THE JOURNAL OF GENE MEDICINE
J Gene Med 2006; **8**: 314–323.
Published online 17 November 2005 in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/jgm.848

RESEARCH ARTICLE

Polyethylenimine-based antisense oligodeoxynucleotides of IL-4 suppress the production of IL-4 in a murine model of airway inflammation

Ji-Hye Seong[1]
Kyung-Mi Lee[2]
Sung Tae Kim[1]
Su-Eon Jin[1]
Chong-Kook Kim[1]*

[1]*National Research Laboratory for Drug and Gene Delivery, College of Pharmacy, Seoul National University, Seoul, Korea 151-742*

[2]*Department of Biochemistry and Division of Brain Korea 21 Program for Biomedical Science, College of Medicine, Korea University, Seoul, Korea 136-705*

*Correspondence to:
Chong-Kook Kim, National Research Laboratory for Drug and Gene Delivery, College of Pharmacy, Seoul National University, Seoul, Korea 151-742.
E-mail: ckkim@plaza.snu.ac.kr

Received: 10 April 2005
Revised: 17 August 2005
Accepted: 18 August 2005

Abstract

Background Interleukin-4 (IL-4) plays a crucial role as an inflammatory mediator in allergic asthma via inducing Th2 inflammation and IgE synthesis. To develop an effective therapeutic agent which specifically inhibits production of IL-4, antisense oligodeoxynucleotides (AS-ODNs) against murine IL-4 mRNA were generated and complexed with polyethylenimine (PEI) to improve intracellular delivery.

Methods AS-ODNs were generated against the translation initiation region of murine IL-4 mRNA, and complexed with linear PEI. *In vitro* efficacy of AS-ODNs/PEI complexes was tested by measuring IL-4 production in the D10.G4.1 cell line, and cytotoxicity was tested by XTT assay. Physicochemical properties of polyplexes were examined using atomic force microscopy (AFM) and DNase I protection assay. *In vivo* effects of IL-4 AS-ODNs/PEI complexes were tested in a murine model of airway inflammation. IL-4 concentrations in the bronchoalveolar lavage (BAL) fluid and circulating IgE levels were measured by ELISA, and histological analysis of lung tissues was performed.

Results IL-4 AS-ODNs/PEI complexes were spheres with an average diameter of 98 nm and resistant to DNase I-mediated degradation. IL-4 AS-ODNs/PEI complexes showed up to 35% inhibition of IL-4 production in D10.G4.1 cells without causing any toxicity, while naked ODNs gave less than 1% reduction. Furthermore, IL-4 AS-ODNs/PEI complexes were effective in suppressing secretion of IL-4 (up to 30% reduction) in the BAL fluid in an ovalbumin-sensitized murine model of airway inflammation. Circulating IgE levels were decreased, and airway inflammation was alleviated by treatment with IL-4 AS-ODNs polyplexes.

Conclusions These data demonstrate that complexation of IL-4 AS-ODNs with PEI provides a potential therapeutic tool in controlling inflammation associated with allergic asthma, and further presents an opportunity to the development of clinical therapy based on combination of multiple AS-ODNs of cytokines and/or signaling effectors involved in Th2 inflammation and eosinophilia. Copyright © 2005 John Wiley & Sons, Ltd.

Keywords antisense oligodeoxynucleotides; polyethylenimine; interleukin-4; allergic asthma; airway inflammation

Introduction

Allergic asthma is shown to be triggered by allergen-induced activation of T helper 2 (Th2) cells and subsequent activation of mast cells producing IgE [1]. One of the key players in the activation of mast cells is interleukin-4

(IL-4) produced mainly by Th2 cells. IL-4 can promote the differentiation and proliferation of Th2 cells and facilitates antibody class switching of B cells to IgE [2]. IL-4 has also been shown to promote eosinophilic inflammation by increasing eotaxin expression and inhibiting apoptosis of eosinophils [3,4]. Therefore, efficient blockade of secretion and/or subsequent signaling of IL-4 would diminish the degree of airway inflammation and hypersensitivity in patients with allergic asthma.

In line with this idea, intranasal administration of soluble IL-4 receptor, which prevents binding of IL-4 to its receptors, reduced pulmonary inflammation that occurred in the murine allergen-challenged asthma model [5]. Likewise, IL-4 receptor blockade using its specific antibodies resulted in a significant decrease of airway responses induced by antigen challenge in mice [6]. Recent advances in delivery of oligodeoxynucleotides into cells provide promising tools to modulate the expression of cellular proteins [7]. Indeed, several antisense-oligodeoxynucleotides (AS-ODNs) have been proven effective *in vitro* and currently are being tested in the clinical trials in patients with cancer, AIDS, as well as hepatitis C [8]. Similarly, AS-ODNs generated against IL-4 have been shown to be effective *in vitro* [9] and in rat models of late airway responses [10].

However, despite its effectiveness, the use of AS-ODNs generated against IL-4 in allergic asthma is still in preclinical stages. As with other ODNs, efficiency of IL-4 AS-ODNs *in vivo* is determined by combination of biological stability, successful uptake into the target cells, resistance to nucleases, and so forth [8,11]. To satisfy these conditions, various approaches have been developed. Modification of phosphodiester bonds in the AS-ODNs to increase stability [12] and incorporation of AS-ODNs into viral or non-viral vectors to increase targeting and cellular uptake [8,13] are among those. Viral vectors have high transfection efficiency but are toxic and immunogenic. Non-viral vectors are relatively safe and over time two main non-viral vehicles have been consolidated as more promising vectors: lipoplexes [14,15], which are nucleic acid/cationic lipid complexes, and polyplexes, which are complexes of nucleic acids and cationic polymers, mainly P(LA-GA) copolymer [16] and polyethylenimine (PEI) [17]. Accumulating data have revealed PEI as one of the most effective DNA-delivery systems both in cell cultures and *in vivo* due to its polycationic property and proton sponge mechanism [17–21]. However, the use of PEI in humans has been limited due to its toxicity. Moreover, when administered systemically, a high amount of PEI activates the compliment system and thus is prone to rapid degradation [22].

Therefore, this study was designed to achieve the optimal concentration of PEI to be complexed with IL-4 AS-ODNs displaying maximum efficacy with minimum toxicity. Stable polyplexes were made without generating any significant cytotoxicity. IL-4 AS-ODNs/PEI complexes were capable of inhibiting IL-4 secretion not only *in vitro* but also *in vivo* in the ovalbumin-sensitized murine model of airway inflammation when administered intranasally. In addition, IgE level and airway inflammation were decreased by IL-4 AS-ODNs. Thus, IL-4 AS-ODNs polyplexes can function as therapeutic agents to down-regulate production of IL-4 in a murine model of allergic asthma.

Material and methods

Reagents

IL-1 alpha was purchased from R&D Systems (Minneapolis, MN, USA). Conalbumin (ConA) and ovalbumin (OVA) were purchased from Sigma (St. Louis, MO, USA), and alum was purchased from Pierce (Rockford, IL, USA). Rat T-STIM factor, recombinant mouse IL-4, purified rat anti-mouse IL-4 mAbs, and biotinylated rat anti-mouse IL-4 mAbs were purchased from Becton Dickinson (Bedford, MA, USA). Linear polyethylenimine (PEI) was purchased from Euromedex (Souffelweiersheim, France).

Cell line and animals

The IL-4-secreting D10.G4.1 murine Th2 cell line was a kind gift from Dr. E. J. Park (Institute of Molecular Biology and Genetics, Seoul, South Korea). Cells were maintained in RPMI medium supplemented with 0.05 mM 2-mercaptoethanol, 10 pg/ml IL-1alpha, 10% fetal bovine serum (FBS), and 10% rat T-STIM factor. Female BALB/c mice (6–8 week of age) were housed in the specific pathogen-free animal facility of Seoul National University (Seoul, South Korea). All animal experiments were conducted in accordance with the Korean National Board for Laboratory Animals.

Preparation of murine IL-4 AS-ODNs/PEI complexes

IL-4 antisense oligodeoxynucleotides (AS-ODNs) were generated against the translation initiation region of murine IL-4 mRNA (+4 to +25). Phosphorothioates were added to the three nucleotides of each end to increase the resistance to nucleases. The sequence corresponds to 5′-CAACTAGCTGGGGGTTGAGACC-3′. Nonsense oligodeoxynucleotides (NS-ODNs), 5′-GGTCTCACCTCCCAACTGCTTC-3′, which possess the same number of GC contents, were used as a negative control.

To prepare IL-4 AS-ODNs/PEI complexes, designed IL-4 AS-ODNs and linear PEI of 22 kDa were diluted in 150 mM NaCl solution (*in vitro*), 5% glucose solution (*in vivo*), or 10 mM Tris-HCl buffer (for DNase I protection assay). A volume of PEI solution (50 μl for *in vitro*, 25 μl for DNA retardation assay, and 20 μl for *in vivo*) was slowly added to an equal volume of IL-4 AS-ODNs solution, and then mixed thoroughly. The mixtures were incubated at room temperature for 20 min prior to being used in the experiment. Polyplexes at various NP (N,

moles of N atoms in PEI; P, moles of phosphate groups in ODNs) ratios were generated. NP ratio = 10 corresponds to a 1 : 1.25 ODN/PEI w/w ratio [23].

DNA retardation assay and fluorescence decay assay

The complexation of IL-4 AS-ODNs with PEI was determined by agarose gel retardation assay. The IL-4 AS-ODNs (10 μg) and increasing amounts of PEI were mixed in 25 μl of 150 mM NaCl and incubated for 20 min. Aliquots of the samples (20 μl) were resolved onto a 0.6% agarose gel and visualized with a UV transilluminator. Initially, when various doses of ODNs were tested, ODNs above 4 μg were found to be optimal to be detected in the gel. Thus, we set the concentration of ODNs at 4 μg in the experiment.

A fluorescence decay assay was performed to confirm the complexation and condensation of the polyplex. In this case, FITC-labeled IL-4 AS-ODNs (2 μg) at different NP ratios were prepared in 150 mM NaCl solution. To perform this, IL-4 AS-ODNs were labeled with FITC prior to complexation with PEI. The samples were loaded in a 96-well microplate, and green fluorescence was measured (Ex = 485 nm, Em = 535 nm) using a microplate fluorimeter.

In vitro transfection experiment

The IL-4-producing D10.G4.1 cell line was seeded onto 24-well plates at 1×10^5 cells/well, and stimulated with ConA (500 ng/well) for 2 days. First, the optimal dose of IL-4 AS-ODNs was selected. Various concentrations of IL-4 AS-ODNs (0, 0.5, 1, 2 or 4 μg) complexed with PEI at a fixed NP ratio of 10 were added to D10.G4.1 cells. After 48 h, cells were harvested and supernatants were analyzed for the presence of IL-4 using a sandwich enzyme-linked immunosorbent assay (ELISA) kit (Becton Dickinson, Bedford, MA, USA).

Then, the optimal NP ratio of the IL-4 AS-ODNs/PEI complex was determined. As a result of selecting the optimal dose of IL-4 AS-ODNs, 2 μg of IL-4 AS (or NS)-ODNs were complexed with various amounts of PEI. Polyplexes at NP ratios of 0, 1, 2, 5, 10, and 20 were added onto D10.G4.1 cells. After 48 h, cells were harvested and supernatants were analyzed using sandwich ELISA (n = 3).

XTT incorporation assay

Cytotoxicity of IL-4 AS-ODNs polyplexes was determined using the XTT assay as described previously [24]. D10.G4.1 cells were plated onto a 96-well plate at 1×10^4 cells/100 μl. A volume of 10 μl of the polyplexes at NP ratios of 0, 1, 2, 5, 10 and 20 was added and incubated at 37 °C for 2 days. Then, 50 μl of XTT reaction mixture prepared with sodium 3′-[1-[(phenylamino)carbonyl]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzenesulfonic acid hydrate (XTT) and activation reagents containing PMS (*N*-methylphenazonium methosulfate) was added to each well and their emission was measured at 450 nm (n = 5).

DNase I protection assay

The IL-4 AS-ODNs (3 μg) and increasing amounts of PEI were mixed for 20 min in 50 μl of 10 mM Tris-HCl buffer. Then, 10 units of DNase I were added and the mixtures were incubated at 37 °C for the indicated time (0, 5, 15, 30, and 45 min). Immediately following incubation, EDTA (0.5 M, 10 μl) was added and the samples were placed on ice to stop DNase activity. Heparin (50 μl, 1250 units) was added and incubated at 4 °C for 1 h to complete the dissociation of polyplexes. Subsequently, ODNs were extracted by phenol and subjected to agarose gel electrophoresis as described above.

Atomic force microscopy

The morphological features of the polyplex were studied by atomic force microscopy (AFM). As a dilution buffer, 10 mM NaCl was used for AFM measurement to prevent salt crystal formation. The polyplex (5 μl) at NP ratio of 10 was deposited onto freshly cleaved mica for 5 min. Then, the sample solution was removed by gently swirling, and a volume of 20 μl of double distilled water (DDW) was spotted onto the mica for 10 s. DDW was removed by gentle swirling and mica was air-dried for 30 min. Images were acquired using scanning probe microscopy (AutoProbe CP™, PSIA, USA). To determine the mean complex size, the diameters of 50 complexes were measured.

Induction of airway inflammation and treatment

Mice were sensitized and rechallenged with OVA as described previously [25]. Briefly, mice received an intraperitoneal injection of 0.2 ml (100 μg) of OVA complexed with alum on days 0 and 14. On day 14, 100 μg OVA in 0.04 ml phosphate-buffered saline (PBS) was administered intranasally and subsequently, on days 25, 26 and 27, 50 μg OVA in 0.04 ml PBS mice was administered intranasally. Where intranasal administration was performed, mice were anesthetized with 0.2 ml 2.5% Avertine (1 g/ml of tribromoethylene alcohol in *tert*-amyl alcohol) in PBS. The negative control animals received PBS intraperitoneally on days 0 and 14, then 0.04 ml of PBS intranasally on days 14, 25, 26 and 27.

To select the optimal dose of IL-4 AS-ODNs, mice received 50 μl of 30, 50, or 70 μg of IL-4 AS-ODNs complexed with a fixed amount of PEI (NP ratio of 5) through intranasal administration at days 25, 26, and 27

(n = 3). IL-4 AS-ODNs/PEI complexes at NP ratios of 0, 5 and 10 were tested to determine the *in vivo* optimal NP ratio (n = 3). After that, negative control, positive control, IL-4 AS-ODNs/PEI complex, IL-4 NS-ODNs/PEI complex, and IL-4 AS-ODN groups were tested (n = 7). Control animals received 50 μl of PBS.

Bronchoalveolar lavage

Twenty-four hours after the last intranasal challenge, mice were anesthetized with 2.5% Avertine and blood samples were obtained using heart puncture. After tying off the left lung at the mainstem bronchus, bronchoalveolar lavage (BAL) of the right lung was performed. The right lung was lavaged three times with 0.4 ml of PBS, and total BAL fluid was collected. The number of cells present in BAL fluid was counted using a hemocytometer after Trypan Blue staining (n = 7). BAL fluid and blood samples were centrifuged at 4 °C for 5 min at 1200 rpm, and the supernatant was stored at −70 °C until analysis by ELISA for IL-4 and IgE protein.

Lung histology

After BAL extraction, the left lung of each group was collected. Lung tissues were fixed in 4% paraformaldehyde at 4 °C for 24 h, and immersed in 30% sucrose solution at 4 °C for 48 h. After embedding in optimal cutting temperature (OCT) compound, the tissues were cut into 6 or 10 μm sections. Sections were stained with hematoxylin/eosin (H&E) and analyzed using a light microscopy.

Statistical analysis

All experiments were performed at least three times. The statistical significance of the results was evaluated by Student's t-test.

Results

Generation of the stable polyplexes using PEI

To determine the optimal ratio of PEI required for formation of stable complexes with IL-4 AS-ODNs, various concentrations of PEI were mixed with a fixed concentration of IL-4 AS-ODNs. Stable incorporation of IL-4 AS-ODNs into PEI was initially tested via gel retardation assay (Figure 1a). The formation of positive complexes with PEI would prevent migration of IL-4 AS-ODNs into the agarose gel and therefore they would be captured on top of the gel. In addition, even for those few that did migrate into the gel, the binding of IL-4 AS-ODNs to EtBr would be sterically hindered by surrounding PEI. As shown in Figure 1a,

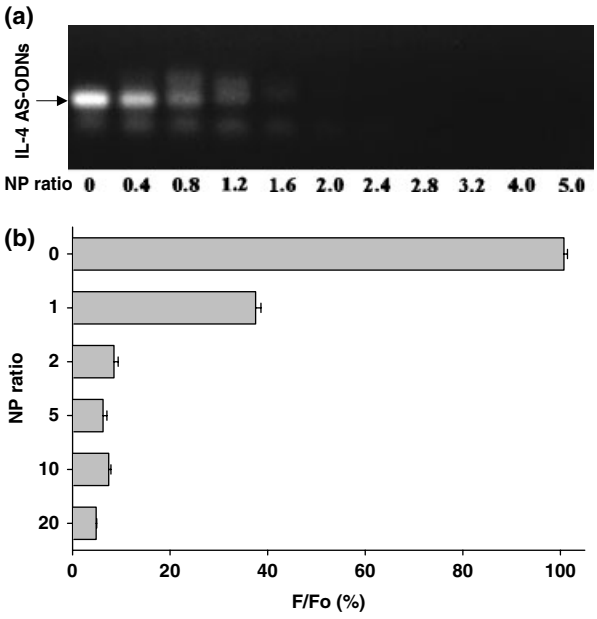


Figure 1. Generation of stable IL-4 AS-ODNs/PEI complexes. (a) Agarose gel electrophoresis was performed using IL-4 AS-ODNs/PEI complexes generated at NP ratios as indicated in the figure. NP ratio 0 indicates no complex formation with PEI. Complexation with PEI results in retardation and disappearance of a band representing ODNs in a NP ratio-dependent manner. (b) Fluorescence decay assay was used to check the level of complexation of IL-4 AS-ODNs with PEI. IL-4 AS-ODNs were labeled with FITC and incorporated into PEI at the NP ratios indicated in the figure. Green fluorescence emitted from each condition was measured (Ex = 485 nm, Em = 535 nm). F/F_0 was determined by dividing fluorescence of ODNs in polyplexes by fluorescence of free FITC-labeled ODNs. The results are expressed as mean values ± standard deviation (s.d.) (n = 3)

migration of polyplexes was significantly retarded at the NP ratios higher than 0.8 and stopped at the NP ratios higher than 2.8. These data suggest that stable complexation and condensation of IL-4 AS-ODNs into PEI could be achieved at the NP ratios above 2.8.

To further confirm the successful incorporation of IL-4 AS-ODNs into PEI, a fluorescence decay assay was performed (Figure 1b). Similar to the gel retardation assay, stable incorporation of IL-4 AS-ODNs into PEI would result in hindrance of fluorescence emission by the fluorochrome-labeled IL-4 AS-ODNs. As shown in Figure 1b, at the NP ratios higher than 2, green fluorescence emitted from FITC-labeled IL-4 AS-ODNs was significantly reduced. These data demonstrate that stable complexation of IL-4 AS-ODNs with PEI could be obtained at the NP ratios higher than 2.

In vitro effect of IL-4 AS-ODNs/PEI complexes in suppressing IL-4 secretion

We next examined the ability of IL-4 AS-ODNs/PEI complexes in inhibiting IL-4 production *in vitro*. For this, we obtained a D10.G4.1 cell line which could produce IL-4 upon stimulation with ConA, and tested the effect of

polyplexes by directly measuring IL-4 concentration in the culture supernatant by ELISA (Figure 2). To determine the optimal dose of IL-4 AS-ODNs, various amounts of ODNs (0, 0.5, 1, 2 or 4 μg) were complexed with a fixed concentration (NP ratio of 10) of PEI (Figure 2a). Cells treated with IL-4 AS-ODNs/PEI complexes underwent dose-dependent inhibition of IL-4 production (up to 35% inhibition achieved with 2 μg of IL-4 AS-ODNs polyplex). However, no further inhibition was observed above 2 μg of ODNs. Thus, we chose 2 μg of IL-4 AS-ODNs to be complexed with PEI. Next, we determined the optimal PEI concentration to be complexed with IL-4 AS-ODNs. As shown in Figure 2b, PEI complexation with IL-4 AS-ODNs resulted in reduction of IL-4 secretion. In the absence of any treatment, the concentration of IL-4 secreted into the medium was approximately 4300 pg/mL. Addition of naked IL-4 AS-ODNs resulted in only slight inhibition of IL-4 secretion (less than 1% reduction). However, IL-4 AS-ODNs complexed with PEI showed significantly higher reduction of IL-4 secretion at all tested NP ratios (Figure 2b). Maximal inhibition was achieved at the NP ratio of 10 and no further inhibition was observed at NP ratios above 10. IL-4 concentration of cells treated with polyplexes at the NP ratio of 10 was reduced to 35% of total IL-4 secretion. When the degree of inhibition was compared with naked ODNs, PEI polyplexes showed 33-fold higher inhibition. In contrast, nonsense ODNs did not result in any significant reduction in IL-4 secretion (data not shown). Together, these data suggest that only IL-4 AS-ODN/PEI complexes effectively enter into the cell and prevent normal process of translation into IL-4 proteins.

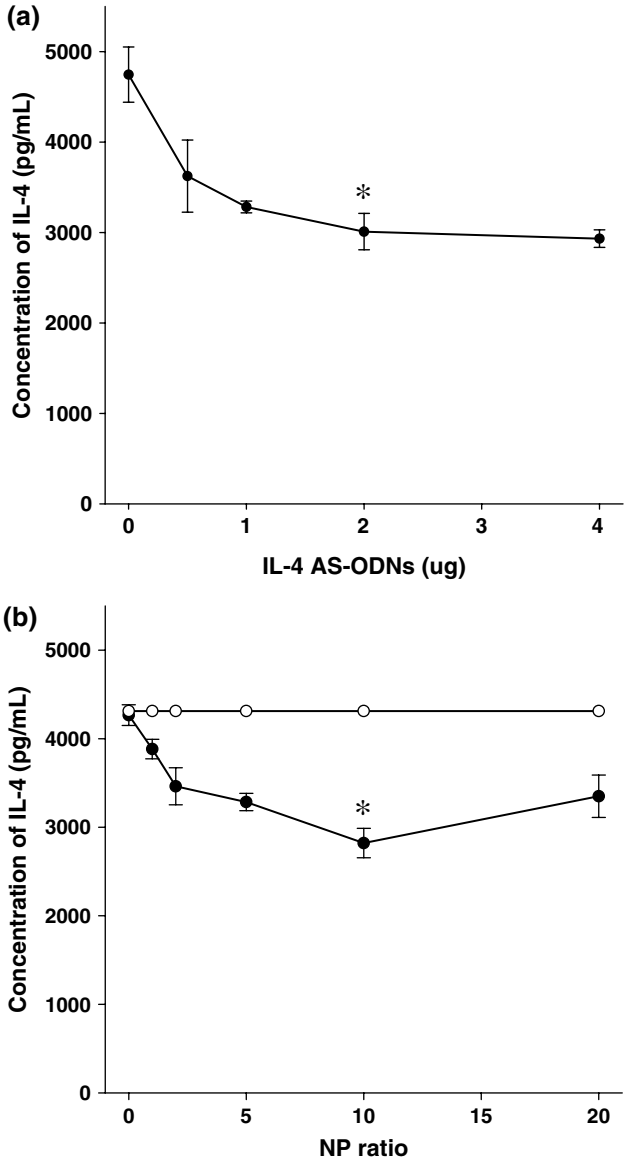


Figure 2. ***In vitro*** **effect of polyplexes in reducing IL-4 secretion. The IL-4-secreting D10.G4.1 cells were seeded onto a 24-well plate at 1 x 10^5 cells/well, and stimulated with 500 ng/ml of ConA for 2 days. To select the optimal dose of IL-4 AS-ODNs, various amounts of ODNs (0, 1, 2, and 4 μg) complexed with PEI at NP ratio of 10 were incubated in D10.G4.1 cells. The concentration of IL-4 of the supernatant was measured using a sandwich ELISA kit (a). Polyplexes at NP ratios of 0, 1, 2, 5, 10, and 20 were incubated at 37 °C for 48 h. IL-4 secretion was measured using sandwich ELISA (closed circles) (b). The open circles represent the concentration of IL-4 in the absence of any treatment. The results are expressed as mean values ± s.d. (n = 3). *$p < 0.05$**

Cytotoxicity of the polyplexes

Since a high concentration of PEI was shown to cause toxicity *in vivo*, we determined if the inhibition of IL-4 secretion observed in Figure 2 might have been attributed to cell death resulting from PEI. To this end, we added XTT reagents to the cells at the end of incubation with polyplexes. Dehydrogenases present in living cells cleave XTT, thus yielding soluble formazan product whose absorbance can be detected at 450 nm. As shown in Figure 3, at all NP ratios lower than 10, there was

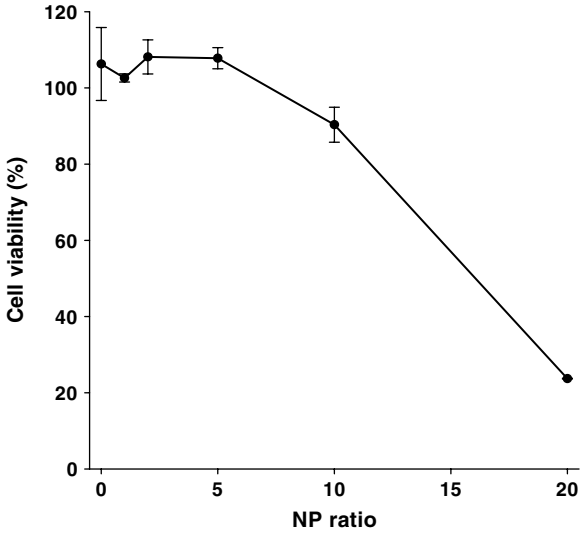


Figure 3. Cytotoxicity of IL-4 AS-ODNs/PEI complexes. D10.G4.1 cells were seeded onto a 96-well plate, and polyplexes at NP ratios of 0, 1, 2, 5, 10 and 20 were added. After incubation for 48 h, XTT reaction mixture (50 μl) was added to each well and the absorbance was measured at 450 nm. The results are expressed as mean values ± s.d. (n = 5)

no significant change in absorbance of XTT. However, polyplexes at NP ratios above 10 were found to decrease viability. At the NP ratio of 20, PEI induces death in up to 80% of cells. Taken together, these data strongly demonstrate that polyplexes generated at the NP ratio of 10 were optimal in the delivery of IL-4 AS-ODNs/PEI complexes to suppress IL-4 secretion without causing any toxicity in D10.G4.1 cells.

Polyplexes are resistant to degradation mediated by DNase I

Degradation of oligodeoxynucleotides by DNases or RNases is one of the serious problems in achieving maximum efficiency. It has been shown previously that the complex formation with PEI can confer protection against endo- or exonucleases [17,26]. Thus, we examined whether polyplexes generated above were resistant to enzymatic degradation. For this, we incubated polyplexes with DNase I for 15 min and ODNs were dissociated from PEI using heparin prior to gel analysis. As shown in Figure 4a, incubation of naked IL-4 AS-ODNs with DNase I resulted in near complete degradation of ODNs (lane 3). However, complexation with PEI markedly increased resistance to DNase I (lanes 5, 7, 9, and 11), and, in particular, ODNs at the NP ratios higher than 5 were completely protected from degradation (lanes 8–11). Interestingly, IL-4 AS-ODNs dissociated from PEI migrated more slowly than naked ODNs, suggesting that PEI complexation might have affected the coiled status of ODNs. Alternatively, residual PEI mixed with ODNs could have hindered migration and condensation of ODNs. To discover whether the resistance of ODNs to DNase I can be maintained for longer period of time, we incubated polyplexes with DNase I for up to 45 min. As shown in Figure 4b, naked IL-4 AS-ODNs were digested even 5 min after addition of DNase I (lane 3), whereas PEI-complexed ODNs were protected up to 45 min of incubation (lanes 7–11).

To visually demonstrate the formation of IL-4 AS-ODNs/PEI complexes, the morphology and size of the polyplexes generated at the NP ratio of 10 were measured using atomic force microscopy (AFM). As shown in Figure 5, polyplexes are distributed homogeneously in solution (Figure 5a) and show spherical shape with an average diameter of 98 nm (n = 50, Figure 5b). In contrast, PEI alone without ODNs did not form any measurable polyplexes (Figure 5c). Together, these data indicate that IL-4 AS-ODNs complexed with PEI can be

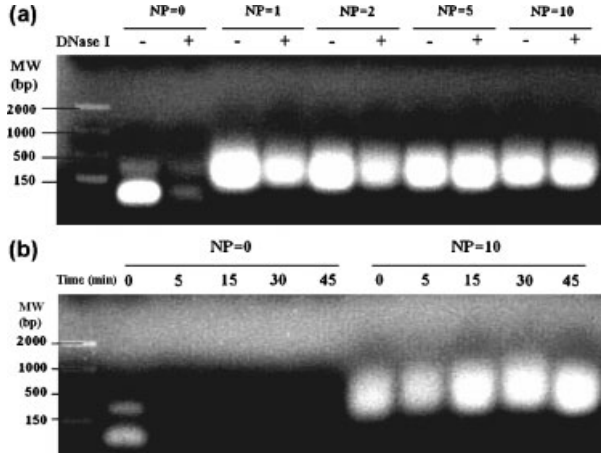


Figure 4. Protection of the polyplexes against DNase I-mediated degradation. (a) IL-4 AS-ODNs/PEI complexes at NP ratios of 0, 1, 2, 5 and 10 were prepared, and incubated with DNase I for 15 min. Enzymatic reaction was stopped with EDTA and ODNs were subsequently dissociated from PEI polyplexes as described in Materials and Methods. IL-4 AS-ODNs extracted were subjected to agarose gel electrophoresis. (b) Time-dependent sensitivity to DNase I was monitored using polyplexes generated at the NP ratio of 10. After incubation with DNase I for 0, 5, 15, 30, and 45 min, the protective effect of the polyplexes (NP = 10) was compared with that of naked IL-4 AS-ODNs (NP = 0)

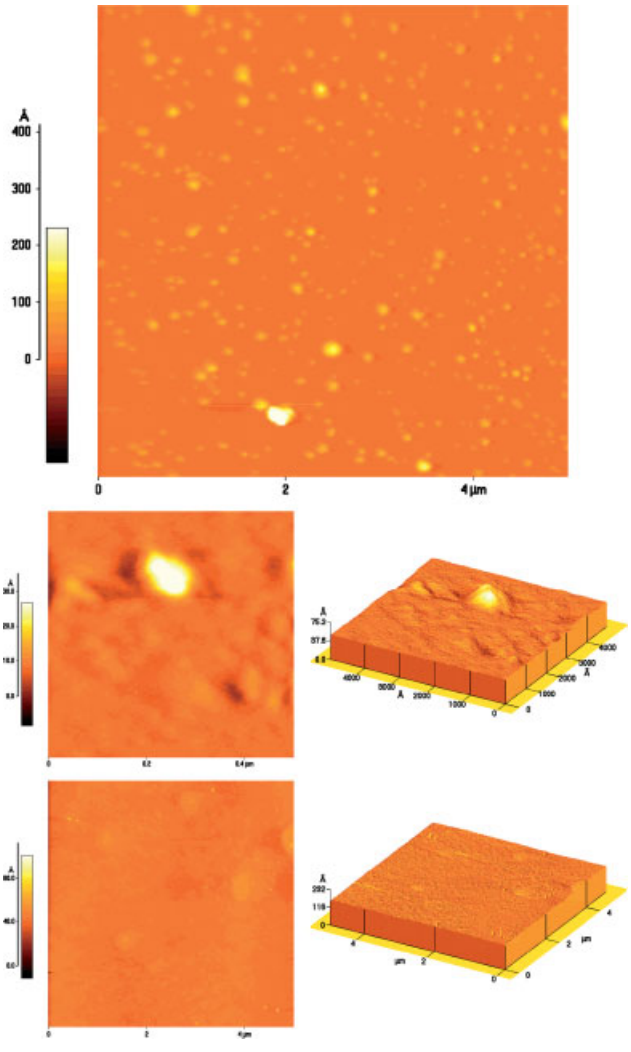


Figure 5. Characterization of polyplexes via atomic force microscopy (AFM). A volume of 5 μl of the IL-4 AS-ODNs/PEI complex generated at the NP ratio of 10 was deposited onto freshly cleaved mica. After washing and air-drying, AFM images were acquired using scanning probe microscopy in tapping mode. The polyplex at NP ratio of 10 was visualized at 5 μm × 5 μm (a), and 0.5 μm × 0.5 μm (b). Image of PEI alone without ODNs was acquired at 5 μm × 5 μm (c)

efficiently protected from enzymatic digestion via forming spherical polyplexes, and further contribute to the efficient blockade of IL-4 secretion as shown in Figure 2.

Intranasal administration of IL-4 AS-ODNs polyplexes suppresses the production of IL-4 in a murine model of airway inflammation

We set out to investigate the efficacy of the IL-4 AS-ODNs polyplexes *in vivo*. To evoke airway inflammation, female BALB/c mice were sensitized with OVA peptide as described previously [25]. After sensitization, PBS, IL-4 AS-ODNs/PEI complex, IL-4 NS-ODNs/PEI complex, or IL-4 AS-ODNs was co-administered intranasally with OVA at days 25, 26 and 27, then IL-4 secreted to BAL fluid was measured by ELISA at day 28.

First, for the optimization of the treatment, various amounts of IL-4 AS-ODNs and various NP ratios of PEI were tested. IL-4 AS-ODNs polyplexes showed dose- and NP ratio-dependent inhibition of IL-4 production (data not shown). However, large amounts of IL-4 AS-ODNs or PEI caused significant *in vivo* toxicity. A previous study had also shown that positively charged polyplexes at the NP ratio of 10 caused acute toxicity, though neutral complexes at the NP ratio of 5 did not [21]. The polyplex at the NP ratio of 10 was most effective in inhibiting the production of IL-4 in the murine Th2 cell line, but toxicity is more critical factor for the *in vivo* trial. Thus, 50 μg of IL-4 AS-ODNs and the NP ratio of 5 were selected for the *in vivo* experiment. As shown in Figure 6, the IL-4 level recorded from the BAL fluid of sensitized mice

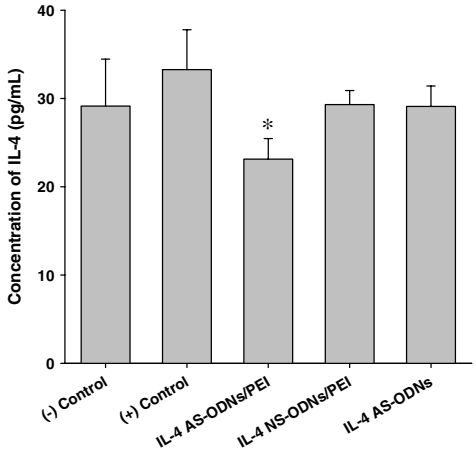
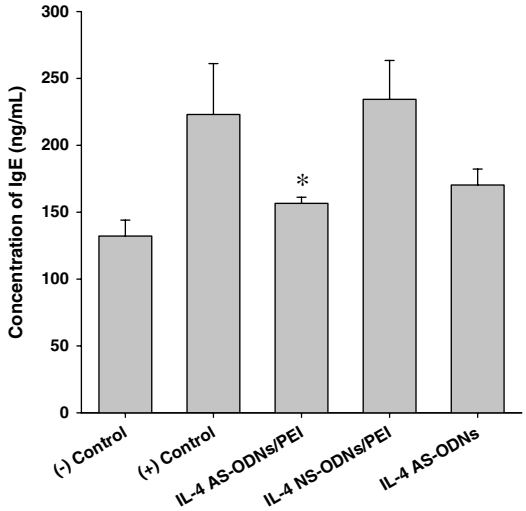


Figure 6. Reduction of IL-4 levels in BALB/c mice sensitized to OVA treated with IL-4 AS-ODNs/PEI complexes. BALB/c mice were sensitized and rechallenged with OVA as described in the Materials and Methods section [25]. Mice received 50 μg of naked IL-4 AS-ODNs, IL-4 AS-ODNs/PEI complex, or NS-ODNs/PEI complex through intranasal administration at days 25, 26, and 27. On day 28, bronchoalveolar lavage (BAL) fluid of the right lung was collected after tying off the left lung at the mainstem bronchus, and the concentration of IL-4 in the BAL fluid was measured by ELISA. The value of R^2 in the standard curve was above 0.99, and the results are expressed as mean values ± s.d. (= 7). *$p < 0.05$

was significantly inhibited (up to 30%) only when the IL-4 AS-ODNs/PEI complex was used. Neither naked IL-4 AS-ODNs nor polyplexes made with nonsense (NS)-ODNs were able to reduce secretion of IL-4 in BAL fluid. These data suggest that IL-4 AS-ODNs/PEI complexes can be applied to allergic asthma to alleviate the inflammation caused by excessive generation of IL-4.

IL-4 AS-ODN polyplexes alleviate allergen-induced airway inflammation

We next determined if administration of IL-4 AS-ODNs/PEI complexes to OVA-sensitized mice can reduce OVA-induced airway inflammation. To this end, blood samples from each group of mice were obtained on day 28 at the same time that BAL fluid was collected, and circulating levels of total IgE were measured by ELISA. As shown in the top panel of Figure 7, mice treated with IL-4 AS-ODNs/PEI complexes show significant reduction of total IgE levels. These results suggest that IL-4 AS-ODNs polyplexes can block IL-4-mediated IgE production. In addition, total cell numbers present in the BAL fluid were also decreased significantly following treatment with IL-4 AS-ODNs/PEI complexes, but not with NS-ODNs/PEI complexes (Figure 7, bottom panel). Thus, it is likely that



Group	Total cell number in BAL
(-) Control	0.95 ± 0.34
(+) Control	1.68 ± 1.12
IL-4 AS-ODNs/PEI	1.04 ± 0.36
IL-4 NS-ODNs/PEI	1.84 ± 0.82
IL-4 AS-ODNs	1.64 ± 0.36

Figure 7. Reduction of IgE levels after treatment with IL-4 AS-ODNs polyplexes and total cell numbers in the BAL fluid. Twenty-four hours after the last intranasal challenge, mice were anesthetized and blood samples were obtained using heart puncture. Blood samples were centrifuged at 4 °C for 5 min at 1200 rpm, and IgE level in the supernatant was measured using ELISA. The results are expressed as mean values ± s.d. (n = 7). *$p < 0.05$. The number of cells present in BAL fluid was counted after Trypan Blue staining. The results are expressed as mean values ± s.d. (n = 7)

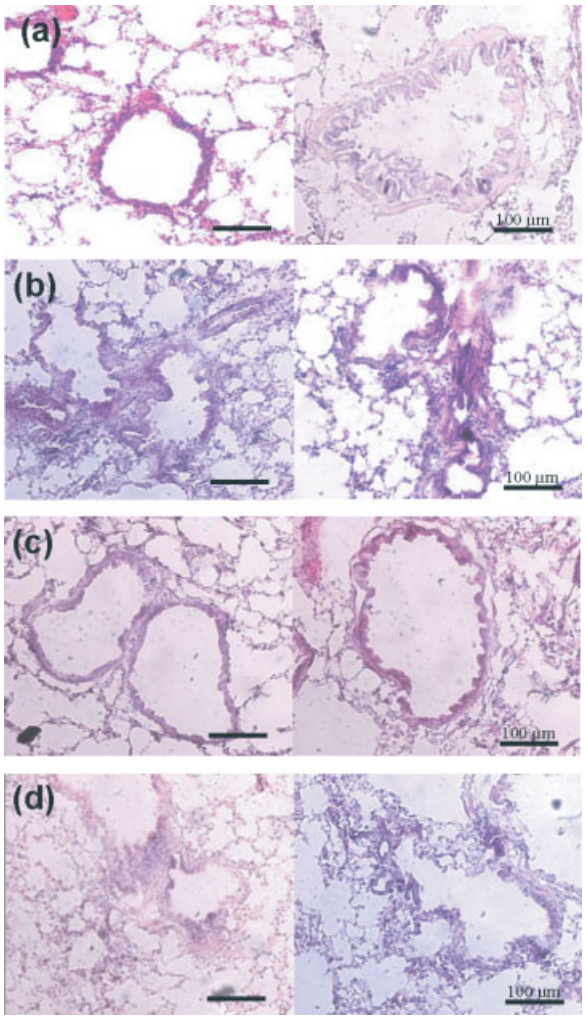


Figure 8. Effects of IL-4 AS-ODNs/PEI complexes on histological analysis of airway inflammation. After BAL extraction, the left lung was obtained from negative control mice (a), positive control mice (b), mice treated with the IL-4 AS-ODNs/PEI complex (c), and mice treated with the IL-4 NS-ODNs/PEI complex (d). Lung tissues were fixed in 4% paraformaldehyde at 4 °C for 24 h, and immersed in 30% sucrose solution at 4 °C for 48 h. After embedding in OCT compound, the tissues were cut into 6 or 10 μm sections. Sections were stained with H&E and analyzed using light microscopy. Bars: 100 μm

IL-4 AS-ODNs polyplexes can inhibit cellular infiltration surrounding airways and pulmonary blood vessels.

To further confirm the effect of IL-4 AS-ODNs/PEI complexes on airway inflammation, the histology of left lung tissues was assessed following treatment with IL-4 AS-ODNs/PEI complexes. As seen in Figure 8, airways became narrow with the recruitment of numerous inflammatory cells in the positive control group (Figure 8b). Following treatment with IL-4 AS-ODNs/PEI complexes, the number of infiltrated inflammatory cells in the airway was significantly decreased and airways became rehabilitated (Figure 8c). In contrast, mice that received IL-4 NS-ODNs/PEI complexes did not show any significant effects (Figure 8d). Taken together, these results strongly suggest that treatment with IL-4 AS-ODNs polyplexes can alleviate allergen-induced airway inflammation *in vivo*.

Discussion

Allergic asthma is a complex inflammatory disease of the airways, characterized by infiltration of inflammatory cells and goblet cell hyperplasia and metaplasia with increased mucin production. The cytokines IL-4, IL-5, IL-9, and IL-13, produced systemically by allergen-reactive Th2 cells and locally by inflammatory cells recruited into the airways, are responsible for many features of allergic asthma [1]. In particular, the critical role of IL-4 in the process of airway inflammation has led to the development of a therapy to block its function. For example, anti-IL4 receptor mAbs were shown to be effective in suppressing antigen-induced IgE synthesis in murine models of inflammation [6]. Soluble IL-4 receptors, now in phase II clinical trials, have been proven to be effective in decreasing inflammation in atopic asthma patients [27].

Antisense oligodeoxynucleotides (AS-ODNs) have been used to specifically inhibit production of target proteins at the level of translation. AS-ODNs function through base pairing and formation of hydrogen bonds with their corresponding complimentary strand of mRNA. Once bound, AS-ODNs inhibit translation by blocking access of ribosomes to the mRNA or by destabilizing mRNA and making it more susceptible to ribonucleases [8]. However, due to the instability and poor cellular targeting of ODNs, large amounts of ODNs were required to obtain visible levels of suppression [28–30]. Thus, the present study was aimed at constructing a targeted nonviral vector based on the PEI backbone for the improved delivery of IL-4 AS-ODNs. At the dose we used in the current study, naked IL-4 AS-ODNs had hardly any effect in inhibiting IL-4 secretion. This might have been due to the poor cellular uptake and high sensitivity to nucleases of the naked AS-ODNs (Figure 4). It is also possible that those few naked ODNs, if any, that entered into the cells could have paired with IL-4 mRNA and reduced the levels of mRNA in the cell. However, it has been shown that down-regulation at the protein level could only be observed when more than 50% of particular mRNA was down-regulated [31]. Thus, the degree of inhibition exhibited by naked ODNs must have been far less than 50% of mRNA down-regulation. Conferring from these data, the PEI formulation used in our study greatly improved the efficiency of AS-ODNs in inhibiting IL-4 production in the murine Th2 cell line and the *in vivo* model of airway inflammation.

PEI exists as two types: a branched or linear form. The size of PEI varies from 2 to 800 kDa. It has been shown that the linear form of PEI is less toxic and has higher transfection efficiency than branched PEI [32,33]. In addition, the smaller size PEI was found to be less toxic as compared to the larger size PEI [34,35]. We also found that linear and smaller PEI was more effective than branched PEI in both transfection and cell viability assay (data not shown), thus linear PEI of lower molecular weight (22 kDa) was chosen for this study. As a result, polyplexes generated in this study did not show any

significant toxicity, and the effect of IL-4 AS-ODNs in inhibiting IL-4 production was maximized.

By varying the amount of PEI to be complexed with IL-4 AS-ODNs, we found that *in vitro* optimal delivery and its suppressive effect in producing IL-4 could be obtained at the NP ratio of 10 (Figure 2b). In addition, at this ratio, the significant *in vitro* toxicity associated with PEI did not develop (Figure 3). However, IL-4 AS-ODNs/PEI complexes the NP ratio of 10 caused slight toxicity in a murine model of allergic asthma (data not shown). A previous study had also shown that positively charged polyplexes at NP ratios of 10 caused acute toxicity, though neutral complexes the NP ratio of 5 did not [21]. Thus, the NP ratio of 5 was more appropriate for the *in vivo* experiments although the NP ratio of 10 was most effective *in vitro*. It was found that intranasal administration of the IL-4 AS-ODNs/PEI complex to OVA-challenged mice resulted in significant reduction of IL-4 concentration in the BAL fluid (Figure 6). Moreover, serum IgE levels and infiltration of inflammatory cells into the airway (Figure 7) were reduced by treatment with IL-4 AS-ODNs polyplexes. Histological study of lung tissues also showed that airway inflammation was alleviated in the mice treated with the IL-4 AS-ODNs/PEI complexes (Figure 8). The *in vivo* effects of the IL-4 AS-ODNs/PEI complexes appeared to be mediated by increased cellular uptake and increased stability of IL-4 AS-ODNs within the cell, as shown in the *in vitro* study (Figure 4). In addition, the ability of PEI polyplexes to escape from the endosome upon entry into the cell might also have contributed to increased cytosolic concentration of IL-4 AS-ODNs, which resulted in efficient blockade of IL-4 translation [17].

It is clear from our data that PEI complexation of IL-4 AS-ODNs significantly increases the ability of AS-ODNs to block translation. While naked IL-4 AS-ODNs showed minimal inhibition (less than 1%), polyplexes containing the same concentration of IL-4 AS-ODNs showed up to 35% decrease in IL-4 secretion. However, with our current regimen of polyplex treatment, complete inhibition of IL-4 secretion was not achieved. Multiple factors might have contributed to this. First, IL-4 AS-ODNs generated against the translation initiation region (+4 to +25) of IL-4 mRNA might not have resulted in complete hybridization. Second, although the resistance to DNase I was achieved by PEI complexation, other destructive enzymes in the cells might have been accessible to polyplexes and reduce the intracellular level of naked IL-4 AS-ODNs. Third, turnover of the IL-4 protein might be much slower than *de novo* synthesis of IL-4 proteins from its mRNA, so that the residual level of IL-4 proteins might have come from those already generated and secreted into the medium. These possibilities might have contributed to the partial inhibition of IL-4 secretion.

Taken together, our studies suggest that the PEI-based AS-ODNs therapy provides a valuable tool in regulating the expression of IL-4 in the treatment of established asthma and in limiting the progression of chronic inflammatory responses in airway remodeling. Furthermore, our data provide a possibility that application of PEI polyplexes can be more broadly applied to other diseases where local delivery can be successfully made.

Acknowledgements

This research was partly supported by a grant from the National Research Laboratory Program (Lab No 2000-N-NL-01-C-171) in the series MOST-NRDP from the Ministry of Science and Technology, Korea. K.-M. Lee is supported by KRF-2004-015-C00409.

References

1. Cohn L, Elias JA, Chupp GL. Asthma: mechanisms of disease persistence and progression. *Annu Rev Immunol* 2004; **22**: 789–815.
2. Steinke JW, Borish L. Th2 cytokines and asthma. Interleukin-4: its role in the pathogenesis of asthma, and targeting it for asthma treatment with interleukin-4 receptor antagonists. *Respir Res* 2001; **2**: 66–70.
3. Chen L, Grabowski KA, Xin JP, *et al*. IL-4 induces differentiation and expansion of Th2 cytokine-producing eosinophils. *J Immunol* 2004; **172**: 2059–2066.
4. Watanabe K, Jose PJ, Rankin SM. Eotaxin-2 generation is differentially regulated by lipopolysaccharide and IL-4 in monocytes and macrophages. *J Immunol* 2002; **168**: 1911–1918.
5. Henderson WR, Chi EY, Maliszewski CR. Soluble IL-4 receptor inhibits airway inflammation following allergen challenge in a mouse model of asthma. *J Immunol* 2000; **164**: 1086–1095.
6. Gavett SH, O'Hearn DJ, Karp CL, *et al*. Interleukin-4 receptor blockade prevents airway responses induced by antigen challenge in mice. *Am J Physiol* 1997; **272**: L253–61.
7. Shoji Y, Nakashima H. Current status of delivery systems to improve target efficacy of oligonucleotides. *Curr Pharm Des* 2004; **10**: 785–796.
8. Akhtar S, Hughes MD, Khan A, *et al*. The delivery of antisense therapeutics. *Adv Drug Deliv Rev* 2000; **44**: 3–21.
9. Fiset PO, Soussi-Gounni A, Christodoulopoulos P, *et al*. Modulation of allergic response in nasal mucosa by antisense oligodeoxynucleotides for IL-4. *J Allergy Clin Immunol* 2003; **111**: 580–586.
10. Molet S, Ramos-Barbon D, Martin JG, Hamid Q. Adoptively transferred late allergic response is inhibited by IL-4, but not IL-5, antisense oligonucleotide. *J Allergy Clin Immunol* 1999; **104**: 205–214.
11. Loke SL, Stein CA, Zhang XH, *et al*. Characterization of oligonucleotide transport into living cells. *Proc Natl Acad Sci U S A* 1989; **86**: 3474–3478.
12. Matsukura M, Shinozuka K, Zon G, *et al*. Phosphorothioate analogs of oligodeoxynucleotides: inhibitors of replication and cytopathic effects of human immunodeficiency virus. *Proc Natl Acad Sci U S A* 1987; **84**: 7706–7710.
13. Phillips MI. Antisense inhibition and adeno-associated viral vector delivery for reducing hypertension. *Hypertension* 1997; **29**: 177–187.
14. Shi F, Nomden A, Oberle V, Engberts JB, Hoekstra D. Efficient cationic lipid-mediated delivery of antisense oligonucleotides into eukaryotic cells: down-regulation of the corticotropin-releasing factor receptor. *Nucleic Acids Res* 2001; **29**: 2079–2087.
15. Maurer N, Mori A, Palmer L, *et al*. Lipid-based systems for the intracellular delivery of genetic drugs. *Mol Membr Biol* 1999; **16**: 129–140.
16. Cleek RL, Rege AA, Denner LA, Eskin SG, Mikos AG. Inhibition of smooth muscle cell growth in vitro by an antisense oligodeoxynucleotide released from poly(DL-lactic-co-glycolic acid) microparticles. *J Biomed Mater Res* 1997; **35**: 525–530.
17. Boussif O, Lezoualc'h F, Zanta MA, *et al*. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine. *Proc Natl Acad Sci U S A* 1995; **92**: 7297–7301.

18. Godbey WT, Wu KK, Mikos AG. Tracking the intracellular path of poly(ethylenimine)/DNA complexes for gene delivery. *Proc Natl Acad Sci U S A* 1999; **96**: 5177–5181.
19. Pollard H, Remy JS, Loussouarn G, Demolombe S, Behr JP, Escande D. Polyethylenimine but not cationic lipids promotes transgene delivery to the nucleus in mammalian cells. *J Biol Chem* 1998; **273**: 7507–7511.
20. Kichler A, Leborgne C, Coeytaux E, Danos O. Polyethylenimine-mediated gene delivery: a mechanistic study. *J Gene Med* 2001; **3**: 135–144.
21. Ferrari S, Moro E, Pettenazzo A, Behr JP, Zacchello F, Scarpa M. ExGen 500 is an efficient vector for gene delivery to lung epithelial cells in vitro and in vivo. *Gene Ther* 1997; **4**: 1100–1106.
22. Chollet P, Favrot MC, Hurbin A, Coll JL. Side-effects of a systemic injection of linear polyethylenimine-DNA complexes. *J Gene Med* 2002; **4**: 84–91.
23. Kircheis R, Kichler A, Wallner G, *et al*. Coupling of cell-binding ligands to polyethylenimine for targeted gene delivery. *Gene Ther* 1997; **4**: 409–418.
24. Roehm NW, Rodgers GH, Hatfield SM, Glasebrook AL. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. *J Immunol Methods* 1991; **142**: 257–265.
25. Henderson WR Jr, Lewis DB, Albert RK, *et al*. The importance of leukotrienes in airway inflammation in a mouse model of asthma. *J Exp Med* 1996; **184**: 1483–1494.
26. Godbey WT, Wu KK, Mikos AG. Poly(ethylenimine) and its role in gene delivery. *J Control Release* 1999; **60**: 149–160.
27. Borish LC, Nelson HS, Lanz MJ, *et al*. Interleukin-4 receptor in moderate atopic asthma. A phase I/II randomized, placebo-controlled trial. *Am J Respir Crit Care Med* 1999; **160**: 1816–1823.
28. Finotto S, Buerke M, Lingnau K, Schmitt E, Galle PR, Neurath MF. Local administration of antisense phosphorothioate oligonucleotides to the c-kit ligand, stem cell factor, suppresses airway inflammation and IL-4 production in a murine model of asthma. *J Allergy Clin Immunol* 2001; **107**: 279–286.
29. Finotto S, De Sanctis GT, Lehr HA, *et al*. Treatment of allergic airway inflammation and hyperresponsiveness by antisense-induced local blockade of GATA-3 expression. *J Exp Med* 2001; **193**: 1247–1260.
30. Allakhverdi Z, Allam M, Renzi PM. Inhibition of antigen-induced eosinophilia and airway hyperresponsiveness by antisense oligonucleotides directed against the common beta chain of IL-3, IL-5, GM-CSF receptors in a rat model of allergic asthma. *Am J Respir Crit Care Med* 2002; **165**: 1015–1021.
31. Mathew E, Hardee GE, Bennett CF, Lee KD. Cytosolic delivery of antisense oligonucleotides by listeriolysin O-containing liposomes. *Gene Ther* 2003; **10**: 1105–1115.
32. Wiseman JW, Goddard CA, McLelland D, Colledge WH. A comparison of linear and branched polyethylenimine (PEI) with DCChol/DOPE liposomes for gene delivery to epithelial cells in vitro and in vivo. *Gene Ther* 2003; **10**: 1654–1662.
33. Wightman L, Kircheis R, Rossler V, *et al*. Different behavior of branched and linear polyethylenimine for gene delivery in vitro and in vivo. *J Gene Med* 2001; **3**: 362–372.
34. Bieber T, Elsasser HP. Preparation of a low molecular weight polyethylenimine for efficient cell transfection. *Biotechniques* 2001; **30**: 74–77, 80, 81.
35. Fischer D, Bieber T, Li Y, Elsasser HP, Kissel T. A novel non-viral vector for DNA delivery based on low molecular weight, branched polyethylenimine: effect of molecular weight on transfection efficiency and cytotoxicity. *Pharm Res* 1999; **16**: 1273–1279.