Therapeutic Effects on Atopic Dermatitis by Anti-RelA Short Interfering RNA Combined with Functional Peptides Tat and AT1002

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ABSTRACT
Atopic dermatitis (AD) has high morbidity and poor prognosis because safe and effective treatments are scarce. Recently, short interfering RNA (siRNA) has shown promise as an effective treatment for targeting specific aberrantly expressed genes. However, naked siRNAs are too inefficient because of various enzymatic, membrane, and cellular barriers. We previously reported that a Tat analog acting as a cell-penetrating peptide, combined with AT1002, which reversibly increases paracellular transport of molecules across the epidermal barrier in epidermis-disrupted mice and enhances the skin permeation of water-soluble siRNA. In the present study, to develop a novel treatment for AD, we determined the intradermal permeation of siRNAs and the antiallergic effects of a siRNA that silences RelA, a member of the nuclear factor-κB family, using Tat and AT1002 peptides in an AD mouse model. We first showed that the Tat analog and AT1002 delivered siRNA into the skin of ICR mice and, upon topical application to the AD-induced ears of NC/Nga mice, changed zonula occludens protein 1 expression. In addition, the silencing effects on the mRNA of RelA in JAWS II cells transfected with siRNA oligonucleotides for mouse RelA, complexed with Tat, were as effective as a commercial vector. Furthermore, the ear thickness, clinical skin severity, topical cytokine levels, and serum IgE production in AD model mice treated with anti-RelA siRNA with Tat and AT1002 were improved.

Introduction
Atopic dermatitis (AD) is a chronic inflammatory pruritic skin disease with a relapsing course that has gross impact on the patient’s lifestyle. In addition, the side effects associated with increased systemic absorption of commercial drugs have led investigative dermatologists to continue with the development of primarily localized therapeutic alternatives for AD (Scha¨cke et al., 2002; Becker et al., 2006). The cutaneous lesions associated with AD result from the local expression of proinflammatory cytokines and chemokines. Cytokines, such as tumor necrosis factor-α (TNF-α), interleukin (IL)-1α, IL-1β, and IL-4 from resident cells, and activation of Toll-like receptors in response to microbial infection, activate the NF-κB pathway, which in turn leads to the coordinated transactivation of key cytokines and adhesion molecules involved with the pathogenesis of inflammatory diseases (Kuper, 1990; Barnes and Karin, 1997). Because NF-κB plays such a significant role in the pathogenesis of AD, there has been an increased effort to develop topical therapies specifically targeting the NF-κB pathway at the upper current of cytokine production (Nakamura et al., 2002; Furukawa et al., 2004). Avenues of investigative research for blocking NF-κB activity include small molecules and decoy oligonucleotides (Morishita et al., 1997; Dajee et al., 2006; Tanaka et al., 2007). siRNA-based therapeutics have entered clinical trials for age-related macular degeneration, cancers, and infectious diseases caused by viruses such as respiratory syncytial virus and human immunodeficiency virus (Hokaiwado et al., 2008; Chen and Zhaori, 2011). However, it is not known whether treatment with siRNA is an effective alternative to present medications, such as corticosteroids, and specific questions regarding the skin penetration of siRNA remain
TABLE 1
Sequences of siRNAs

<table>
<thead>
<tr>
<th>Name</th>
<th>Sense</th>
<th>Antisense</th>
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<tbody>
<tr>
<td>FAMsiRNA</td>
<td>5’ UUC UUC CUG CAC CdTdT 3’</td>
<td>5’ C GU GUA GAA AGA CAU U TdT 3’</td>
</tr>
<tr>
<td>siRelA</td>
<td>5’ GGU GCA GAA AGA AGA CAU U TdT 3’</td>
<td>5’ A AU GUC UUC UUU CUG CAC COTdT 3’</td>
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Unclear. Topical application of naked siRNA does not exert strong therapeutic effects because of its low permeation efficiency owing to various skin barriers and its degradation by enzymes in the body. The most important function of the skin is to form an effective barrier between the internal and external layers of the organism. Although the stratum corneum (SC) is recognized as an important physical barrier, nucleated epidermal layers are also significant for barrier function, and so, too, are tight junctions (TJs) (Proksch et al., 2008). TJs are cell-cell junctions that connect neighboring cells and control the paracellular pathway of molecules. Zonula occuluta toxin (Zot) has been shown to reversibly open the TJs and thereby increase the paracellular transport of several drugs in a nontoxic manner (Fasano et al., 1995; Watts et al., 2005). Moreover, as an alternative to Zot, advances in the study of structure-activity relationships have led to the identification of AT1002, a novel TJ modulator peptide (Song et al., 2008). AT1002 is a six-mer synthetic peptide that retains nontoxic biological activity by reversibly opening TJs by changing the zonula occludens protein 1 (ZO-1), like Zot, and increases paracellular transport of drugs and even peptides across the epithelial barrier.

In skin diseases such as AD, where the SC is disrupted, the transport of molecules through the SC is considered to be relatively easy. However, in the epidermis, the granular layer has low permeation because of the barriers that are formed by TJs. Even though paracellular delivery to the epidermal layer in AD skin is considered to be a good method, it has proven difficult to achieve. As a result, physical enhancement methods, for example, iontophoresis, sonophoresis, and electroporation, have been developed to improve transdermal delivery. However, these methods are difficult to use because of their invasiveness and topical damage; therefore, less-invasive and easier topical delivery methods are required.

Several proteins with cell-penetrating peptides are capable of passing through animal skin (Robbins et al., 2002; Hou et al., 2007). In addition, we reported previously that a combination of a cell-penetrating peptide (the Tat peptide) and AT1002 strongly increases siRNA stability against RNaseA and accelerates transdermal siRNA delivery both widely and effectively (Uchida et al., 2011). We also found that ZO-1 disappeared from the skin after treatment with AT1002 and gradually recovered with time after washing.

In the present study, to determine the penetration mechanism of our transdermal siRNA delivery system, we observed the codistribution of FAMsiRNA and immunostained rhodamine-ZO-1 in mouse skin applied with FAMsiRNA using the Tat analog combined with AT1002. In addition, the intradermal penetration of FAMsiRNA in the ear skin of atopy-like mice upon application of both naked FAMsiRNA and FAMsiRNA/Tat + AT1002 was determined. Furthermore, we evaluated the therapeutic effects of siRNA silencing RelA, which is a NF-κB family member, using this transdermal system in AD model mice.

**Materials and Methods**

**Animals, Cells, and siRNAs.** Six-week-old female ICR mice were purchased from SLC (Hamamatsu, Japan), and 6-week-old male NC/Nga mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan). The mice were housed under standard conditions of temperature (22–24°C), humidity (40–60%), and 12-h light/dark cycles with the light period starting at 8:00 AM. 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. PAM212 keratinocytes were provided by Prof. Hiroshi Matusuda (Tokyo University of Agriculture and Technology). The siRNA oligonucleotides for mouse RelA (siRelA) were designed and synthesized by Nippon Gene (Tokyo, Japan), and FAM-labeled nonsilencing oligonucleotides for mouse RelA (siRelA) were designed and synthesized by Nippon Gene (Tokyo, Japan). The sequences of the sense and antisense oligos are shown in Table 1. Each siRNA was diluted to a final concentration of 1 mg/ml.

**Peptide Carriers.** The Tat analog, which consists of Cys-Gly-NH2 added to the N terminus of HIV-Tat (48–57), and the AT1002 analog, which consists of Gly and Cys-Gly-NH2 added to the C and N termini of AT1002 (Table 2), were synthesized as PTD or permeabilizing peptides using the 9-fluorenylmethoxycarbonyl solid-phase peptide synthesis method with an ABI 433A peptide synthesizer (Applied Biosystems, Tokyo, Japan) as reported previously (Tanaka et al., 2010; Uchida et al., 2011). Both analogs were used after purification by reverse-phase high-performance liquid chromatography. The molecular weight of each analog was determined by matrix-assisted laser desorption ionization time-of-flight mass spectrometry; Tat analog, 1627; AT1002 analog, 939.

**In Vitro Study.** JAWS II cells were cultured to 70 to 80% confluence in α-minimum essential medium (Cambrex Co., East Rutherford, NJ) supplemented with mouse granulocyte macrophage–colony-stimulating factor (Wako Pure Chemicals, Osaka, Japan), 10% fetal bovine serum (Invitrogen, Carlsbad, CA), and 1% penicillin/streptomycin (stock solution 10,000 U/ml, 10,000 mg/ml, respectively; Invitrogen). JAWS II cells were seeded into 24-well plates at a density of 5 × 105 cells per well with 1 ml of fetal bovine serum (+1% minimum essential medium and incubated at 37°C in a humidified 5% CO2 atmosphere for 48 h before the transfection study. After a 48-h incubation, the cells were rinsed with PBS, and 1 ml of Dulbecco’s modified Eagle’s medium without FBS was added to each well. Either naked siRelA (naked) or siRelA/Tat complex (Tat) solution (100 μl containing siRelA: 0.5 μg) was applied to each well.

**TABLE 2**
Sequences of Tat and AT1002 analogs

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tr>
<td>AT1002 analog (AT1002)</td>
<td>Phe-Cys-Ile-Gly-Arg-Leu-Cys-Gly</td>
</tr>
<tr>
<td>Tat analog (Tat)</td>
<td>Gly-Arg-Lys-Lys-Arg-Gln-Arg-Arg-Cys-Gly</td>
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After 24 h, cells were stimulated with lipopolysaccharide (100 ng) for 8 h. After 8 h, cells were washed twice with PBS, and total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. The primers were designed for PCR amplification of cDNA and the quantification of RelA by real-time PCR. cDNA was synthesized using total RNA and the PrimeScript reagent kit (Takara, Kyoto, 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 (Applied Biosystems), and primers. The expression level of glyceroldehyde-3-phosphate dehydrogenase was determined as the internal control. The PCR primers to detect mouse glyceraldehyde-3-phosphate dehydrogenase were from TaqMan gene expression assays (Applied Biosystems). To detect mouse RelA (forward, 5′-gtgccagacttgccgaaga-3′; reverse, 5′-cgatgccatcagcttgag-3′), the primers were designed by Nippon EGT Co., Ltd. (Toyama, Japan). According to the cycle threshold value and standard curve equation, the relative content of mRNA was calculated and normalized as the mRNA expression of RelA in each sample. The data are expressed as the relative RelA mRNA ratio versus the control value.

**siRNA Application and Observation of FAMsiRNA and ZO-1 in Mouse Skin.** Six-week-old male ICR mice were used in this study. All mice were anesthetized intraperitoneally with pentobarbital (50 mg/kg). The backs of the mice were shaved clean by an electric clipper, and the hair was removed by a cream-based hair remover (Kanebo, Tokyo, Japan). Furthermore, the backs of mice were tape-removed 20 times by surgical tape (Transpose; Sumitomo 3M Limited, Tokyo, Japan), and 25 μl of FAM-labeled siRNA samples (naked siRNA or siRNA/Tat complex with AT1002) were applied to the backs of the mice. After 2 to 24 h, the mice were sacrificed, and then dermal tissues were washed by phosphate-buffered saline and resected in 1-cm² cross-sections of samples at the application site. The tissues were soaked in Tissue Mount (Shiraimatsu, Osaka, Japan) at 4°C in the dark overnight and mounted with Tissue Mount in cold acetone. The tissues were preserved at 4°C in the dark overnight and mounted with Fluorescent-G. FAMsiRNA in the left ear lobes was observed by confocal laser microscopy (450-nm channel).

**Treatment of AD Model Mice with siRNA.** AD model mice (n = 4 per group) were administrated naked siRelA or Tat analog/siRelA (molar ratio of the amine groups in cationic carriers and phosphate groups in siRNA (N/P ratio): 10) complex with AT1002 (400 μg) in 20 μl of PBS (siRelA: 5 μg) on days 11, 13, 15, 18, 20, and 22. Control mice were not treated.

**Evaluation of AD Model Mice Treated with siRelA Samples.** Total IgE concentrations in serum, clinical skin severity score, and ear thickness were periodically examined throughout the study. The clinical skin severity score of dermatitis was measured by summing the individual clinical severity of redness/hemorrhage, edema, acacia/excoriation, dryness, and anoma as 0 or 1. Edema was evaluated by measuring ear thickness. The skin lesions were evaluated by macroscopic observation. Total clinical skin severity score was calculated from the sum of the individual scores graded as none, redness/hemorrhage, edema, acacia/excoriation, dryness, and anoma. Ear thickness was measured using a micrometer. Finally, cytokines (interferon-γ, TNF-α, and IL-13) in the tested ear area and IgE production in serum were measured with an ELISA kit (cytokines) or sandwich ELISA (IgE).

**Statistical Analysis.** All values are expressed as the mean ± S.E. Statistical analysis of the data were performed using an unpaired Student’s t test. Statistical significance was defined as p < 0.05 and < 0.01.

**Results**

**Intradermal siRNA Delivery Using Tat and AT1002 Through the Back Skin of Tape-Stripped Mice.** The distributions of FAM-labeled siRNA (green) and immunostained ZO-1 (red), the latter of which is a TJ protein, in the mouse epidermis after administration of naked siRNA or siRNA/Tat+AT1002 were observed by CLISM. As shown in Fig. 1, ZO-1 was observed at the bottom of the epidermis, and FAMsiRNA was not observed in mice treated with naked siRNA. In contrast, in mice treated with siRNA/Tat + AT1002, FAMsiRNA was observed even in the deep epidermis, and the immunostained ZO-1 was never observed. The penetration of FAMsiRNA was examined on day 11 after sensitization. In untreated control mouse ears, no fluorescence in the epidermis was observed except in the hair follicles. In mice treated with naked siRNA, a small amount of fluorescence of FAMsiRNA was observed. In contrast, in mice treated with siRNA/Tat + AT1002, the fluorescence of FAMsiRNA was observed strongly and widely in the ear skin (Fig. 2).

**The Silencing Effects of siRelA/Tat Complexes.** The silencing effects on the mRNA of RelA in JAWS II cells (mouse dendritic cells) transfected with siRNA for RelA (siRelA) complexed with Tat or LipoTrust are shown in Fig. 3.

As shown in Fig. 3, in JAWS II cells, the mRNA of RelA was silenced by naked siRNA because the uptake activity of foreign
particles by dendritic cells was much higher than that of normal cells. Furthermore, much higher silencing effects by siRNA/LipoTrust or siRNA/Tat (N/P = 10) were observed. These results indicate that siRelA designed in this study can strongly silence the mRNA of RelA, and Tat is as good a carrier as LipoTrust for siRelA delivery into cells with phenotypes related to atopic diseases.

Ear Thickness and Clinical Score in NC/Nga AD Model Mice by siRelA Treatment. We examined whether siRelA had a therapeutic effect on the clinical symptoms of AD. Picryl chloride was repeatedly applied topically to the left ear lobe of NC/Nga mice on days 4, 11, and 18 after first sensitization, and siRelA was applied on days 11, 13, 15, 18, 20, and 22. The ear thickness in NC/Nga AD model mice on days 0, 4, 11, 13, 15, 18, 20, and 22 and 25 is shown in Fig. 4a and b. The ear thickness gradually increased until day 25 in untreated control mice. In contrast, the ear hypertrophy of mice treated by siRelA was strikingly suppressed compared with that of untreated control mice (Fig. 4a). There were no differences in ear thickness between naked siRelA- and

Fig. 1. Effect of functional peptides on localization of ZO-1 and permeability of FAMsiRNA. Naked FAMsiRNA (5 μg) (a) or FAMsiRNA (5 μg) with Tat + AT1002 (32 μg + 400 μg) (b) was applied to 20 times tape-stripped back skin of ICR mice. The skin was harvested after lapses of 5, 10, and 24 h. The skin sections were fixed, and ZO-1 in the skin after processing by immunofluorescence and FAMsiRNA were detected using a confocal laser microscope. Magnification, ×20.

Fig. 2. Permeability of FAMsiRNA on left ear skin in NC/Nga AD model mice. Middle and bottom, the naked FAMsiRNA (5 μg) (middle) or FAMsiRNA (5 μg) with Tat + AT1002 (32 μg + 400 μg) (bottom) was applied to left ear skin of AD model mice for 10 h. Top, control means nonapplied mice. The skin sections (20 μm) were observed using a confocal laser microscope. Magnification, ×20.

Fig. 3. RelA mRNA expression in JAWS II cells after transfection of siRelA. The naked siRelA and siRelA/Tat complex (N/P = 1, 10) were transfected in JAWS II cells (0.5 μg siRelA) for 24 h. After transfection, the cells were stimulated with lipopolysaccharide for 8 h. The RelA mRNA was measured by RT-PCR. The relative RelA mRNA was calculated based on the RelA of the mRNA control group. Each bar represents the mean ± S.D. (n = 4). * P < 0.05; ** P < 0.01 versus control (t test).
siRNA/Tat + AT1002-treated mice. Furthermore, the clinical score of dermatitis was also measured on days 0, 4, 11, 13, 15, 18, 20, 22, and 25 (Fig. 4b) by summing the individual clinical severity of redness/haemorrhage, edema, aconia/excoriation, dryness, and anthoma as 0 or 1. The appearance of ears in NC/Nga AD model mice treated with siRelA samples at days 0 or 25 is shown in Fig. 4c. The total clinical score gradually increased between days 11 and 25 in untreated control mice. Only slight erythema and edema were observed in the ear lobes of mice treated by naked siRelA. Clinical symptoms on day 25 were seldom seen in mice treated by siRelA/Tat + AT1002 (Fig. 4c).

**Histological Observation of Ears in NC/Nga AD Model Mice Treated with siRelA.** The sections of left ear lobes of NC/Nga model mice, with or without siRelA treatment, were examined histologically using hematoxylin and eosin (HE) staining and toluidine blue (TB) staining on day 25 (Fig. 5). HE staining preparations demonstrated obvious hyperplasia of the epidermis and dermis, and infiltration of eosinophils in the dermis, in AD-induced mice. TB staining preparations indicated that mast cells were degranulated in AD-induced mice. Epidermal hyperplasia with areas of parakeratosis and severe infiltrations of eosinophils and a small number of mononuclear cells in the dermis of ears were broadly observed in untreated mice, but scarcely observed in both siRelA-treated mice groups. We also observed an increased number of mast cells in control mice, and most of the mast cells were degranulated in the skin lesions, but this was not the case in siRelA-treated mice (Fig. 5b).

**Topical Cytokines and Serum IgE in AD-Induced Mice with Applied siRelA.** We measured topical cytokines such as TNF-α and IL-4 in ear skin, and total IgE production in serum, in topically induced NC/Nga AD model mice on day 25 by ELISA. As shown in Fig. 6a and b, in the AD-uninduced right ear, there was no difference in the production of TNF-α and IL-4 between all groups. However, both inflammatory (TNF-α) and T-helper 1 type (IL-4) cytokines increased in the left ear lobe of AD model mice, and the application of siRelA clearly decreased the production of both cytokines. In particular, the suppression of cytokine production was significantly
higher in AD-induced mice treated by siRelA/Tat + AT1002 than in those treated by naked siRelA, indicating that Tat and AT1002 played an important role in skin treated by topical siRelA application. Serum IgE at day 25, which is strongly associated with symptoms in AD mice, was decreased in siRelA-treated mice compared with that in untreated mice. The serum levels of total IgE in mice treated with siRelA/Tat + AT1002 were the lowest of all groups (Fig. 6c).

Discussion

Inflammatory stimuli lead to the activation of NF-κB in immune-related cells. Some reports have speculated that NF-κB plays a critical role in the immunological disturbance that is observed in AD. Moreover, NF-κB decoy oligodeoxynucleotides and an NF-κB small-molecule inhibitor, when applied topically, have been reported to be effective in regressing atopic skin lesions in NC/Nga mice (Nakamura et al., 2002; Tanaka et al., 2007). Topical use of siRNA has been increasingly studied because of the importance of treating skin diseases, topical vaccination, and improving skin properties (Partidos et al., 2003; Lopes et al., 2005). In particular, siRNA has been investigated as a novel drug for allergic skin diseases because of its target-factor silencing effect. Therefore, in this study, we selected siRelA, which is one of the NF-κB subdomains, because siRNA can silence disease factor targets selectively, and RelA is both the major NF-κB subdomain and the most important one for inducing the production of inflammatory cytokines.

Topical application of naked siRNA does not exert strong therapeutic effects because of its low permeation efficiency owing to various skin barriers, such as the SC and epidermis, and its degradation by enzymes in the body. To achieve the
therapeutic effects of siRNA in topical applications, it is necessary to overcome various transmembrane and transcellular barriers. The transport of water-soluble and large molecules such as siRNAs through the skin might be difficult even in degenerated skin, such as in AD, where the SC is mostly disrupted. This is because the granular layer in the epidermis shows low permeation through the paracellular barriers that are formed by TJs. Methods to deliver molecules that treat AD must therefore overcome these paracellular barriers. We previously reported that the intradermal delivery of siRNA was strikingly accelerated using a combination of two functional peptides, cell-penetrating peptide (Tat) and TJ modulator (AT1002). In the present study, to develop a noninvasive and effective topical treatment system for AD, we applied siRelA using our combination system of Tat and AT1002.

First, to assess the penetration route of siRNA in the epidermis, we observed the permeation of FAMsiRNA (green) and the distribution of immunostained ZO-1 (red), a TJ protein, in mice epidermis administered with naked siRNA or siRNA/Tat. As shown in the naked siRNA-treated mice, in siRNA/Tat + AT1002-treated mice, FAMsiRNA was observed in the deep epidermis (Fig. 1). Meanwhile, ZO-1 in the epidermis was eliminated or reduced by AT1002 treatment. These results suggest that TJs are disrupted because of the degeneration of the TJ protein ZO-1 and are clearly a barrier for siRNA transdermal delivery during the 24 h after AT1002 application. It has been reported that AT1002 can inhibit the phosphorylation of ZO-1 in mucosal cells and reversibly disrupt the TJs in epithelial membranes, resulting in the enhancement of mucosal absorption of peptides (Song et al., 2008).

As shown in Fig. 2, in AD model mice treated with naked siRNA without peptides, the fluorescence of siRNA was observed in the ear lobes. This result is caused by the disruption of SC and epidermis in AD-induced mice. However, the fluorescence in siRNA/Tat + AT1002-treated mice was obviously stronger than that in the naked siRNA-treated mice, suggesting that the combination of Tat and AT1002 is a good delivery system for water-soluble large molecules such as siRNA.

To determine whether siRNA can penetrate into cells and interact with the mRNA in target cells, the mRNA silencing effects of RelA in mouse dextric cells (JAWS II) transfected with siRelA with or without Tat was measured (Fig. 3). The mRNA silencing effects in JAWS II cells transfected with siRelA complexed with Tat was markedly stronger than in control cells. However, there was hardly a difference between siRelA complexed with Tat and siRNA only, suggesting that the N/P ratio of Tat/siRelA used in this study (JAWS II cells) was not enough to deliver into cells.

The therapeutic efficacy of siRelA was assessed in NC/Nga AD model mice induced by topical application of picryl chloride. Both naked siRelA and siRelA complexed with cell-penetrating peptides, the Tat analog and TJ modulator AT1002, exerted strong therapeutic and preventive effects for AD symptoms (Figs. 4 and 5). Furthermore, siRelA combined with Tat and AT1002 significantly suppressed local TNF-α and IL-4 and serum IgE production in AD model mice compared with naked siRelA (Fig. 6). In a previous report, we showed that Tat increases siRNA stabilization against RNaseA and improves intracellular delivery (Uchida et al., 2011), and in this article, we show that AT1002 enhances siRNA intraderal delivery through the granular layer by modulation of TJs (Fig. 1). In this study, ear thickness of naked siRelA-treated mice almost did not increase as well as that of Tat + AT1002/siRelA-treated mice because skin barriers of AD-induced mice were disrupted and siRelA could enter to under the epidermis without functional peptides in AD-induced mice. Thus, there was no difference between naked siRelA and siRelA combined with Tat + AT1002 in this study. However, naked siRelA can not suppress the inflammatory cytokine production and clinical score completely. Therefore, we believe that functional peptides that can enhance transdermal siRNA delivery and siRNA stabilization are needed for complete therapy for AD.

In conclusion, siRelA could be expected to be the prospective anti-AD drug, and then combination of functional peptides, Tat and AT1002, can improve the treating force of siRelA.

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Authorship Contributions

Participated in research design: Kanazawa and Okada.
Conducted experiments: Uchida, Kanazawa, Kawai, Takashima, and Okada.
Contributed new reagents or analytic tools: Uchida, Kanazawa, Kawai, and Okada.
Performed data analysis: Uchida, Kanazawa, and Okada.
Wrote or contributed to the writing of the manuscript: Kanazawa and Okada.

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Immunity under the skin: potential application for topical delivery of vaccines.


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