



Pharmaceutical nanotechnology

Functional peptide nanocarriers for delivery of novel anti-RelA RNA interference agents as a topical treatment of atopic dermatitis



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ABSTRACT

Small interfering RNAs (siRNAs) are a potential treatment of atopic dermatitis (AD) because they can specifically silence the gene expression of AD-related factors. However, siRNA alone cannot exert a sufficiently strong therapeutic effect due to low delivery efficiency to the target tissues and cells; simply increasing the amount used is not possible due to the possibility of off-target effects. We previously reported a novel class of therapeutic RNA interference (RNAi) agents called nkRNA[®] and PnkRNA[®], which have been shown to be effective in several disease models, have greater resistance to nuclease degradation than canonical siRNAs, and do not induce any immunotoxicity. In the present study, we describe a non-invasive and effective transdermal RNAi therapeutic system for atopic dermatitis that uses the functional cell-penetrating stearyl-oligopeptide OK-102 as a cytoplasm-responsive nanocarrier for nkRNA[®] and PnkRNA[®]. The two RNAi agents were targeted against RelA, a subclass of NF-κB (nuclear factor kappa B), and, as part of OK-102 complexes, they strongly silenced RelA mRNA in macrophage cells and demonstrated a significant therapeutic effect in a mouse model of AD. It was shown that OK-102-complexed RNAi agents were an efficient therapeutic system for AD and caused no adverse reactions.

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1. Introduction

Atopic dermatitis (AD) is a chronic inflammatory disease of the skin that is characterized by an itchy red rash; its pathogenesis is complex and relates to both genetic and environmental factors (Leung, 2000). In its most potent form, AD results in a very low quality of life and, as a result, an effective, non-toxic treatment is necessary.

The use of topical corticosteroids, in combination with aggressive moisturizing, is the most commonly used and most effective treatment of AD. However, the use of high-strength corticosteroids combined with an occlusive dressing can cause side effects such as an increase in the risk of skin thinning. In addition, the long-term use of steroids can result in complications such as weight gain, reduced bone density, and suppression of the immune

system. An alternative, non-steroidal, treatment is the tacrolimus ointment, which is effective and has a minimal side-effect profile (Cheer and Plosker, 2001).

Nuclear factor kappa B (NF-κB) plays a critical role in the immunological disturbances that are observed in AD, and a small molecule NF-κB inhibitor and NF-κB decoy oligodeoxynucleotides (ODNs) have been reported to ameliorate atopic skin lesions in NC/Nga mice (Morishita et al., 1997; Tanaka et al., 2006; Dajee et al., 2006). Additionally, an ointment containing NF-κB decoy ODNs was shown to be an effective treatment of atopic dermatitis lesions in NC/Nga mice (Nakamura et al., 2002). The topical administration of NF-κB decoy ODNs twice a month resulted in a significant reduction in clinical skin condition scores, and a marked improvement was observed according to histological data. The topical application of NF-κB decoy ODNs appears to be a potent therapeutic that does not cause significant side effects.

Small interfering RNA (siRNA) allows the specific silencing of genes related to AD pathogenesis through a process called RNA interference (RNAi). Therefore, it is also expected to offer a means of treating AD without causing side effects. In our previous study, we endeavored to treat AD using siRNA that was targeted against

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RelA—the most important NF- κ B subdomain with respect to the production of inflammatory cytokines (Paul, 1999; Uchida et al., 2011a,b). We found that the use of cell-penetrating peptides to deliver anti-RelA siRNA was an effective therapeutic in a mouse model of AD. In general, the therapeutic effectiveness of siRNA is hampered by low delivery efficiency and poor specificity to target tissues and cells. The half-life of naked siRNA is very short because they are rapidly degraded by nucleases in the serum and extracellular fluids. Furthermore, there are concerns regarding the possibility of adverse effects and immunotoxicity caused by off-target delivery. The effectiveness of RNAi could be improved by the use of the novel agents, nkRNA[®] and PnkRNA[®], which were developed by Hamasaki et al. (2012) who also demonstrated their effectiveness in mouse models of acute lung injury and pulmonary fibrosis. Low-cost, large-scale production of these RNAi agents is simple, because they are synthesized as single-stranded RNAs (ssRNAs) that spontaneously self-anneal. In addition, these novel RNAi agents have been shown to be effective in several disease models and have higher stability towards nuclease degradation than canonical siRNA (Hamasaki et al., 2012). Furthermore, these RNAi agents have not been found to elucidate immunotoxic side effects based on an evaluation of proinflammatory cytokine induction (Hamasaki et al., 2012). Therefore, these novel RNAi agents can be regarded as safe and might be used to replace siRNA in clinical therapies (Hamasaki et al., 2012; Fujita et al., 2013; Nakama et al., 2015).

Although, nkRNA and PnkRNA are more resistant to degradation than siRNA, a carrier is still required to improve cellular uptake, endosomal escape and release in the cytoplasm. OK-102 is a cytoplasm-responsive peptide that we developed for use as a gene carrier in a previous study (Tanaka et al., 2010a,b). OK-102 has the peptide sequence Cys-His-His-Arg-Arg-Arg-His-His-Cys and features an N-terminal stearyl group. We previously demonstrated that OK-102 strikingly enhanced *in vitro* and *in vivo* siRNA silencing efficiency. It was also shown that OK-102 enhanced local gene expression in vaginal mucosa in an *in vivo* study (Kanazawa et al., 2013). This carrier peptide forms a complex with nucleotides because of ionic interactions between its cationic arginine groups and the anionic phosphate groups in RNA. Additionally, rigidity is imparted to the complex by the formation of intermolecular disulfide cross-links between Cys residues. In environments such as the extracellular matrix, tissue fluids, and blood there is a low concentration of the tripeptide reducing agent glutathione (GSH, γ -glutamyl-cysteinyl-glycine), the Cys-Cys disulfide bonds remain intact, and the nucleic acids in the complex are effectively protected against nucleases. In contrast, the intracellular environment has a GSH concentration that is approximately 1000 times higher than the extracellular environment; this promotes the cleavage of the disulfide linkages and triggers the release of the siRNA payload. The other residues of OK-102 also contribute to its effectiveness as a gene delivery agent. The oligoarginine moiety induces higher endocytic uptake by target cells, and endosomal escape is triggered by histidine. Therefore, these carrier peptides have enable high transfection efficiency without cytotoxicity, as extensively reviewed previously (Okada et al., 2014).

Topical application of therapeutic peptides, proteins and nucleic acids, such as plasmid DNA and siRNA, has been increasingly studied because of the medical importance of treating skin diseases, topical vaccination, and improving skin properties (Partidos et al., 2003; Lopes et al., 2005). However, the effectiveness of topically administered therapeutics is reduced by low permeation efficiency across the skin. The stratum corneum, nucleated epidermal layers, and tight junctions are all important physical barriers (Proksch et al., 2008). To improve transdermal delivery, physical enhancement methods—including, iontophoresis, sonophoresis,

and electroporation—have been developed. However, these methods are invasive and cause topical damage and, therefore, less-invasive and easier topical delivery methods are required. One such method is the use of cell-penetrating peptides. Several previous studies have reported that arginine-rich peptides and transcriptional activator (TAT)-derived peptide are capable of passing through animal skin (Rothbard et al., 2000; Hou et al., 2007; Robbins et al., 2002). Additionally, we previously reported that TAT peptide widely and effectively accelerates transdermal siRNA delivery (Uchida et al., 2011a,b). Therefore, it is expected that OK-102 can also be used to deliver RNA into the skin.

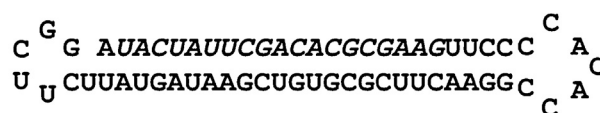
In the present study, OK-102-complexed nkRNA and PnkRNA were investigated as a novel transdermal RNAi therapeutic system and its effectiveness was evaluated against AD-like ear lesions induced in NC/Nga mice. We determined the *in vitro* silencing efficiency of OK-102-complexed anti-RelA nkRNA and PnkRNA in mouse macrophages and evaluated the anti-inflammatory effects in AD model mice following topical application of the therapeutic system.

2. Materials and methods

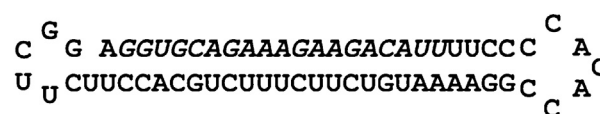
2.1. RNAs, peptides, cells and animals

The long RNA oligomers anti-RelA nkRNA (62-mer) and PnkRNA (51-mer) were synthesized using a solid-phase method as single-stranded RNAs (ssRNAs) that self-anneal into a unique helical structure containing a central stem and two loops according to a previously reported procedure (Hamasaki et al., 2012; Fujita et al., 2013; Nakama et al., 2015). The sequences and structures of the RNAi agents are shown in Fig. 1. PnkRNA incorporates a proline-derived molecule. OK-102 peptide was

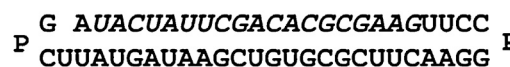
(a) Scrambled nkRNA



(b) Anti-RelA nkRNA



(c) Scrambled PnkRNA



(d) Anti-RelA PnkRNA

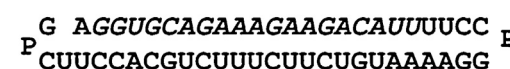


Fig. 1. Sequences and structures of RNAi agents.

(a) Scrambled nkRNA, (b) anti-RelA nkRNA, (c) scrambled PnkRNA, (d) anti-RelA PnkRNA. P, proline-derived molecule; RNAi, RNA interference.

purchased from BEX Co., Ltd. (Tokyo, Japan). OK-102-complexed RNAi agents with a nitrogen to phosphorous (N/P) ratio of 10 were prepared by mixing both solutions for 30 min at room temperature before use. RAW264.7 murine macrophage cells were purchased from American Type Culture Collection (WA, USA). Cell culture medium, Dulbecco's modified Eagle's medium (DMEM), certified fetal bovine serum (FBS), and penicillin/streptomycin stock solutions were purchased from Life Technologies Japan (Tokyo, Japan).

Six-week-old male NC/Nga mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The mice were housed under controlled conditions of 22–24 °C, 40–60% humidity, and 12-h light/dark-cycles with the light period starting at 8:00 a.m. Food and water were supplied ad libitum. All experiments with animals were carried out in accordance with a protocol approved by the Animal Care and Ethics Committee of Tokyo University of Pharmacy and Life Sciences.

2.2. In vitro silencing study

RAW264.7 cells were cultured to 70–80% confluence in DMEM with 10% FBS, and 1% penicillin/streptomycin (stock solution 10,000 U/mL; 10,000 µg/mL). The cells (1×10^5 cells/well) were seeded into 24-well culture plates. After 24–48 h incubation in serum-containing DMEM, the cells were washed with phosphate-buffered saline (PBS), and then serum-free DMEM (900 µL) was added to each well. A solution of RNAi agent complex (100 µL; 50 nM RNAi agent) was applied to each well. After 24 h, the medium was removed and replaced with serum-containing DMEM (900 µL), and lipopolysaccharide (LPS) solution (100 µL; 10 mg/mL) was added. After 8 h, the cells were washed twice with PBS, and total RNA was isolated using the TRIzol reagent (Life Technologies Japan) according to the manufacturer's instructions. Real-time polymerase chain reaction (PCR) was used to quantify the levels of RelA in the cells. cDNA was synthesized using the isolated total RNA and the PrimeScript™ reagent kit (Takara Bio Inc., Shiga, Japan) at 42 °C for 15 min and then at 85 °C for 5 s. Real-time PCR was performed with cDNA, TaqMan® Universal PCR Master Mix No AmpErase® UNG (Life Technologies Japan), and primers. The expression level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the internal control. The PCR primers to detect mouse GAPDH were purchased from Life Technologies Japan (product code, Mm99999915_g1). To detect mouse RelA mRNA (Forward 5'-gtgcgacaaggtgcagaaag-3'; reverse 5'-ccgatgcacatcagcttgag-3'), primers were designed by Nippon Gene Material Co., Ltd. (Toyama, Japan). The relative content of mRNA was calculated according to the cycle threshold (Ct) value and standard curve equation. Data were normalized to give the mRNA expression of RelA in each sample. Relative RelA mRNA was calculated based on the RelA mRNA of the control group (LPS-treated cells that received no RNAi agent).

2.3. Evaluation in AD model mice treated with RNAi agents

AD-like skin lesions were induced in NC/Nga mice by repeated topical application of 2,4-dinitrofluorobenzene (DNFB) (Wako Pure Chemical Ind., Ltd.). NC/Nga mice were first sensitized with DNFB dissolved in acetone by topical application onto foot pads and shaved back on days 0 and 3. After the first sensitization, DNFB dissolved in acetone/olive oil was applied to the left ear on days 7 and 10. Three days after the first sensitization, scrambled or anti-RelA RNAi agents with OK-102 complex solutions were administered to the left ears of AD-model mice two or three times a week for 2 weeks. The left ear was covered with olive oil after every administration. The ear thickness and clinical skin severity score were periodically examined throughout the study. Ear thickness was measured using a digital thickness gauge

(Mitutoyo Co., Kanagawa, Japan). The skin lesions were examined by macroscopic observation. The clinical score was measured by assessing the individual clinical severity of redness and deformation between 0 and 1, and assessing hemorrhage, dryness and thickness from 0 to 2. The levels of the local inflammatory cytokines tumor necrosis factor alpha (TNF-α) and interleukin (IL)-6 in the left ear lobe were measured using an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Inc., MN, USA). For ELISA analysis, the left ear lobe was excised and homogenized in lysis buffer (ethylenediaminetetraacetic acid, polyoxyethylene octylphenyl ether, Tween 80, NaCl, and Tris-HCl).

2.4. Statistical analysis

All values are expressed as means ± S.E. Statistical analysis of the data was performed using analysis of variance (ANOVA) followed by Dunnett's test. Statistical significance was defined as ** $P < 0.01$ and * $P < 0.05$.

3. Results

3.1. Silencing efficiency of OK-102-complexed RNAi agents

The expression of RelA mRNA in RAW264.7 cells that were transfected with OK-102-complexed anti-RelA RNAi agents for 24 h was determined following LPS-stimulation for 8 h. As shown in Fig. 2, both nkRNA and PnkRNA induced significant, strong silencing effects as compared to non-transfected cells. Treatment of cells with OK-102-complexed scrambled RNAi agent did not induce any silencing effects.

3.2. Therapeutic effect of RNAi agents in AD model mice

The ear thickness (Fig. 3) and macroscopic observations of clinical skin severity (Fig. 4) of AD model mice were evaluated after treatment with OK-102-complexed nkRNA (Figs. 3 and 4(a)) and PnkRNA (Figs. 3 and 4(b)). From day 7, ear thickness increased in untreated AD model mice. The ear thickness in mice treated with both forms of anti-RelA RNAi agent increased less than in the untreated mice. Furthermore, the ear thickness in the anti-RelA

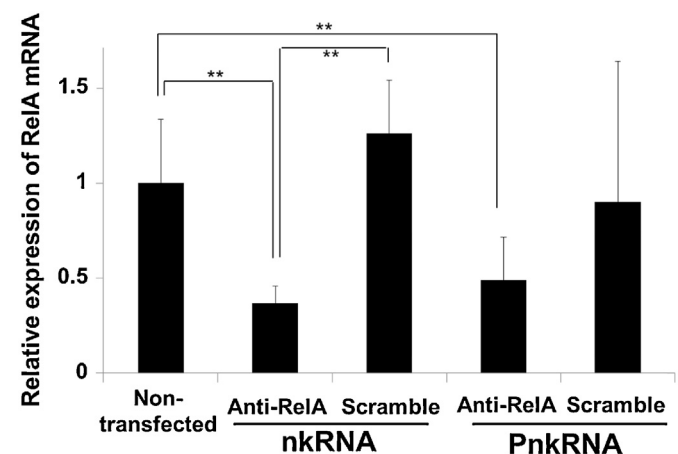


Fig. 2. RelA mRNA expression in RAW264.7 cells after transfection with OK-102-complexed RNAi agents.

RAW264.7 cells were transfected with OK-102-complexed RNAi agents (N/P = 10; 50 nM RNAi agent) for 24 h. After transfection, cells were stimulated with LPS for 8 h. RelA mRNA level was measured using real-time PCR. The relative RelA mRNA was calculated based on the RelA mRNA level of a non-transfected control group. Each bar represents the mean ± S.D. ($n = 4$). ** $P < 0.01$. LPS, lipopolysaccharide; N/P, ratio of nitrogen to phosphorous; PCR, polymerase chain reaction; RNAi, RNA interference.

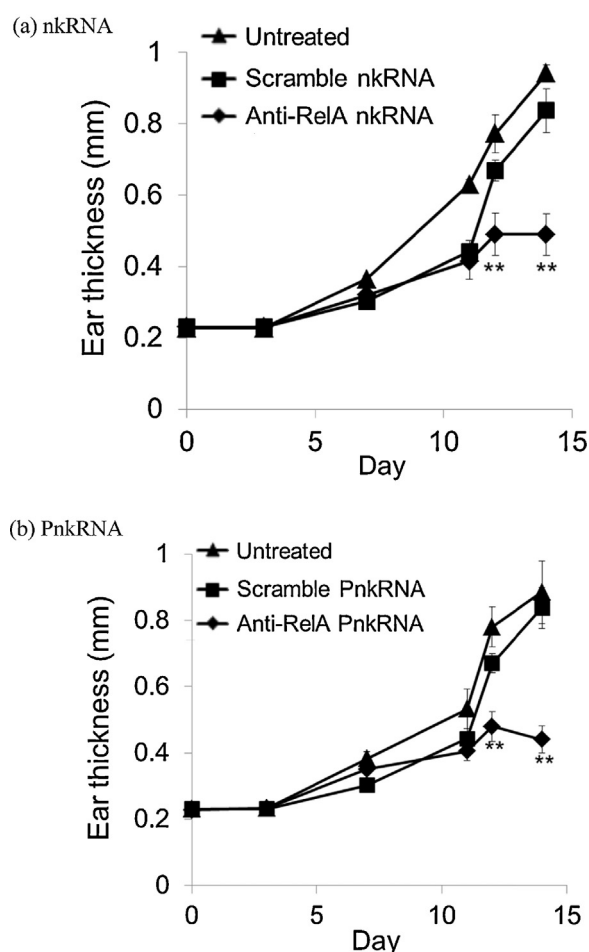


Fig. 3. Thickness of ear lobes in AD model mice treated with OK-102-complexed RNAi agents.

OK-102-complexed (a) nkRNA or (b) PnkRNA was administered to the left ear of AD model mice on days 3, 4, 7, 11 and 12. Ear thickness was measured on days 0, 3, 7, 11, 12 and 14. Each data point represents the mean $\pm$ S.E. ($n=4$). ** $P < 0.01$ vs untreated and scrambled groups on the same day. AD, atopic dermatitis; RNAi, RNA interference.

PnkRNA-treated mice decreased between days 12 and 14. There was no significant difference in the ear thickness between the untreated AD mice and the mice that were treated with OK-102-complexed scrambled RNAi agents.

As shown in Fig. 4, from day 7, the clinical skin severity score in AD-like mice treated with either OK-102-complexed anti-RelA RNAi agent was significantly lower than in untreated AD-like mice. Most notably, the clinical skin severity score in the OK-102-complexed anti-RelA PnkRNA-treated group almost never increased (Fig. 4(b)). There was no significant difference in the clinical score between the untreated AD mice and OK-102-complexed scrambled RNAi agent-treated AD mice.

Fig. 5 shows photographs of AD model mice after treatment with scrambled and anti-RelA RNAi agents that were complexed with OK-102. As shown in Fig. 5(a) and (c) scabs, hemorrhages, dryness, and hypertrophy were observed in AD model mice that had been treated with the scrambled RNAi agents. In contrast, except for slight redness no symptoms were observed in the mice treated with OK-102-complexed anti-RelA nkRNA or PnkRNA (Fig. 5(b) and (d)).

The levels of TNF- α and IL-6 in the left ear of the AD model mice on day 14 were determined using ELISA. As shown in Fig. 6, the concentration of both inflammatory cytokines was increased strikingly in the untreated AD model mice. However, treatment

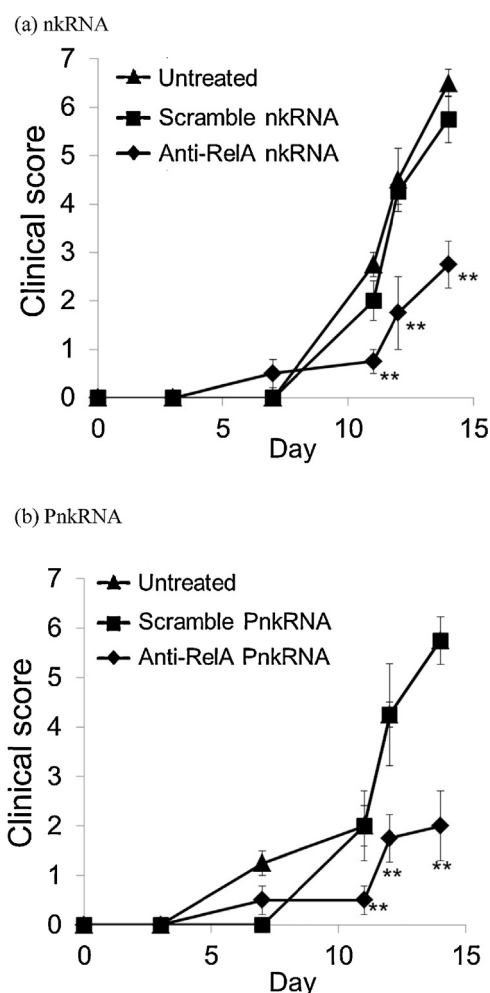


Fig. 4. Clinical score assessment of ear lobes of AD model mice treated with OK-102-complexed RNAi agents.

OK-102-complexed (a) nkRNA or (b) PnkRNA was administered to the left ear of AD model mice on days 3, 4, 7, 11 and 12. Clinical scores were measured on days 0, 3, 7, 11, 12 and 14. Each point represents the mean $\pm$ S.E. ($n=4$). ** $P < 0.01$ vs untreated and scrambled groups on the same day. AD, atopic dermatitis; RNAi, RNA interference.

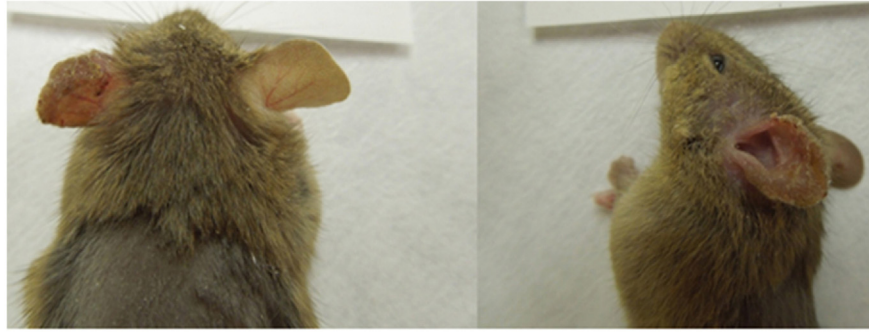
with OK-102-complexed RNAi agents suppressed this increase dramatically and significantly. There was no significant difference in the levels of TNF- α and IL-6 between the untreated AD model mice and the mice treated with OK-102-complexed scrambled RNAi agents.

4. Discussion

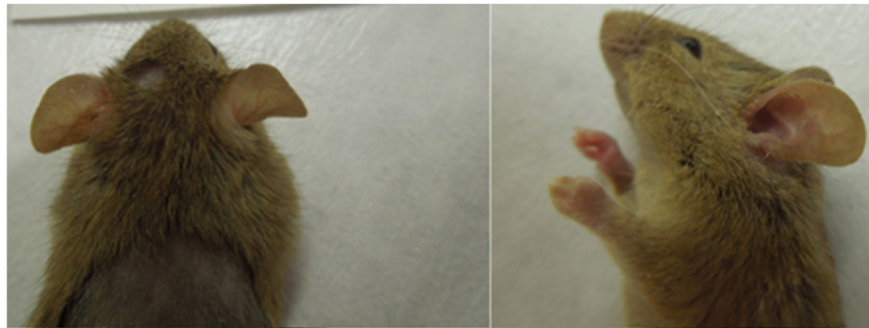
Several investigations have indicated that NF- κ B plays a critical role in the immunological disturbances that accompany AD. Recently, the gene silencing effect of siRNA has led to it being investigated as a novel treatment of allergic skin diseases. We also previously reported that the administration of siRNA targeted against RelA, a subclass of NF- κ B, is an effective treatment of AD-like symptoms in model mice (Uchida et al., 2011a,b). In general, siRNA alone is not able to exert a sufficiently effective therapeutic effect due to only small amounts of the material reaching the target tissue.

In this study, we sought to develop a novel intradermal RNAi therapeutic system for the treatment of AD. We selected the novel RNAi agents, nkRNA and PnkRNA, and complexed them with the peptide OK-102 to improve their stability and delivery efficiency.

(a) OK-102–complexed scrambled nkRNA



(b) OK-102–complexed anti-RelA nkRNA



(c) OK-102–complexed scrambled PnkRNA



(d) OK-102–complexed anti-RelA PnkRNA

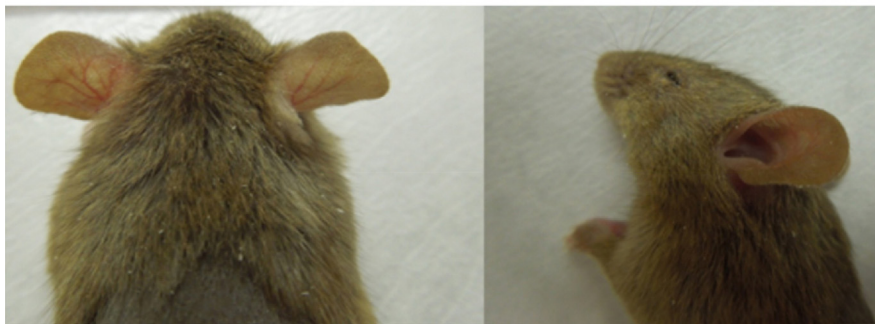


Fig. 5. Photographs of left ear lobe in AD model mice after intradermal application of OK-102–complexed RNAi agents. OK-102–complexed (a) nkRNA or (b) PnkRNA was administered to the left ear of AD model mice on days 3, 4, 7, 11 and 12. Photographs were taken on day 14. (a) Scrambled nkRNA; (b) anti-RelA nkRNA; (c) scrambled PnkRNA; (d) anti-RelA PnkRNA. AD, atopic dermatitis; RNAi, RNA interference.

For the *in vitro* experiments, we used RAW264.7 mouse macrophages as the target cells because macrophages are known to play an important role in controlling the allergic inflammatory responses in diseases such as AD (Arango Duque and Descoteaux,

2014; Valledor et al., 2010). We first determined the RelA mRNA silencing efficiency of OK-102–complexed nkRNA and PnkRNA in LPS-treated RAW264.7 cells (Fig. 2). Real-time PCR indicated that the level of RelA mRNA in cells treated with either anti-RelA RNAi

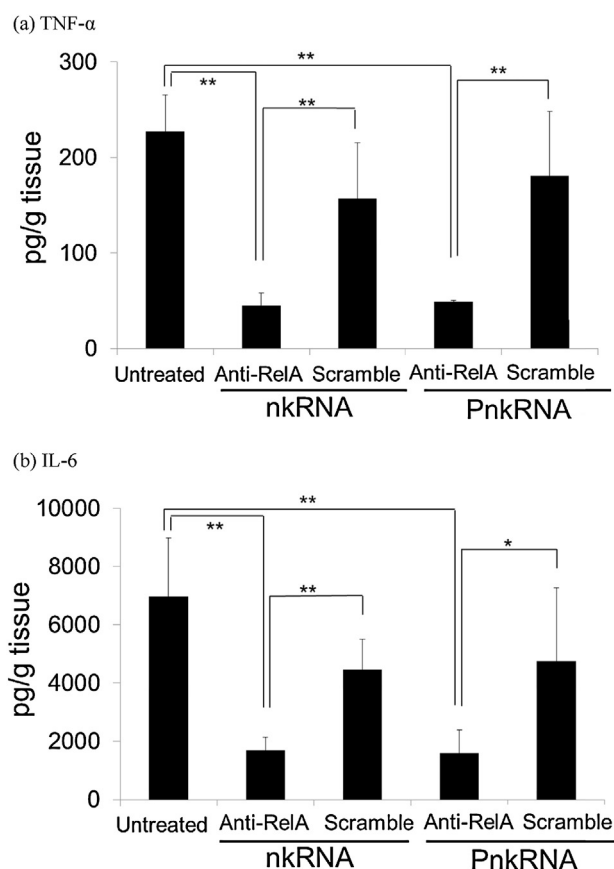


Fig. 6. Levels of inflammatory cytokines in ear lobes of AD model mice after transdermal administration of OK-102-complexed RNAi agents. AD model mice were treated with OK-102-complexed nkRNA (nkRNA, 0.375 nmol) or OK-102-complexed PnkRNA (PnkRNA, 0.375 nmol) with olive oil on the left ear of the on days 3, 4, 7, 11 and 12. Untreated mice received only olive oil. (a) TNF- α and (b) IL-6 in the ear lobe at 14 days after first sensitization were measured by ELISA. Each bar represents the mean $\pm$ S.E. ($n=3$). * $P<0.05$, ** $P<0.01$.

agent was markedly lower than in cells that were not treated with RNAi agents. In addition, it was confirmed that the effect was sequence-specific because OK-102-complexed scrambled RNAi agents failed to silence RelA mRNA expression. This result also indicated that the OK-102 peptide did not silence the genes itself. All of these results suggested that topical application of OK-102-complexed anti-RelA RNAi agents would be effective in *in vivo* experiments using AD-like model mice.

The therapeutic efficacy of RNAi agents *in vivo* was assessed in NC/Nga AD mice that had AD-like symptoms induced using topical DNFB treatment. As shown in Figs. 3 and 4, both OK-102-complexed anti-RelA RNAi agents strongly suppressed DNFB-induced increases in ear thickness and clinical score. No severe dryness, redness and hypertrophy were observed in OK-102-complexed anti-RelA PnkRNA-treated mice. In contrast, scrambled RNAi agents did not suppress the inflammatory symptoms. These results suggest that OK-102-complexed RNAi agents are able to cross the skin barriers reach therapeutically relevant cells such as macrophages and dendritic cells. In several previous reports, arginine-rich peptides improved the transdermal delivery of proteins, peptides, and RNA because of their basic amine groups (Rothbard et al., 2000; Hou et al., 2007; Robbins et al., 2002; Uchida et al., 2011a,b). Therefore, we propose that OK-102 improved the delivery of RNAi agents in this study because of its arginine content. However, the exact mechanism of peptide-mediated delivery of RNAs into epidermal tissue

through the skin barrier layers is not fully understood and further experiments are required.

In addition, we measured the levels of the inflammatory cytokines TNF- α and IL-6 in the ear lobes of the AD model mice. Cytokine levels were evaluated using ELISA on day 14. In the ear lobes of AD model mice, OK-102-complexed anti-RelA RNAi agents significantly suppressed TNF- α and IL-6 production as compared to untreated and scrambled RNAi agent-treated mice (Fig. 6). This indicated that OK-102-complexed anti-RelA RNAi agents suppress the expression of RelA mRNA at inflamed sites since RelA induces the production of inflammatory cytokines. This reduction of cytokine expression then leads to a suppression of the increase of ear thickness and clinical score.

In conclusion, both nkRNA and PnkRNA can be used as anti-RelA RNAi agents for AD therapy, and the OK-102 peptide can be used as the basis of an effective topical therapeutic system. In addition, the use of OK-102-complexed RNAi agents as a topical therapeutic avoids systemic side effects and can therefore be expected to enhance patient compliance and improve patient satisfaction.

Disclosure

T. Hamasaki (TH) is employee of BONAC Corporation. T. Ohgi (TO) is member of the Board of Directors of BONAC Corporation. The patent on nkRNA (WIPO Patent WO/2012/005368A1) and PnkRNA (WIPO Patent WO/2012/017919A1) became public, and in these patents, the name of TH is included. T. Kanazawa, T. Hamasaki, Y. Seta, T. Ohgi and H. Okada have a pending patent (Patent 2014-062686) on this study. The remaining authors have no conflict of interest. The products described above have not been as yet marketed. There are no further patents, products in development or marketed products to declare.

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