and HRP-labeled rat-anti-mouse IgA (Southern Biotech Associates Inc., Birmingham, AL) as the detecting secondary antibody. To test OVA-sIgA, undiluted samples were added to OVA (1 μg/ml) coated plate, followed by incubation with HRP-labeled rat-anti-mouse IgA secondary antibody. The substrate 3,3',5,5'-tetra-methyl-benzidine (TMB) was used for color development. The OD₄₅₀ values were read by an automated reader, and then analyzed statistically.

Assay of OVA-specific IgE (OVA-sIgE) in serum

OVA-sIgE was assayed using ELISA (Li et al., 2000). Undiluted serum samples were assayed with OVA-coated plates (10 μg/ml; 100 μl OVA/well) and HRP-labeled rat-anti-mouse IgE (1:1000) as the secondary antibody (Southern Biotechnology Associates Inc., Birmingham, AL). After color development with TMB, OD₄₅₀ values were measured and analyzed.

Assay of residual histamine in the small intestine

A tissue fluorescence method was used to examine a 5 cm length of small intestine collected from below the

Table 3. Relevant parameters for standard curve of each gene by real-time quantitative RT-PCR

Amplified gene	Slope	Intercept	Coefficient	Amplification efficacy	Range of Ct value
β-Actin	−3.37	11.70	−0.99	1.98	12–29
TGF-β1	−3.40	8.35	−0.99	1.97	6–27
IFN-γ	−3.45	8.30	−1.00	1.94	4–27
IL-4	−3.00	13.16	−0.99	2.07	8–29

The slope, coefficient (r), intercept, amplification efficiency ($E = 10^{(-1/\text{slope})}$), and range of Ct values were obtained by LightCycler software. The standard curve of mRNA amplification is $Y/\text{or } Ct_{\text{target gene}} = \text{slope} \times X_{\text{target gene}} + a_{\text{target gene}}$ or $Y/\text{or } Ct_{\text{internal control gene}} = \text{slope}_{\text{internal control gene}} \times X_{\text{internal control gene}} + b_{\text{internal control gene}}$. $a_{\text{target gene}}$ or $b_{\text{internal control gene}}$ is referred to intercept. Y was the Ct value of the amplified gene; $\log X$ was the mRNA copy number of the amplified gene. mRNA value of target gene ($\text{mRNA}_{\text{target gene}}$) was normalized to the value for β-actin mRNA ($\text{mRNA}_{\text{internal control gene}}$). The log ratio ($\log \text{mRNA}_{\text{target gene}}/\text{mRNA}_{\text{internal control gene}}$) was used to compare samples from the control and experimental groups.

Treitz ligament (van Halteren et al., 1997). Increase in the amount of residual histamine in the small intestine tissues reflects reduction of the amount of histamine released by mast cells into the intestinal tract thus reduced damage of the intestinal tract.

Statistical analyses

SAS 8.0 statistical analysis package software was utilized. Levene's test was used to evaluate data between groups for homogeneity of variance. If the variance was homogeneous, the results were presented as mean $\pm$ SD ($\bar{x} \pm s$). Single-variable analysis of variance was then applied. If the difference between group means was significant, the Student–Newman–Keuls method (q test) was used to make pairwise comparisons between the mean values of groups. $P < 0.05$ was set as the threshold for significance. Levene's test showed that the data from quantitative RT-PCR analysis of relative mRNA expression level of each gene ($\text{mRNA}_{\text{target gene}} / \text{mRNA}_{\text{internal control gene}}$ Ratio) had inhomogeneous variance. Statistical analysis of this parameter was carried out on log-transformed (log ratio) data, which resulted in homogeneous variance.

Results

Construction and characterization of a TGF-beta1 expressing vector

Plasmid pBudCE4.1/TGF-beta1 was constructed to express TGF-beta1 in mouse tissue. Construction and characterization of the vector by restriction enzyme

digestion is shown in Fig. 1A. Briefly a 1.6 Kb *Hind*III and *Bam*HI fragment containing mouse TGF-beta1 cDNA was cloned into expression vector pBudCE4.1. The expression of TGF-beta1 was verified by DNA sequencing (data not shown).

To test the effectiveness of pBudCE4.1/TGF-beta1, we transfected COS-7 cells with the plasmid and analyzed TGF-beta1 mRNA expression by RT-PCR. As shown in Fig. 1B, a 285 bp PCR product was found in COS-7 cells transfected with pBudCE4.1/TGF-beta1 plasmid (Fig. 1B, lane 2), but no PCR product was found in COS-7 cells transfected with insert-free vector (Fig. 1B, lanes 3). TGF-beta1 protein expression was analyzed by Western blot analysis. Consistent with the RT-PCR results, a 25 kDa TGF-beta1 band was found in the culture supernatant of COS-7 cells that were transfected with pBudCE4.1/TGF-beta1 (Fig. 1C, lane 1), but not in that of COS-7 cells transfected with insert-free vector (Fig. 1C, lanes 2). These results confirm that pBudCE4.1/TGF-beta1 expresses TGF-beta1 in mammalian cells and that the expressed TGF-beta1 was secreted from cells into media.

Administration of pBudCE4.1/TGF-beta1 to BALB/c mice results in TGF-beta expression

TGF-beta expression was induced in mice by feeding the OVA-induced allergic mice with pBudCE4.1/TGF-beta1 plasmid by gavage that was encapsulated with chitosan nanoparticles to improve the stability and delivery efficiency. To evaluate the efficacy of oral delivery of chitosan-encapsulated pBudCE4.1/TGF-beta1 DNA (eTGfO), we used the following controls: oral administration of encapsulated empty plasmid

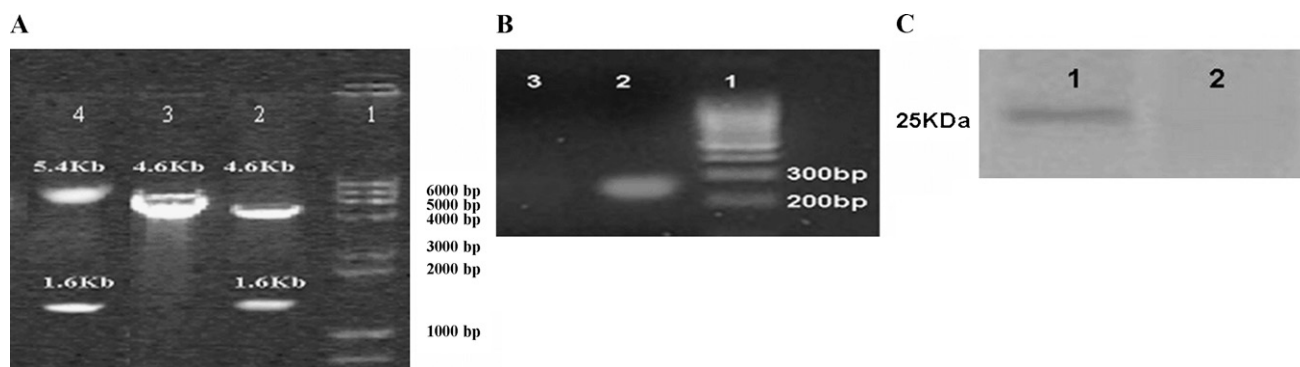


Fig. 1. pBudCE4.1/TGF-beta1 plasmid construction and TGF-beta1 expression in COS-7 cells. (A) Electrophoretic analysis of enzyme digests of pcDNA3.1⁽⁺⁾/TGF-beta1 and pBudCE4.1/TGF-beta1. Lane 1, DNA molecular size marker; lane 2, pBudCE4.1/TGF-beta1 digested by *Hind*III and *Bam*HI (4.6 kb and 1.6 kb); lane 3, empty plasmid pBudCE4.1 (4.6 kb); lane 4, pcDNA3.1⁽⁺⁾/TGF-beta1 digested by *Hind*III and *Bam*HI (5.4 kb and 1.6 kb). (B) RT-PCR analysis of transfected COS-7 cells. 285 bp PCR product is detected from COS-7 cells transfected with pBudCE4.1/TGF-beta1 plasmid (lane 2), no PCR products are detected from COS-7 cells transfected with insert-free vector (lane 3), molecular size marker is shown in the right (lane 1). (C) Western-blot analysis with polyclonal anti-TGF-beta1. 25 kDa band was detected from the cultural supernatant in COS-7 cells transfected with pBudCE4.1/TGF-beta1 (lane 4), no band was detected in the supernatants of COS-7 cells transfected with insert-free vector (lane 2), protein molecular weight marker is shown in the left (lane 1).

pBudCE4.1 (eVo) by gavage as an allergic positive control, oral administration of naked pBudCE4.1/TGF- β 1 plasmid (nTGfO) by gavage, intramuscular injection of pBudCE4.1/TGF- β 1 plasmid (nTGfM) and oral administration of TGF- β 1 protein (pTGfO) by gavage to compare efficiencies of different delivery approaches. We also used non-allergic wild-type mice as the negative control for the allergy symptom (NC group). As shown in Fig. 2, at day 45, significant TGF- β 1 expression was observed in the eTGfO, nTGfM, prTGfO and NC groups and mainly localized in the lymphocytes, macrophages, and epithelial cells of the intestinal villi. In the nTGfO and eVo group, however, the TGF- β 1 level was either greatly reduced or undetectable. Our results support those published in previous reports (Okamoto et al., 2005) in that the TGF- β 1 expression was decreased in OVA-allergic mice. The latter decrease was effectively rescued by the administration of TGF- β 1 DNA encapsulated with chitosan nanoparticles and protein. Because of the nature of two different methods, we compared the

temporal effects of TGF- β 1 deliveries, oral intake of chitosan-encapsulated TGF- β 1-DNA, and oral intake of TGF- β 1 protein. Two weeks after the final delivery (day 59 overall), TGF- β 1 expression was re-evaluated. While the TGF- β 1 protein level was drastically reduced in the prTGfO group where TGF- β 1 protein was delivered, in both the eTGfO and nTGfM group, a significant amount of TGF- β 1 expression can be readily detected with the highest level being present in the eTGfO group (Fig. 2). Thus, our data suggest that compared to the previously reported approaches involving oral intake of TGF- β 1, oral intake of chitosan-encapsulated TGF- β 1 DNA has the advantage of being significantly long lasting.

Expression of TGF- β 1, IFN- γ , and IL-4 mRNA in Peyer's Patches in the small intestine

TGF- β 1 expression was also monitored at the mRNA level by real time RT-PCR. We found that on

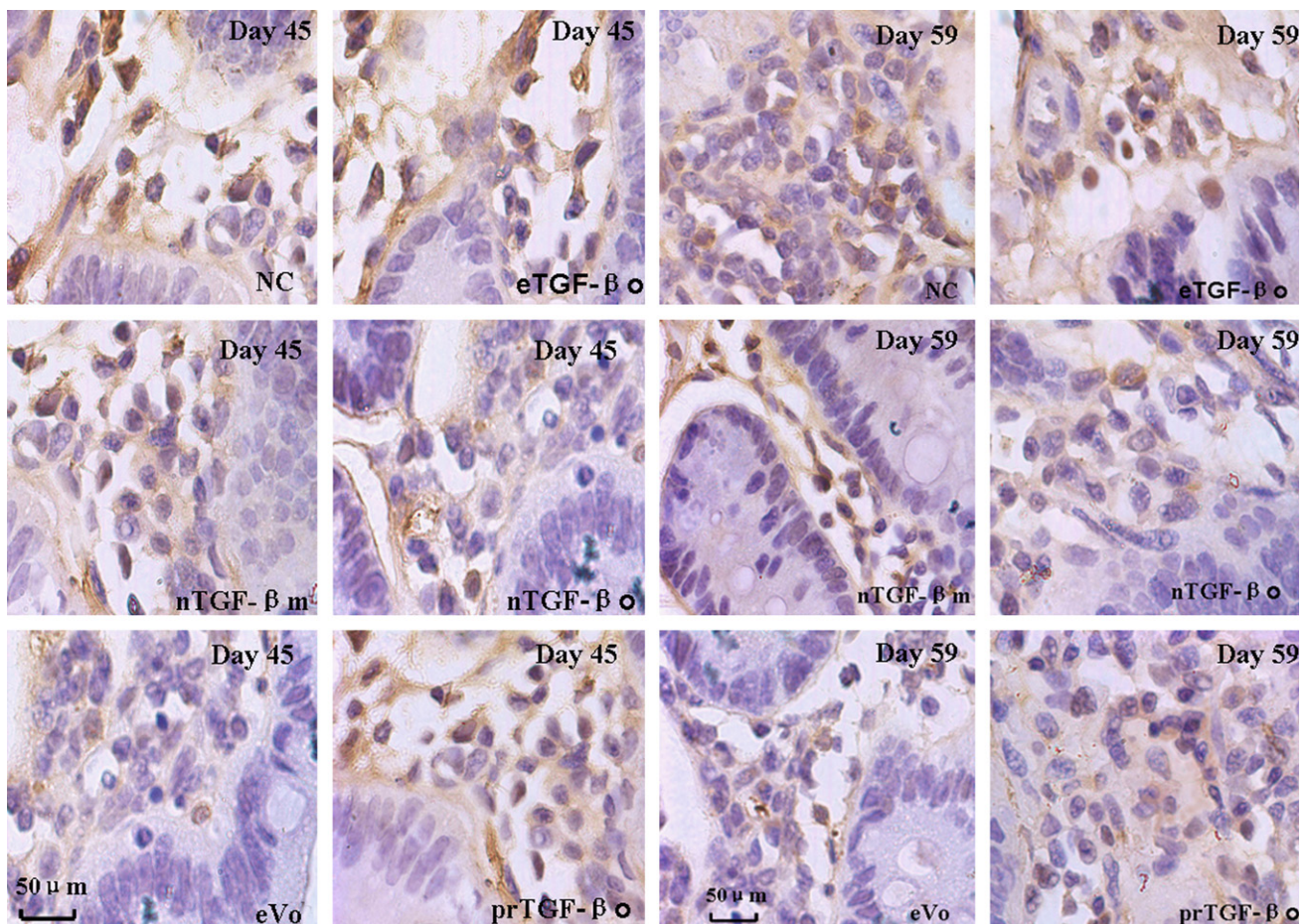


Fig. 2. TGF- β expression in the villus in different groups (10×100) (bar = $50 \mu\text{m}$). Immunoreactivity was mainly localized in the lymphocytes, macrophages, and epithelial cells of the intestinal villi. The pattern of TGF- β 1 staining was high in the eTGfO and nTGfM group, higher in eTGfO than in nTGfM group on both the 45th day, and the 59th day; TGF- β immunoreactivity in the prTGfO group was strongly positive on the 45th day, but significantly reduced on the 59th day.

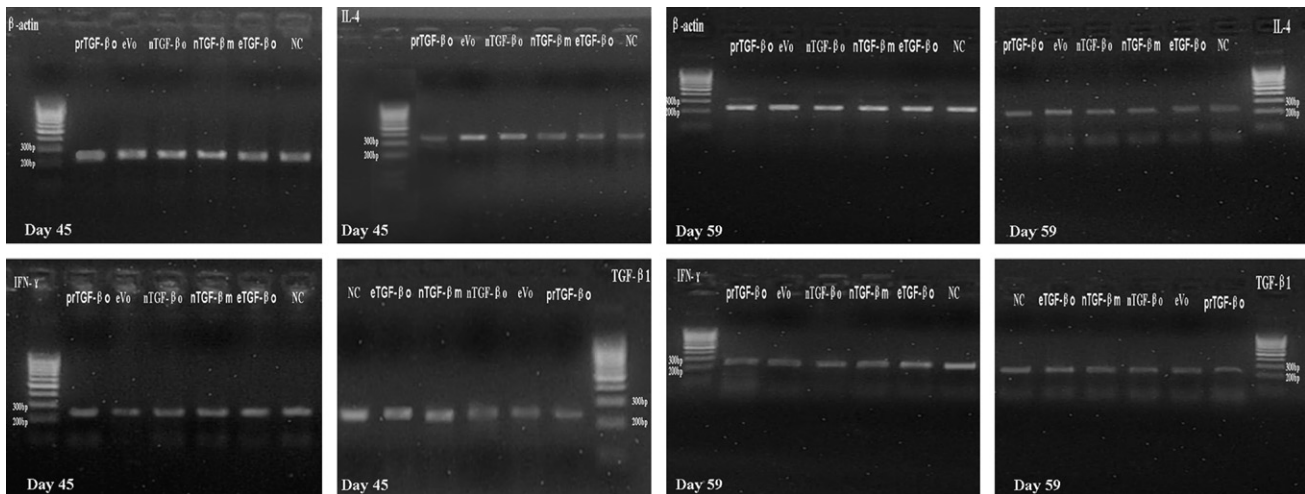


Fig. 3. Electrophoretic separation for identification of RT-PCR products of target genes in tissue of the small intestine. The IL-4 product was 359 bp, while the other gene products including IFN-gamma, TGF-beta1 and beta-actin were 200–300 bp.

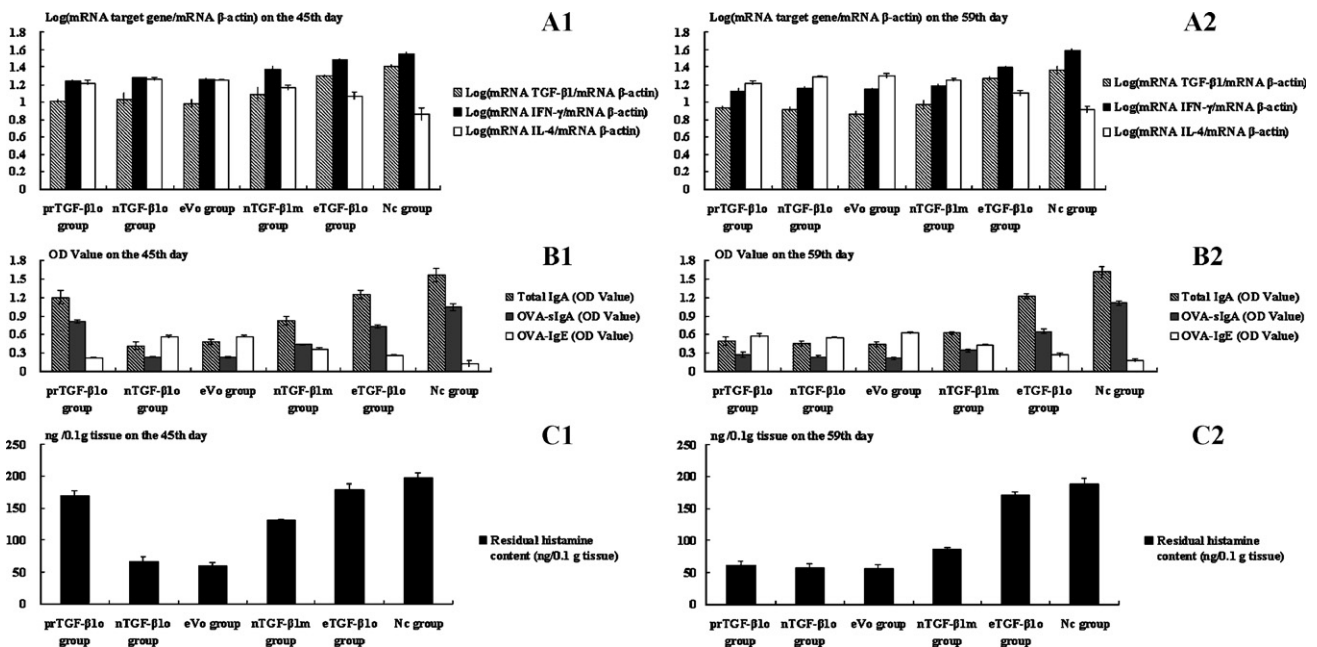


Fig. 4. Therapeutic effects of the different TGF-beta1 treatments for food allergy on the 45th day and the 59th day ($P < 0.05$ indicated significant different groups). Panel A1 and A2: quantitative real-time RT-PCR analysis of TGF-beta1, IFN-gamma, and IL-4 mRNA expression in Peyer's Patch of the small intestine. The TGF-beta1 and IFN-gamma expression level was $NC > eTGF\beta > nTGF\beta m > eVo = nTGF\beta = prTGF\beta$, respectively, whereas the IL-4 expression was $NC < eTGF\beta < nTGF\beta m < eVo = nTGF\beta = prTGF\beta$ on the day 45 (A1) and day 59 (A2), respectively. Panel B1 and B2: ELISA evaluation of total IgA and OVA-sIgA in intestinal lavage fluids and OVA-IgE in serum. On day 45 (B1), the total IgA and OVA-sIgA level was $NC > eTGF\beta = prTGF\beta > nTGF\beta m > eVo = nTGF\beta$, whereas the OVA-IgE level was $NC < eTGF\beta = prTGF\beta < nTGF\beta m < eVo = nTGF\beta$. On day 59 (B2), the total IgA and OVA-sIgA level was $NC > eTGF\beta > nTGF\beta m > eVo = nTGF\beta = prTGF\beta$, whereas the OVA-IgE level was $NC < eTGF\beta < nTGF\beta m < eVo = nTGF\beta = prTGF\beta$. Panel C1 and C2: fluorescence assay of residual histamine in intestinal tissue. On day 45 (C1), residual histamine was $NC > eTGF\beta = prTGF\beta > nTGF\beta m > eVo = nTGF\beta$, and on day 59 (C2), $NC > eTGF\beta > nTGF\beta m > eVo = nTGF\beta = prTGF\beta$.

both the 45th day and the 59th day, TGF-beta1 expression in the eTGF β , nTGF β m, and NC group was higher than those in the eVo, nTGF β , and prTGF β group with the NC group showing the highest level and

eTGF β group showing a higher level than that in the nTGF β m group (Figs. 3 and 4A1, A2, Tables 4 and 5). Corresponding to the TGF-beta1 expression, real time RT-PCR indicated a similar distribution of IFN-gamma

Table 4. Therapeutic effects of the different TGF- β 1 treatments for food allergy on the 45th day

Indices	nTFG- β 0 group	eVo group	nTFG- β m group	eTFG- β 0 group	NC group	prTFG- β 0 group
Log(mRNA _{TGF-β1} /mRNA _{β-actin})	1.03 $\pm$ 0.08 □▲●	0.98 $\pm$ 0.06 □▲●	1.14 $\pm$ 0.08 ■□▲●	1.30 $\pm$ 0.02 ■□□●	1.41 $\pm$ 0.01 ■□□▲	1.01 $\pm$ 0.03 □▲●
Log(mRNA _{IFN-γ} /mRNA _{β-actin})	1.28 $\pm$ 0.01 □▲●	1.26 $\pm$ 0.02 □▲●	1.37 $\pm$ 0.04 ■□▲●	1.48 $\pm$ 0.02 ■□□●	1.55 $\pm$ 0.01 ■□□▲	1.51 $\pm$ 0.02 ■◆□●
Log(mRNA _{IL-4} /mRNA _{β-actin})	1.26 $\pm$ 0.02 □▲●	1.25 $\pm$ 0.01 □▲●	1.16 $\pm$ 0.03 ■□▲●	1.07 $\pm$ 0.04 ■□□●	0.86 $\pm$ 0.07 ■□□▲	1.05 $\pm$ 0.03 ■◆□●
Total IgA (OD value)	0.41 $\pm$ 0.06 □▲●	0.47 $\pm$ 0.04 □▲●	0.82 $\pm$ 0.07 ■□▲●	1.25 $\pm$ 0.07 ■□□●	1.56 $\pm$ 0.11 ■□□▲	1.21 $\pm$ 0.15 ■□□●
OVA-sIgA (OD value)	0.24 $\pm$ 0.01 □▲●	0.23 $\pm$ 0.02 □▲●	0.43 $\pm$ 0.01 ■□▲●	0.72 $\pm$ 0.03 ■□□●	1.04 $\pm$ 0.05 ■□□▲	0.80 $\pm$ 0.03 ■□□●
OVA-sIgE (OD value)	0.56 $\pm$ 0.03 □▲●	0.57 $\pm$ 0.02 □▲●	0.36 $\pm$ 0.02 ■□▲●	0.26 $\pm$ 0.01 ■□□●	0.12 $\pm$ 0.02 ■□□▲	0.21 $\pm$ 0.03 ■□□●
Residual histamine content (ng/0.1 g tissue)	66.15 $\pm$ 8.03 □▲●	59.43 $\pm$ 5.28 □▲●	130.14 $\pm$ 2.08 ■□▲●	178.81 $\pm$ 8.99 ■□□●	197.68 $\pm$ 7.32 ■□□▲	170.08 $\pm$ 7.11 ■□□●

Compared with nTFG- β 0 group ■ $P<0.01$; with eVo group □ $P<0.01$; with nTFG- β m group □ $P<0.01$; with eTFG- β 0 group ▲ $P<0.01$; with NC group ● $P<0.01$; with prTFG- β 0 group ◇ $P<0.01$.

Table 5. Therapeutic effects of the different TGF- β 1 treatments for food allergy on the 59th day

Indices	nTFG- β 0 group	eVo group	nTFG- β lm group	eTFG- β lo group	NC group	prTFG- β lo group
Log(mRNA _{TGF-β1} /mRNA _{β-actin})	0.91 $\pm$ 0.04 □▲●	0.86 $\pm$ 0.04 □▲●	0.97 $\pm$ 0.06 □▲●	1.27 $\pm$ 0.03 ■□□●	1.36 $\pm$ 0.05 ■□□▲	0.93 $\pm$ 0.03 ▲●
Log(mRNA _{IFN-γ} /mRNA _{β-actin})	1.15 $\pm$ 0.03 ▲●	1.18 $\pm$ 0.02 ▲●	1.18 $\pm$ 0.04 ▲●	1.39 $\pm$ 0.02 ■□□●	1.59 $\pm$ 0.02 ■□□▲	1.12 $\pm$ 0.04 ▲●
Log(mRNA _{IL-4} /mRNA _{β-actin})	1.29 $\pm$ 0.01 □▲●	1.30 $\pm$ 0.03 □▲●	1.25 $\pm$ 0.02 □▲●	1.10 $\pm$ 0.03 ■□▲●	0.91 $\pm$ 0.04 ■□□▲	1.22 $\pm$ 0.02 ▲●
Total IgA (OD value)	0.45 $\pm$ 0.04 □▲●	0.43 $\pm$ 0.04 □▲●	0.63 $\pm$ 0.02 ■□▲●	1.22 $\pm$ 0.04 ■□□●	1.61 $\pm$ 0.09 ■□□▲	0.49 $\pm$ 0.07 ▲●
OVA-sIgA (OD value)	0.24 $\pm$ 0.02 □▲●	0.21 $\pm$ 0.02 □▲●	0.34 $\pm$ 0.02 ■□▲●	0.65 $\pm$ 0.03 ■□□●	1.10 $\pm$ 0.03 ■□□▲	0.28 $\pm$ 0.04 ▲●
OVA-sIgE (OD value)	0.55 $\pm$ 0.02 □▲●	0.63 $\pm$ 0.02 □▲●	0.42 $\pm$ 0.01 ■□▲●	0.27 $\pm$ 0.03 ■□□●	0.17 $\pm$ 0.03 ■□□▲	0.58 $\pm$ 0.04 □▲●
Residual histamine content (ng/0.1 g tissue)	57.22 $\pm$ 6.12 □▲●	55.43 $\pm$ 6.30 □▲●	86.15 $\pm$ 3.04 ■□▲●	170.87 $\pm$ 5.93 ■□□●	187.82 $\pm$ 8.65 ■□□▲	61.03 $\pm$ 6.04 □▲●

Compared with nTFG- β 0 group ■ $P<0.01$; with eVo group □ $P<0.01$; with nTFG- β lm group □ $P<0.01$; with eTFG- β lo group ▲ $P<0.01$; with NC group ● $P<0.01$; with prTFG- β lo group ◇ $P<0.01$.

mRNA level in the eVo and nTGfO groups, while in contrast IL-4 mRNA level followed the opposite trend; eVo and nTGfO group displayed stronger IL-4 expression than the eTGfO, nTGfM, and NC group. The IL-4 expression was lower in the eTGfO than in nTGfM group with the NC group displaying the lowest expression. In the prTGfO group, while the TGF- β 1 mRNA level was low on both day 45 and day 59, due to treatment with TGF- β 1 protein, IFN- γ mRNA expression increased transiently on day 45, but diminished markedly on day 59. Concomitantly, the IL-4 mRNA level remained low until day 45 and increased on day 59. Our results suggest that administration of TGF- β 1, either orally or through muscle injection, can increase the expression of TGF- β 1 and IFN- γ while reducing the IL-4 expression in the intestinal Peyer's Patch. Remarkably, these effects could last for at least 2 weeks.

Measurement of total IgA and OVA-sIgA in intestinal lavage fluids

IgA and OVA-sIgA have been reported to provide protection to the intestine while relieving allergy. To measure the therapeutic effects of TGF- β 1, we measured their levels in intestinal lavage fluids. ELISA assays showed that total IgA and OVA-sIgA levels in intestinal lavage fluids were highest in the NC group both on the 45th day and on the 59th day ($P < 0.01$). On the 45th day, after sensitization and challenge with OVA, total IgA and OVA-sIgA levels in intestinal lavage fluids dramatically decreased (NC versus eVo groups). Total IgA and OVA-sIgA levels were partially recovered by the TGF- β 1 plasmid and protein treatment in the prTGfO, eTGfO, and nTGfM groups ($P < 0.01$). The highest levels of total IgA and OVA-sIgA were found in the prTGfO group and the second highest levels were found in eTGfO among all treatment groups, including nTGfM group ($P < 0.01$) (Table 4, Fig. 4B). On the 59th day, whereas the total IgA and OVA-sIgA levels remained high in both eTGfO and nTGfM groups, especially significantly higher in eTGfO group than in nTGfM group ($P < 0.01$) (Table 5; Fig. 4B), the levels of total IgA and OVA-sIgA were markedly suppressed in the prTGfO group (Table 5; Fig. 4B). Thus, our data indicated that IgA secretion lasted longer when TGF- β 1 was delivered in the form of plasmid encapsulated with chitosan nanoparticles.

OVA-sIgE evaluation

We further evaluated the effects of different TGF- β 1 treatments by measuring OVA-sIgE in the serum from OVA-allergic mice. On day 45 (the final treatment day), the OVA-sIgE level was found to be the lowest in

the NC group which was not induced by OVA ($P < 0.01$) (Table 4, Fig. 4B). The OVA-sIgE levels were lower in the eTGfO, prTGfO, and nTGfM group as compared to the control eVo and nTGfO group indicating that TGF- β 1 therapy-based on either protein or DNA may inhibit the IgE-mediated food allergy ($P < 0.01$) (Table 4, Fig. 4B). While the OVA-sIgE levels were similar between the eTGfO and prTGfO groups, both are lower than that in nTGfM group ($P < 0.01$) (Table 4, Fig. 4B). To evaluate the temporal effects of different delivery approaches, the OVA-sIgE levels were investigated again on day 59, 2 weeks from the final TGF- β 1 treatments after OVA challenge. This time, the eTGfO and nTGfM groups still showed a significantly lower level of OVA-sIgE as compared with the control eVo and nTGfO groups ($P < 0.01$) (Table 5, Fig. 4B). The prTGfO group, in contrast, exhibited a drastic increase in serum OVA-sIgE to a level similar to that seen in the eVo and nTGfO groups (Table 5, Fig. 4B). Clearly, these results indicated that a relatively long lasting and highly efficient IgE inhibition may be induced in allergic mice when TGF- β 1 was delivered as orally administered chitosan-encapsulated DNA.

Level of residual histamine in the small intestinal segment

For further confirmation, we then measured the level of residual histamine in the small intestinal segment. An increase in the amount of residual histamine in the small intestine reflects a reduction of the amount of histamine released by mast cells into the intestinal tract indicating reduced damage of the intestinal tract. As shown in Fig. 4C1 and C2, Tables 4 and 5, the residual histamine in small intestine segment of mice was the highest in the NC group among all the groups on either day 45 or day 59 ($P < 0.01$). The level of residual histamine was higher in the eTGfO and nTGfM group than in the nTGfO and eVo groups on both day 45 and 59 ($P < 0.01$) (Fig. 4C1 and C2, Tables 4 and 5), suggesting that TGF- β 1 plasmid treatment, especially when orally administered by chitosan-encapsulated nanoparticles, significantly inhibited the histamine release within 2 weeks after the last TGF- β 1 treatment. In the prTGfO group, the level of the histamine release varied significantly between day 45 and 59. On the 45th day, the residual histamine level in prTGfO group stayed high and was not significantly different from that observed in the eTGfO group (Fig. 4C1, Table 4). On the 59th day however, it was reduced dramatically to the level as low as that in nTGfO and eVo group (Fig. 4C2, Table 5). These results indicate that, albeit very effective in inhibiting the histamine release within a short period of time, oral administration of TGF- β 1 protein did not produce a long-lasting effect if not repeatedly administered.

Discussion

Food allergy is often associated with aberrant Th2-type immune responses and breakdown in oral tolerance to food antigens. The inhibitory cytokine TGF- β , which plays important roles regulating the balance between Th1 and Th2 cells, inhibiting IL-4 secretion by Th2 cells (Seder et al., 1998), as well as promoting the production of secretory IgA antibody in the intestinal tract (Strober et al., 1998), is suggested to be an important molecule in the anti-allergy machinery. Several groups have found that an exogenous increase of the TGF- β level in the intestinal tract could ameliorate food allergy by employing oral delivery of TGF- β protein (Okamoto et al., 2005; Bellinghausen et al., 2006; Wan and Flavell, 2007). Albeit effective, as a practical therapy, these treatments were limited by their requirements of frequent deliveries of high-dosage TGF- β protein. Furthermore, because of its high turn over rate, the TGF- β level could drop quite significantly between treatments (Mowat, 1999; Okamoto et al., 2005; Howell and McAnulty, 2006). Obviously, these limitations need to be addressed when developing future therapeutic strategies.

Gene therapy via *in vivo* transfection has shown a lot of promise in treating different diseases. In the case of delivering TGF- β , it may suit even better as TGF- β secreted from the transfected population of cells may contribute enough to relieve the symptom of food allergy. *In vivo* transfected cells are more likely to maintain a steady TGF- β level over time. Moreover, comparing with TGF- β protein, plasmid DNA expression vector is much more stable when being stored. In this study, we aimed to deliver a plasmid encoding TGF- β 1 (pBudCE4.1/TGF- β 1) to determine whether its *in vivo* application could stably enhance TGF- β expression in the intestinal tract of allergic mice and alleviate symptoms of allergy.

The plasmid DNA was encapsulated in chitosan nanoparticles in order to enhance the efficiency of *in vivo* transfection. It has been reported that chitosan, a deacetylated product of natural chitin, is compatible with tissue, degradable, non-immunogenic, and can form polyelectrolyte complexes that protect plasmid DNA from digestion by DNase (Roy et al., 1999). It represents a highly effective controlled release system for delivering therapeutic drugs given orally because of its high rate of absorption, and is effective for treating diseases of the stomach and intestinal tract (Roy et al., 1999). Moreover, chitosan can form microcapsules with DNA. These microcapsules exhibit a better transfection efficacy than traditional intramuscular injection of DNA vaccines. It is considered as the most promising, safe, and effective non-viral gene carrier available, and it is increasingly used for therapeutic gene transport and vaccine research (Koping-Hoggard et al., 2001). Because

of these features, and intestine being where the symptoms reside, we investigated the oral delivery of chitosan packaged DNA. Based on the observations presented herein, chitosan-encapsulated DNA proved to be much more effective than the naked DNA when administered orally.

When comparing different delivery approaches, we focused on two factors, including the efficiency with which TGF- β is delivered to the intestine and the efficiency and duration of the therapeutic functions. Analysis of both mRNA and protein revealed significantly induced TGF- β expression in both eTGf α and nTGf α groups, indicating the effectiveness of *in vivo* transfection. In both groups, the TGF- β level stayed high on the 45th day and lasted at least through the 59th day which was 2 weeks from the last treatment. These results demonstrated that both approaches could generate a high level of TGF- β expression that lasted for a relatively long period of time. In comparison, oral DNA-chitosan proved to be more effective than intramuscular injection, possibly because the former approach allowed a better absorption by intestine cells.

In our study, the OVA-induced mouse allergic model was used to evaluate the therapeutic functions. We measured the allergy-relieve-related biomarkers total IgA and OVA-sIgA, and compared Th1/Th2 cells balance related genes such as IL-4 and IFN- γ mRNA expressions in the Peyer's Patch. We also evaluated allergy onset biomarkers, including the OVA-sIgE level in serum and histamine residual level in intestine.

Our data showed that the levels of total IgA and OVA-sIgA in small intestine were higher in the prTGf α , eTGf α and nTGf α groups than in other TGF- β treatment groups on the 49th day. On the 59th day, they were reduced significantly in the prTGf α group, but remained high in eTGf α and nTGf α group, especially higher in eTGf α group. Their levels paralleled TGF- β 1 protein expression in different groups, suggesting that TGF- β 1 expression facilitated IgA secretion in the intestinal tract, since the TGF- β 1 level in the intestinal tract was highly associated with secretion of sIgA (van Ginkel et al., 1999). It is reported that CD3⁺ cells in Peyer's Patch are primarily involved in favoring IgA production through the release of IL-10, and secretory IgA facilitates thereby inducing the oral tolerance to foods and suppressing the serum IgE response and systemic anaphylaxis in the food allergy model (Frossard et al., 2004; Okamoto et al., 2005). In our study, plasmid-based TGF- β therapy showed a higher capability of inducing total IgA and OVA-sIgA expression over a relatively long period compared with protein-based TGF- β therapy. Moreover, oral plasmid-chitosan appeared to be more effective than intramuscular injection in enhancing the total IgA and OVA-sIgA expression and alleviating symptoms of food allergy (Saarinen et al., 2002).

One mechanism by which TGF- β relieves food allergy is to restore the imbalance between Th1 and Th2 cells. This imbalance induces overpolarization of Th2 cells and brings on increased IL-4 secretion and decreased IFN- γ secretion and results in elevation of IgE antibodies in serum and release of histamine in intestinal mast cells. On day 45 and day 59, both the eTGF β and nTGF β groups displayed a significant inhibition of allergic inflammation with low OVA-sIgE level, low level of IL-4 expression, and low level of intestinal histamine release, but high level of IFN- γ expression. The therapeutic effects were even better in eTGF β than in nTGF β group. Similar results were found in the prTGF β group on day 45, but almost completely vanished when tested on day 59. These data support previous reports (Okamoto et al., 2005; Ando et al., 2007) about the therapeutic effects of the oral intake of TGF- β protein on relieving Th2-cell-induced immune inflammation, and explained why frequent and high-dosage applications would be necessary, which affects its use as an efficient therapy.

Taken together, we found that the therapeutic effects positively correlate with the amount of increased TGF- β . When measurements were conducted shortly after the treatments, oral administration of chitosan-encapsulated DNA, like oral administration of TGF- β protein, effectively relieved OVA-induced food allergy in the mouse model, thereby supporting the importance of TGF- β in suppressing allergy reaction as reported previously (Kalliomaki et al., 1999; Oddy et al., 2003; Okamoto et al., 2005; Ando et al., 2007). However, measurements of the long-term effects clearly demonstrated the power of DNA-based therapy. Two weeks after treatments, the group treated with TGF- β protein exhibited little relief from OVA-induced allergy while mice administered with chitosan-encapsulated TGF- β plasmid remained protected. Thus, the oral intake of chitosan-encapsulated TGF- β expressing plasmid could promote a long lasting TGF- β expression in the intestine and provide a less expensive, but more durable alleviation of allergic inflammation.

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