

Potential Use of Iontophoresis for Transdermal Delivery of NF- κ B Decoy Oligonucleotides

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Abstract

Topical application of nuclear factor- κ B (NF- κ B) decoy appears to provide a novel therapeutic potency in the treatment of inflammation and atopic dermatitis. However, it is difficult to deliver NF- κ B decoy oligonucleotides (ODN) into the skin by conventional methods based on passive diffusion because of its hydrophilicity and high molecular weight. In this study, we evaluated the *in vitro* transdermal delivery of fluorescein isothiocyanate (FITC)-NF- κ B decoy ODN using a pulse depolarization (PDP) iontophoresis. *In vitro* iontophoretic experiments were performed on isolated C57BL/6 mice skin using a horizontal diffusion cell. The apparent flux values of FITC-NF- κ B decoy ODN were enhanced with increasing the current density and NF- κ B decoy ODN concentration by iontophoresis. Accumulation of FITC-NF- κ B decoy ODN was observed at the epidermis and upper dermis by iontophoresis. In mouse model of skin inflammation, iontophoretic delivery of NF- κ B decoy ODN significantly reduced the increase in ear thickness caused by phorbol ester as well as the protein and mRNA expression levels of tumor necrosis factor- α (TNF- α) in the mice ears. These results suggest that iontophoresis is a useful and promising enhancement technique for transdermal delivery of NF- κ B decoy ODN.

Keywords: Iontophoresis, NF- κ B decoy, Oligonucleotides, Transdermal delivery, Skin inflammation

1. Introduction

Atopic dermatitis (AD) is a chronically relapsing inflammatory disorder with pruritic eczematous skin lesions usually associated with elevated serum IgE levels (Cooper, 1994, Rudikoff and Lebwohl, 1998). Both genetic and environmental factors are known to be involved in the pathogenesis of AD (Abramovits, 2005). The skin lesions of AD patients are characterized by the presence of an inflammatory infiltrate consisting of T lymphocytes, monocytes/macrophages, eosinophils, and mast cells (Uehara et al., 1990, Soter, 1989). These cells are involved in the pathogenesis and development of AD through the release of various cytokines and chemokines including interleukin-4 (IL-4), IL-5, IL-13 (Homey et al., 2006), and granulocyte macrophage colony-stimulating factor (GM-CSF) (Pastore et al., 1997). Tumor necrosis factor- α (TNF- α) is also implicated in the pathogenesis of AD and plays an important role in the early stage of AD (Homey et al., 2006).

Recent progress in molecular biology has provided new insights into the pathophysiology of AD. Numerous cytokines related to AD seem to be regulated by certain transcriptional factors. In particular, NF- κ B is the center of interest, since it regulates the expression of genes related to immunostimulatory cytokines such as IL-1 β , IL-2, IL-6, IL-12, GM-CSF and TNF- α as well as adhesion molecules such as intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule (VCAM) (Brennan et al., 1990, Morishita et al., 1994, Banchereau and Steinman, 1998) or MHC class II co-stimulatory molecules (CD80, CD86 and CD40) on antigen-presenting cells (Yoshimura et al., 2001, O'Sullivan and Thomas, 2002). Sustained activation of NF- κ B is reported in inflammatory lesions in diverse diseases like rheumatoid arthritis, inflammatory bowel, psoriasis, asthma and atopic dermatitis (Makaroy, 2000, Isomura

and Morita, 2006). Therefore, regulation of the NF- κ B signaling pathway may offer a significant target to control those immune disorders.

Recently, the decoy strategy has been developed and considered a useful tool as a new class of anti-gene method (Morishita et al., 1998, Morishita et al., 2000). Transfection of double-stranded oligodeoxynucleotides (ODN) as a decoy corresponding to cis-sequence results in the attenuation of authentic cis-trans interaction, leading to the removal of trans-factors from endogenous cis-elements with subsequent modulation of gene expression (Morishita et al., 1998, Morishita et al., 2000). Therefore, the decoy approach enables us to treat diseases by modulating endogenous transcriptional regulation (Morishita et al., 1997, Kawamura et al., 1999, Tomita et al., 1999, Tomita et al., 2000, Kawamura et al., 2001). It was hypothesized that the skin's allergic response in AD might be improved by the transfection of NF- κ B decoy ODN. If the expression of cytokines and adhesion molecules involved in the development of atopic skin conditions, the most important step in the process of AD might be suppressed by NF- κ B decoy ODN *in vivo* (Nakamura et al., 2002).

In general, antisense ODN, siRNA and decoy ODN have difficulty permeating the skin through the stratum corneum due to their high ionization, low lipophilicity, and high molecular weight (Aramaki et al., 2003, De Rosa et al., 2005, Kigasawa et al., 2010). Previously, it has been reported that ointment containing NF- κ B decoy ODN can be used for the treatment of AD in NC/Nga mice by a conventional method based on passive diffusion. However, a high dose, with frequent applications for over than 20 weeks was needed (Nakamura et al., 2002).

Iontophoresis is known to accelerate transdermal permeation of charged molecules by applying a low level electric current to the skin, as a noninvasive enhancement

technique (Kalia et al., 2004). Iontophoresis is classified by the current flow pattern into continuous direct current (CDC) and pulse depolarization current (PDP). CDC iontophoresis is the most common, but its use is sometimes restricted by irritation attributed to the skin polarization. PDP iontophoresis has desirable electrical characteristics having a depolarization process between high electric pulses. The depolarization process restores the electrical polarized state of the skin to its initial state and contributes to the elimination of skin irritation (Okabe et al., 1986, Zakzewski et al., 1992). Successful delivery of hydrophilic macromolecules, such as antisense ODN (Aramaki et al., 2003) and siRNA (Kigasawa et al., 2010) by iontophoresis has been reported. However, there is no report on the iontophoretic delivery of decoy ODN including NF- κ B decoy ODN up till now.

In the present study, we examined the *in vitro* transdermal delivery of FITC-NF- κ B decoy ODN using PDP iontophoresis and evaluated the parameters affecting the efficacy of the enhancement technique. In addition, the stability and localization of FITC-NF- κ B decoy ODN in the skin were examined. Moreover, the effect of iontophoretic delivery of NF- κ B decoy ODN on the expression level of TNF- α in phorbol ester-induced skin inflammation animal model was also investigated.

2. Materials and Methods

2.1. Mice

Male C57BL/6 mice (7 weeks old) and female BALB/c mice (5 weeks old) housed under specific pathogen free (SPF) conditions were purchased from SLC (Shizuoka, Japan).

2. 2. *Chemicals and reagents*

Proteinase K was purchased from Wako Pure Chemical Industries (Osaka, Japan). 12-*O*-Tetradecanoylphorbol-13-acetate (TPA) was obtained from Sigma-Aldrich (St. Louis, MO). NF- κ B decoy ODN, FITC-NF- κ B decoy ODN (NF- κ B decoy ODN labeled with FITC at the 5' end) and scrambled decoy ODN were synthesized by Hokkaido System Science (Sapporo, Japan). The sequences of the NF- κ B decoy ODN and scrambled decoy ODN were as follows (consensus sequences are underlined): NF- κ B decoy ODN, 5'-CCTTGAAGGGATTCCCTCC-3', 3'-GGAACTTCCCTAAAGGGAGG-5', and scrambled decoy ODN, 5'-TTGCCGTACCTGACTTAGCC-3', 3'-AACGGCATGGACTGAATCGG-5'. All other chemicals and solvents were of analytical reagent grade.

2.3. *In vitro iontophoretic permeation experiments*

The hair was shaved from the abdominal skin region of male C57BL/6 mice using an animal hair clipper 24 h before the start of the experiment. The abdominal skin was isolated, and the adhered fat as well as visceral tissues were removed using a blunt scalpel. Finally, the skin was washed with distilled water and observed physically for any damage then equilibrated for 1 h with phosphate-buffered saline (PBS, pH 7.4). The skin of 2.5 cm² surface area was mounted between the donor and receptor compartments of a horizontal diffusion cell with the stratum corneum side facing the donor compartment. The cathodal (donor) chamber contained 2 mL of FITC-NF- κ B decoy ODN solution (2.5-16.5 μ g/mL) in Tris-EDTA buffer (TE, pH 8.0), and the anodal (receptor) chamber was filled with 4 mL of PBS (pH 7.4) with constant stirring

at 600 rpm. Ag/AgCl (anodal/cathodal) electrodes were then connected to an ADIS (Ver 6.0) power supply apparatus (ADVANCE, Tokyo, Japan). Experiments were performed using PDP with a constant voltage of 20 V, current densities of 0-0.5 mA, frequency of 40 kHz, and duty of 30% for 6 h at 25°C. At appropriate time intervals, 100 μ L samples were withdrawn from the receptor compartment and replaced by an equal volume of fresh buffer solution. The concentration of FITC-NF- κ B decoy ODN in each receiver sample was determined by measuring the fluorescence intensity using a fluorescence spectrophotometer (F-4500, Hitachi, Tokyo, Japan) at an excitation wavelength of 495 nm and emission wavelength of 520 nm.

2. 4. Stability of FITC-NF- κ B decoy ODN in the skin

FITC-NF- κ B decoy ODN were extracted from the skin as described before (Sakamoto et al., 2004). Briefly, the whole skin after 6 h PDP iontophoresis was added to 2 mL of DNA extraction buffer (0.5% SDS, 10 mM NaCl, 20 mM Tris-HCl, 10 mM EDTA, pH 7.6) and was homogenized with a Polytron homogenizer (ULTRA-TURRAX T25, IKA Works, Wilimington, NC). Proteinase K solution (final concentration; 1 mg/mL) was added to the homogenate followed by incubation at 55°C for 4 h. After addition of an equal volume of phenol/chloroform (1: 1 v/v) solution to the homogenate, the mixture was centrifuged at 12,000 x g for 20 min at 4°C to extract FITC-NF- κ B decoy ODN into the supernatant. Then, the supernatant was mixed with a half-volume of chloroform and centrifuged. The upper fraction was mixed with a 1/10 volume of sodium acetate (3.5 M, pH 5.2) and 2.5 times the volume of 100% ethanol then kept on ice for 15 min. The mixture was centrifuged at 12,000 x g for 20 min at 4°C. The supernatant was removed and the pellet was washed with 1 mL of 70% ethanol followed

by centrifugation at 12,000 x g for 10 min at 4°C. The supernatant was aspirated and the pellet was dried at 37°C for 10 min then resuspended in TE buffer (pH 8.0). Aliquots of the donor, receptor and skin-extracted solutions were electrophoresed using 2% agarose gel.

2.5. *Localization of FITC-NF-κB decoy ODN in the skin*

Localization of FITC-NF-κB decoy ODN in the skin was confirmed by a confocal laser scanning microscopy (CLSM) through measuring of FITC of the decoy ODN, according to the method with some modifications (Cullander and Guy, 1992, Turner et al., 1997, Sakamoto et al., 2004). The hair on the abdominal and dorsal skin of C57BL/6 mice was shaved 24 h before application of FITC-NF-κB decoy ODN. Mice were anaesthetized by intraperitoneal injection of 120 μL of urethane (25% in PBS, pH 7.4). A cathode (AgCl) with absorbent cotton (0.79 cm²) containing 100 μL of FITC-NF-κB decoy ODN (33 μg/mL) was set on the abdominal skin. While, an anode (Ag) with absorbent cotton moistened with 100 μL of PBS (pH 7.4) was set on the dorsal skin. Each electrode was connected to ADIS (Ver 6.0) power supply (ADVANCE, Tokyo, Japan) run in the pulse depolarized mode. Iontophoresis of FITC-NF-κB decoy ODN was carried out with a constant voltage of 20 V, constant current of 0.3 mA, frequency of 40 kHz, and duty of 30% for 2.5 h. As a control, passive diffusion of FITC-NF-κB decoy ODN was performed. The skin under the cathode was excised immediately after iontophoresis, embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), and snap frozen in liquid nitrogen. The skin was then cut by a cryostat (Leica CM3050S, Leica Microsystems, Japan) into 10 μm sections and examined by light microscope for histological changes. To analyze

the distribution of FITC-NF- κ B decoy ODN in the skin, the cross sections were rinsed three times with PBS (pH 7.4) and mounted without fixation with glycerol/water (9/1 (v/v)). The sections were examined by CLSM (Olympus Fluoview FV300, Olympus Optical, Tokyo, Japan).

2.6. Induction of skin inflammation in BALB/c mice

TPA was used as an inducer of skin inflammation (Kang et al., 2007). The ear thickness of untreated mice was measured using dial thickness gauge (Teclock Corporation, Nagano, Japan) as a reference. One hundred microliters of NF- κ B decoy ODN (30, 60 or 120 μ g), TE buffer or scrambled decoy ODN (120 μ g) was pretreated on the dorsal surface of the mouse ear for 2.5 h by cathodal iontophoresis in the pulse depolarized mode (constant voltage of 20 V, constant current of 0.3 mA, frequency of 40 kHz, and duty of 30%). Next, 3 μ L of TPA (2 mg/mL) was applied to the dorsal surface of the ear to induce skin inflammation. At 4 h after the topical application of inducer, ear thickness was measured again, and the changes in ear thickness (ear swelling) were calculated.

2.7. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was isolated using TRIzol[®] Reagent (Invitrogen, Carlsbad, CA) according to manufacturer's procedure. Briefly, the mouse ear was homogenized in 1 mL TRIzol[®] Reagent. Chloroform (0.2 mL) was then added to the homogenate, and the mixed suspension was centrifuged at 12,000 x g for 15 min at 4°C. The total RNA was recovered in the aqueous phase, and precipitated by an addition of 2-propanol. After centrifugation, the pellet was washed once with 80% ethanol, dried, and redissolved in

50 μ L of diethyl pyrocarbonate-treated water. RNA was then treated with DNase I (Takara, Tokyo, Japan) according to the manufacturer's instructions. The synthesis of the first strand cDNA was carried out with high capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster city, CA). Approximately, 2.5 μ M random primer was annealed to 2 μ g of total RNA and extended with 200 U of Multiscribe Reverse Transcriptase in 20 μ L of reaction containing 2 μ L of first strand buffer and 0.8 μ L of deoxyribonucleotide triphosphate (dNTPs). Reverse transcription was carried out at 25°C for 10 min followed by 37°C for 2 h, and heating at 85°C for 5 min. The expression of mRNA transcripts of TNF- α (forward: 5'-CAAAGGGATGAGAAGTTCCCAA-3', reverse: 5'-CTCCTGGTATGAGATAGCAAA-3'), and β -actin (forward: 5'-TGGAATCCTGTGGCATCCATGAAAC-3', reverse: 5'-TAAAACGCAGCTCAGTAACAGTCCG-3') was determined by the reverse transcriptase-polymerase chain reaction (RT-PCR). PCR amplification was carried out in a PCR Thermal Cycler (Takara Bio, Shiga, Japan). PCR was conducted in a total volume of 30 μ L with 200 ng of the cDNA, 200 μ M of each dNTP, 1.25 U of TaKaRa Ex Taq DNA polymerase and 200 nM of both forward and reverse primers. The thermal cycling conditions were set to 94°C for 8 min, followed by 45 cycles of amplification at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min for denaturing, annealing, and extension. After the last cycle, the samples were incubated at 72°C for 7 min. The amplified products were separated on 2% agarose gels by electrophoresis and visualized with 0.1% ethidium bromide under UV light.

2.8. Real-time quantitative RT-PCR for mouse TNF- α

TNF- α mRNA in the mice ear skin was quantified by real-time quantitative RT-PCR. Real-time quantitative PCR was carried out with TaqMan Universal PCR Master Mix by 7500 Real-Time PCR System (Applied Biosystems, Foster city, CA) under the following conditions: 95°C for 10 min followed by 45 cycles at 95°C for 15 s, and 60°C for 1 min. TaqMan primer pair, probe set for mouse TNF- α , and the primers for mouse β -actin were designed using Primer Express® 3.0 software (Applied Biosystems, Foster city, CA). Primer and TaqMan probe sequences for TNF- α ; F: 5'-CATCTTCTCAAAATTCGAGTGACAA-3', R: 5'-TGGGAGTAGACAAGGTACAACCC-3', P: 5'-CACGTCGTAGCAAACCACCAAGTGGA-3'. β -actin primer pair (forward: 5'-TGGAATCCTGTGGCATCCATGAAAC-3', reverse: 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'). Additional reactions were performed on each 96-well plate using known dilutions of mouse genomic DNA as a PCR template to allow construction of a standard curve relating threshold cycle to template copy number where the amount of TNF- α was normalized by the amount of β -actin cDNA.

2.9. Enzyme-linked immunosorbent assay (ELISA)

The protein levels of TNF- α in the ear of the TPA-induced inflammation model mice in the absence and presence of NF- κ B decoy ODN after 2.5 h iontophoresis were assessed using a commercially available ELISA kit for mouse TNF- α (BD OptEIA™, BD Biosciences Pharmingen, San Diego, CA), as previously reported (Pizarro et al., 1993, Kubota et al., 1997). Briefly, resected ear (35-45 mg) was homogenized with a Polytron homogenizer (ULTRA-TURRAX T25, IKA Works, Wilmington, NC) in 500

μL of ice-cold PBS containing protease inhibitors (1 mmol/L phenylmethylsulfonyl fluoride, 0.3 μmol/L pepstatin A, and 2 μmol/L leupeptin) (Nacalai Tesque, Kyoto, Japan). The homogenate was centrifuged and the supernatant was collected. Total protein levels were determined using a Bio-Rad Protein Assay system (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin as a standard (0-2.0 mg/mL). The samples were standardized to 40 μg of total proteins in 100 μL of diluent buffer for each immunoassay. Mouse TNF-α provided by the manufacturer was used as a standard (0-5000 pg/mL). Results were analyzed spectrophotometrically at a wavelength of 450 nm with a microplate reader.

2.10. Statistical analysis

Data are given as the mean±S.E.M. Statistical significance of means for the studies was determined by analysis of variance followed by Scheffe's test. *p*-Values for significance were set at 0.05.

3. Results and discussion

3.1. *In vitro* iontophoretic permeation experiments

It is difficult to deliver NF-κB decoy ODN into skin by conventional methods based on passive diffusion, because of its hydrophilicity and high molecular weight. Therefore, we evaluated the *in vitro* transdermal delivery of FITC-NF-κB decoy ODN using PDP iontophoresis. At first, we examined the effects of current densities on the permeation of FITC-NF-κB decoy ODN through isolated mice skin, using a horizontal diffusion cell. The cumulative amount of FITC-NF-κB decoy ODN (pmol/cm²) in the

receptor solution was estimated, then plotted against time (h) and the apparent flux was calculated from the slope of the regression line (Fig. 1). As shown in Fig. 2, the fluxes of FITC-NF- κ B decoy ODN increased proportionally with the increase in the current density ($R^2=0.9873$). The mean flux values (J_{ss}), permeability coefficient (P_{app}) and enhancement ratio (ER_{flux} , flux with iontophoresis/flux without iontophoresis) are summarized in Table 1. The J_{ss} , P_{app} and ER_{flux} values significantly increased with increasing the current density, suggesting that FITC-NF- κ B decoy ODN are permeated through skin by iontophoresis in a current density-dependent manner. These results were in accordance with previous reports on other compounds such as verapamil (Wearley and Chien, 1990), diclofenac (Hui et al., 2001), ketorolac (Tiwari and Udupa, 2003) and [32 P]-labeled phosphodiester ODN (Aramaki et al., 2003). From the safety point of view, 0.5 mA/cm² of current density has been suggested as the upper limit in transdermal drug delivery (Singh and Maibach, 1994). Therefore, we decided to set the current density at 0.3 mA in the subsequent experiments.

Next, we investigated the effects of FITC-NF- κ B decoy ODN concentrations in a donor phase on their permeation. As shown in Fig. 3, the cumulative amount of FITC-NF- κ B decoy ODN permeated was increased in a decoy ODN concentration-dependent manner. In sharp contrast, without iontophoresis (control), the total permeated amount and the flux values of FITC-NF- κ B decoy ODN were negligible even with increasing the decoy ODN concentration. A linear relationship was observed between the concentrations of FITC-NF- κ B decoy ODN and the fluxes with a good correlation coefficient ($R^2=0.9958$) (Fig. 4). The J_{ss} , P_{app} and ER_{flux} are summarized in Table 2. Unlike the passive permeation result, the J_{ss} and ER_{flux} values augmented as the concentration of FITC-NF- κ B decoy ODN increased, suggesting that

FITC-NF- κ B decoy ODN permeate through skin according to Faraday's law (Phipps et al., 1989) under the experimental conditions. In sharp contrast, the P_{app} values in the decoy ODN concentration of 5, 10 and 16.5 μ g/mL in a donor phase tended to be lower than that of 2.5 μ g/mL in a donor phase. This may be due to the lower activity of the decoy ODN in more concentrated solution (Santi et al., 1993). Similar findings were reported by other researchers for metoprolol (Thysman et al., 1992), atenolol HCl (Jacobsen, 2001), [32 P]-labeled phosphodiester ODN (Aramaki et al., 2003), rotigotine (Nugroho et al., 2004) and dopamine agonist 5-OH DPAT (Nugroho et al., 2005). Therefore, these results indicate that PDP iontophoresis enhances the *in vitro* transdermal delivery of FITC-NF- κ B decoy ODN as other ODN and drugs.

The Nernst-Planck equation, a trinomial equation, consisting of the passive flux, the electrophoretic movement, and the convective flow (electro osmosis), describes the flux of an ion under the influence of both a concentration gradient and an electric field, and has been often used to describe iontophoretic transport of ions (Banga and Chein, 1988). In the present study, the passive flux is probably negligible, since little permeation was observed without current (Figs. 1 and 3). At physiological pH, the skin is negatively charged and cation permselective (Burnette and Ongpipattanakul, 1987). Thus, current passage causes a net convective solvent flow in the anode-to-cathode direction, facilitating cation transport and inhibiting that of anions, in addition to being a major mode of transport for neutral molecules (Pikal, 1992). The effect of convective flow is also negligible, since no flux was observed from [32 P]D-ODN anodal side which is a negatively charged molecule similar to NF- κ B decoy ODN (Aramaki et al., 2003). Brand and Iversen (1996) have also reported that electrorepulsion is the predominant mechanism for iontophoretic ODN penetration. Therefore, the enhanced permeation

of FITC-NF- κ B decoy ODN by PDP iontophoresis may be well attributed to the electrophoretic movement. This consideration may be supported by the fact that the apparent flux of FITC-NF- κ B decoy ODN increased proportionally to the current density (Fig. 2) and to its initial concentration (Fig. 4).

3.2. *Stability of FITC-NF- κ B decoy ODN in the skin*

It has been reported that NF- κ B decoy ODN applied to skin can be absorbed through hair follicles, and persist within the epidermis and dermis for at least 4 days (Nakamura et al., 2002). Considering the delivery of NF- κ B decoy ODN which selectively inhibit the expression of targeted cytokine genes (Tomita et al., 1998), intact NF- κ B decoy ODN retained in the shallow layer of the skin would be crucial for its effect. Therefore, we examined the stability of FITC-NF- κ B decoy ODN during and after PDP iontophoresis by agarose gel electrophoresis. Sample solutions withdrawn from the donor and receptor compartments periodically and the skin extracted solution after iontophoresis for 6 h were subjected to agarose gel electrophoresis. As shown in Fig. 5, intact NF- κ B decoy ODN were detected in the donor compartment and in the skin during and after 6 h iontophoresis. On the other hand, faint band for NF- κ B decoy ODN was observed in the receptor compartment. These results suggest that NF- κ B decoy ODN applied to skin by iontophoresis retain their intact form in the skin.

3.3. *FITC-NF- κ B decoy ODN localization in the skin*

To evaluate the localization of NF- κ B decoy ODN in the skin, abdominal skin was cross-sectioned after iontophoresis of FITC-NF- κ B decoy ODN. Fluorescence of FITC was observed at the epidermis and upper dermis, and iontophoresis allowed

FITC-NF- κ B decoy ODN to penetrate the stratum corneum, although FITC-NF- κ B decoy ODN remained in the stratum corneum (Fig. 6). On the other hand, no fluorescence was observed in the stratum corneum, when no current was applied (Fig. 6). These observations suggest that the permeation of NF- κ B decoy ODN through the skin was enhanced by iontophoresis. Similar to our findings using CLSM, iontophoresis has been found to enhance the transdermal permeation of other ODN such as rhodamine-labeled antisense ODN (Sakamoto et al., 2004) and Cy3-labeled siRNA (Kigasawa et al., 2010).

3.4. *Effects of NF- κ B decoy ODN on induction of skin inflammation in mice*

Induction of acute skin inflammation by skin irritants causes vasodilation, leukocyte infiltration and edema, resulting in the increase of skin thickness (Kang et al., 2007). Therefore, in our experiment, change in ear thickness was used as a parameter of skin inflammation caused by TPA, which is a specific activator of protein kinase C (PKC) and induce NF- κ B activation (Chang et al., 2005). Figure 7 shows that TPA (2 mg/mL) caused a substantial increase in ear thickness, and pretreatment of NF- κ B decoy ODN by iontophoresis inhibited the increase in ear thickness in a concentration-dependent manner. In contrast, application of scrambled decoy ODN or TE buffer showed no significant effect on the ear thickness. These results suggest that pretreatment of NF- κ B decoy ODN by iontophoresis suppresses the inflammation caused by TPA in mouse skin.

Next, we investigated whether NF- κ B decoy ODN delivered via iontophoresis could alter the level of expression of a target gene involved in inflammation. Activation of NF- κ B protein is known to transactivate targeted genes such as IL-1 β , IL-2, IL-6, IL-12,

TNF- α , CD54, and vascular cell adhesion molecule-1 (VCAM), responsible for inflammation and AD (Banchereau and Steinman, 1998). Then, we investigated the effects of NF- κ B decoy ODN on the *TNF- α* mRNA level in mice ears using RT-PCR. As shown in Fig. 8A, *TNF- α* mRNA was hardly detected in the ear of the non-treated group. In contrast, significant induction of *TNF- α* mRNA was observed by TPA application. The *TNF- α* mRNA induced by TPA was significantly suppressed by the treatment of NF- κ B decoy ODN in a dose-dependent manner. As anticipated, the *TNF- α* mRNA level was not significantly different in mice received scrambled decoy ODN or TE buffer, compared to those treated with TPA alone. To determine the *TNF- α* mRNA level more quantitatively, we examined using a real time qRT-PCR. As shown in Fig. 8B, the *TNF- α* mRNA level in the group of iontophoresis of 120 μ g of NF- κ B decoy ODN was significantly reduced to $38.54 \pm 3\%$ of control (the TPA-treated group) (Fig. 8B). In contrast, scrambled decoy ODN and TE buffer had no inhibitory effect on *TNF- α* mRNA level throughout this study.

Next, we performed ELISA to determine the amount of TNF- α protein in the mice ears. As shown in Fig. 9, substantial amount of TNF- α protein in the TPA-treated skin in mice was significantly reduced by iontophoresis of 120 μ g of NF- κ B decoy ODN. In contrast, no apparent differences in the TNF- α protein level were observed in the mice treated with scrambled decoy ODN or TE buffer, compared to TPA-treated group. These results suggest that pretreatment of NF- κ B decoy ODN by iontophoresis suppresses the inflammation caused by TPA through the impairment of TNF- α induction in mouse skin.

The skin irritation such as erythema, eschar and edema during iontophoresis are known as its major side effects. Under the *in vivo* experimental conditions, the skin

irritation was not observed (data not shown). The negligible skin irritation could be attributed to the adequate iontophoresis condition, because no significant skin irritation was observed in a current density of 0.5 mA/cm² for up to 4 h in rabbits (Anigbogu et al., 2000). The similar results were reported by Kumar and Lin (2008). Hence, current density of 0.38 mA/cm² used in this study should be well tolerated in mice.

The NF- κ B is activated immediately after aggregation of Fc ϵ RI on mast cells and dendritic cells, and also activated in B cells and T cells, when they are stimulated via CD40 or T-cell receptors, respectively (Tanaka et al., 2007). Meanwhile, TNF- α is an inflammatory cytokine produced primarily by activated macrophages but also by other cell types including epidermal keratinocytes (Chen and Goeddel, 2002), and then TNF- α triggers a series of intracellular events, resulting in the activation of transcription factors, including NF- κ B, AP-1, CCAAT enhancer-binding protein β , and others (Banno et al., 2004). Thus, NF- κ B is likely to activate in various cells in skin. Therefore, it is speculated that NF- κ B decoy ODN were taken up by various inflammatory cells including mast cells, dendritic cells, lymphocyte, macrophages and keratinocytes in the TPA-treated skin in mice. Hereafter, the elaborate study is further required for cellular localization of NF- κ B decoy ODN in skin after iontophoresis.

In conclusion, we report the development of a PDP iontophoretic delivery system for NF- κ B decoy ODN. As far as we know, this is the first report on transdermal delivery of decoy ODN using iontophoresis as well as the suppression of TNF- α expression by NF- κ B decoy ODN introduced by iontophoresis into skin. These results suggest that iontophoresis is a useful and promising enhancement technique for transdermal delivery of NF- κ B decoy ODN.

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Figure Legends

Figure 1. Effects of current densities on the permeation of FITC-NF- κ B decoy ODN through isolated mouse skin. Iontophoresis was performed with various current densities (0, 0.1, 0.5 and 0.5 mA) at 10 μ g/mL of FITC-NF- κ B decoy ODN for 6 h. Each point represents the mean \pm S.E.M. of 3 experiments.

Figure 2. Effects of current densities on the flux of FITC-NF- κ B decoy ODN through isolated mouse skin. Iontophoresis was performed with various current densities (0, 0.1, 0.5 and 0.5 mA) at 10 μ g/mL of FITC-NF- κ B decoy ODN for 6 h. Each point represents the mean \pm S.E.M. of 3 experiments.

Figure 3. Effects of FITC-NF- κ B decoy ODN concentrations on its permeation through isolated mouse skin. Iontophoresis was performed with a constant current density (0.3 mA) at various concentrations of FITC-NF- κ B decoy ODN (2.5, 5, 10 and 16.5 μ g/mL) for 6 h. Each point represents the mean \pm S.E.M. of 3 experiments.

Figure 4. Effects of FITC-NF- κ B decoy ODN concentrations on the flux through isolated mouse skin. Iontophoresis was performed with a constant current density (0.3 mA) at various concentrations of FITC-NF- κ B decoy ODN (2.5, 5, 10 and 16.5 μ g/mL) for 6 h. Each point represents the mean \pm S.E.M. of 3 experiments.

Figure 5. Stability of FITC-NF- κ B decoy ODN in mouse skin, donor and receptor phases during *in vitro* permeation study.

Figure 6. Cross sections showing the *in vivo* distribution of FITC-NF- κ B decoy ODN in mouse skin after iontophoresis.

Figure 7. Effects of NF- κ B decoy ODN on TPA induced skin inflammation in mice. NF- κ B decoy ODN were pretreated on mouse ear for 2.5 h by iontophoresis before treatment with TPA. Ear thickness was measured after treatment with TPA for 4 h and ear swelling was determined. Data are presented as mean \pm S.E.M. of 4-6 experiments. *, $p < 0.05$ versus TPA alone.

Figure 8. Effects of NF- κ B decoy ODN on *TNF- α* mRNA induction caused by TPA in mouse ear. NF- κ B decoy ODN were pretreated on mouse ear for 2.5 h by iontophoresis before treatment with TPA. Total mRNA was isolated after treatment with TPA for 4 h. (A) *TNF- α* mRNA levels in the mice ears were determined by semi-quantitative RT-PCR. (B) Relative *TNF- α* mRNA expression level was determined using real time qRT-PCR and normalized by that of *β -actin* mRNA. Data are presented as mean \pm S.E.M. of 3 experiments. *, $p < 0.05$ versus TPA alone.

Figure 9. Effects of NF- κ B decoy ODN on TNF- α induction caused by TPA in mouse ear. NF- κ B decoy ODN was pretreated on mouse ear for 2.5 h by iontophoresis before treatment with TPA. Total protein was separated after treatment with TPA for 4 h. TNF- α protein level was determined by ELISA. Data are presented as mean \pm S.E.M. of 3 experiments. *, $p < 0.05$ versus TPA alone.

Table Legends

Table 1. Skin permeation parameters of FITC-NF- κ B decoy ODN after iontophoretic application of different current densities.

Table 2. Skin permeation parameters after passive or iontophoretic application of different concentrations of FITC-NF- κ B decoy ODN.