Anti-RelA siRNA-Encapsulated Flexible Liposome with Tight Junction-Opening Peptide as a Non-invasive Topical Therapeutic for Atopic Dermatitis

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INTRODUCTION

Atopic dermatitis (AD) is a very common allergic skin disease affecting up to 20% of all children and 10% of all adults in industrialized countries. The underlying mechanism of AD involves activation of the inflammatory cytokine transcription factor nuclear factor kappa B (NF-κB) in the epidermis and dermis, which leads to chronic skin inflammation and failure of the stratum corneum barrier. Therefore, it is believed that siRNA therapy could cure AD.

Small interfering RNA (siRNA) has been proposed as a novel treatment for atopic dermatitis (AD) because it suppresses sequence-specific mRNA expression. Indeed siRNA-based therapy achieves an almost complete cure with fewer side effects than currently available treatments. However, the tight junctions in the granular layer of the epidermis in the atopic skin are barriers to siRNA delivery. We previously reported the potential clinical utility of AT1002, a peptide that opens tight junctions. In the present study, we evaluated a topical siRNA-based therapy for AD using AT1002 in combination with a flexible liposome. The 1,2-dioleoylsn-glycero-3-phosphoethanolamine (DOPE)/cholesteryl hemisuccinate (CHEMS) liposome was chosen as a carrier for siRNA because of its highly flexible structure and permeability. We prepared siRNA-encapsulated DOPE/CHEMS liposomes and examined their physical properties, safety, uptake into RAW264.7 cells, and topical application in healthy and AD-affected skin. We then assessed the efficacy of anti-nuclear factor-kappa B (NF-κB) (RelA) siRNA (siRelA)-encapsulated DOPE/CHEMS liposomes with AT1002 in AD model mice. The siRNA-DOPE/CHEMS liposomes were absorbed significantly better than siRNA alone and they enhanced siRNA skin penetration without toxicity. Moreover, siRelA-DOPE/CHEMS liposomes with AT1002 alleviated AD symptoms and reduced the levels of inflammatory cytokines in AD model mice. Therefore, the combination of AT1002 and DOPE/CHEMS liposomes could be a dermally applied RNA interference therapeutic system for effective RNA delivery and AD treatment.

Key words: atopic dermatitis; small interfering RNA (siRNA); RelA; topical siRNA application; liposome; AT1002 peptide

The preferred administration route for siRNA in AD therapy is dermal application because it is simple and noninvasive for patients, acts directly on the affected areas, and reduces systemic side effects. The drugs used to treat skin allergic diseases must be transferable to the deep granular layer containing immunocompetent cells. For this reason, drug delivery must be optimized. However, there are two obstacles that must first be overcome if dermally applied siRNA is to be effective in treating AD. First, siRNA is enzymatically degraded in vivo and has low membrane permeability. Therefore, carrier genes may be needed to stabilize the siRNA, improve its delivery to target dermal cells, and effectively suppress the target gene. Second, the intercellular lipids of the stratum corneum and the tight junction (TJ) are strong barriers to siRNA and impede it from penetrating the cutaneous immunocompetent cells. The stratum corneum is a far less effective barrier in AD skin than it is in healthy skin. However, TJs still effectively block siRNA delivery even in AD. Hence, an appropriate siRNA carrier is required that can break through the TJs and deliver the siRNA to its target.

Arginine-rich, cell-penetrating peptides such as Tat are known to enhance drug penetration. Our previous study demonstrated the potential of the cytoplasm-responsive, arginine-rich peptide STR-CH2R4H2C as a carrier for siRNA in dermal applications. STR-CH2R4H2C consists of cysteine, arginine, and histidine modified with stearic acid, and was found to be highly effective at intradermal siRNA
delivery. Moreover, STR-CH2R4H2C significantly enhanced the gene-silencing effect of siRNA relative to that of siRNA administered alone. The arginine residue forms a complex with siRNA, the histidine moiety enables the complex to exit endosomes, while the cysteine constituent stabilizes and releases the siRNA in a reducing environment. Peptide modification with stearic acid further stabilizes the complex via hydrophobic interaction. As arginine is cationic, there was concern regarding the potential for cytotoxicity and skin irritation with long-term application. Therefore, a newly topicalized applied siRNA carrier with high dermal penetration and low toxicity is needed. Recent studies have focused on noninvasive dermal application systems using nanocarriers. Thus, in the present study, liposomes were investigated as siRNA carriers in skin applications.

Liposomes are closed spherical vesicles composed of phospholipids, and have been used as nucleic acid carriers to improve in vivo systemic drug delivery for cancer therapy. In recent years, this technology has been adapted for dermal applications such as in cosmetics and skin disease therapy.

Liposomes have a high affinity for cell membranes and are biocompatible and degradable. Moreover, many drugs can be encapsulated in liposomes whose physical properties can be modulated by altering their lipid composition. Lipid vesicles have been used to facilitate the delivery of large molecules such as siRNAs into the deeper skin layers where they form a reservoir for the slow, sustained release of the drug, thereby allowing for a reduction in the administration frequency. Flexible liposomes, ethosomes, and transfersomes are the most effective vesicles at breaching the skin barrier. For this purpose, we chose the 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE)/cholesteryl hemisuccinate (CHEMS) liposome, which we previously demonstrated to be a highly flexible and effective siRNA delivery agent in both mouse and human model skin without toxicity. The DOPE/CHEMS liposome forms a stable lamella layer at physiological pH. The CHEMS protonates and the DOPE moiety shifts from the liposome structure collapses. This pH-sensitive property of the DOPE/CHEMS liposome, is advantageous for cellular delivery.

To deliver siRNA to immunocompetent cells, the siRNA carrier must be able to penetrate the TJs in the granular layer of the epidermis. The TJs form intercellular connections and control the paracellular pathway. The AT1002 peptide consists of six amino acids (FCIGRL) and can open the TJ of the granular layer by phosphorylating the TJ structural protein ZO-1. AT1002 also activates ΔG and the zonula occludens toxin. Our previous study confirmed that AT1002 enhances the delivery of topical applied siRNA, and deeper dermal siRNA penetration can substantially improve its efficacy at treating skin diseases such as AD. The target protein selected for AD treatment in this study was NF-xB (RelA). NF-xB is overproduced in immunocompetent dendritic cells and macrophages, and regulates the expression of various inflammation-related molecules, including the inflammatory cytokines interleukin (IL)-6 and tumor necrosis factor (TNF)-α. RelA (p65) is one of the two subunits of NF-xB along with p50, and is closely associated with NF-xB transcriptional activation. The siRelA suppresses RelA, which is closely associated with allergy induction. We also confirmed the therapeutic efficacy of siRelA in model mice with immuno-disorders using rheumatoid arthritis and AD models. In this study, we developed an siRNA-encapsulated DOPE/CHEMS flexible liposome with AT1002 as an effective dermally applied siRNA delivery and RNAi therapeutic system, and determined the intracellular siRNA uptake ability and cytototoxicity in macrophages of siRNA-encapsulated flexible liposomes. In addition, we assessed the siRNA permeability and localization in the tape-stripped dorsal skin of normal mice and in the auricle skin of AD-induced mice, and evaluated the therapeutic effects of AD in model mice topically treated with siRNA-encapsulated flexible liposome with AT1002. The therapeutic effects were estimated based on changes in ear thickness, clinical score, and observations of the mouse auricle and histologically stained sections, and inflammatory cytokine secretion in AD-induced tissue.

MATERIALS AND METHODS

Materials, Cells, and Animals The AT1002 (Phe-Cys-Ile-Gly-Arg-Leu-NH$_2$) and STR-CH2R4H2C (CH3(CH2)6CO-Cys-His-His-Arg-Arg-Arg-His-His-Cys) peptides were purchased from BEX Co. Ltd. (Tokyo, Japan).

DOPE and 1,2-distearyl-sn-glycero-3-phosphothanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG$\text{}_{2000}$) were purchased in powder form from Avanti Polar Lipids (Alabaster, AL, U.S.A.). CHEMS was purchased from Sigma-Aldrich Corp. (St. Louis, MO, U.S.A.). The rotary evaporator system used to prepare the liposomes consisted of the following: a CCA-1111 recirculating chiller, DPE-1120 solvent recovery unit, NYC-2100 vacuum controller, N-1100 rotary evaporator, OSB-2100 water bath (all from Eylea, Tokyo Rikakikai Co., Ltd., Tokyo, Japan), and DIVAC 1.2-L vacuum pump (Oerlikon, Pfäffikon, Switzerland). A probe-type sonicator was also used.

Fluorescence 6-carboxyfluorescein-aminohexylphosphoramidite (FAM)-labeled siRNA (FAMsiRNA) and mouse anti-RelA (siRelA) were obtained from CosmoBio Co., Ltd. (Tokyo, Japan). The FAMsiRNA sequence was sense 5'-carboxyfluorescein-aminohexylphosphoramidite-AUC CGC GCG AUA GUA CGU AdTdT and antisense 5'-UAC GUA CUA UCG CGC GGA UdTdT. The RelA sequence was sense 5'-GGU GCA GAA ACA GAU UdTdT-3' and antisense 5'-AUU GUC UUU CUG CAC CdTdT-3'. A universal negative control siRNA (scControl) was purchased form Nippon Gene (Tokyo, Japan). These sequences are non-public and confirmed to have no homology with any eukaryote genes. SYBR® Green Nucleic acid gel stain (Cambrex Corporation, Rutherford, NJ, U.S.A.), Triton-X (Sigma-Aldrich Corp.), and dextran sulfate sodium salt from Leuconostoc sp. (molecular weight (MW) 6500–10000) (Sigma-Aldrich Corp.) were used for confirming siRNA liposome encapsulation.

RAW264.7 murine macrophages were obtained from American Type Culture Collection (Manassas, VA, U.S.A.). Dulbecco’s modified Eagle medium (DMEM; Nacalai Tesque, Kyoto, Japan), fetal bovine serum (FBS; Life Technologies, Carlsbad, CA, U.S.A.), penicillin (10000U/mL)–streptomycin (10000g/mL) solution (Nacalai Tesque, Kyoto, Japan), Lipotrust™ (Hokkaido System Science, Sapporo, Japan),
and CCK-8 (Dojindo Laboratories, Kumamoto, Japan) solution were used in the cytological assays. Surgical tape (3M Japan Ltd., Tokyo, Japan), rabbit immunoglobulin G (IgG) anti-mouse ZO-1 (Thermo Fisher Scientific, Waltham, MA, U.S.A.), vectastain ABC-AP rabbit kit (Vector Laboratories, Burlingame, CA, U.S.A.), avidin D with AMCA conjugated (Vector Laboratories), Dako fluorescence measuring medium (Agilent Technologies, Santa Clara, CA, U.S.A.), Cryostat HM550 (Thermo Fisher Scientific), a fluorescence microscope (BZ8100; Keyence, Osaka, Japan), and a confocal laser microscope (FV1000D IX81; Olympus, Tokyo, Japan) were used to study FAMsiRNA permeability and ZO-1 expression. Patch testers (Torii Pharmaceutical Co., Ltd., Tokyo, Japan), hair removal cream (depilatory; Kracie, Tokyo, Japan), a cryostat HM550 (Thermo Fisher Scientific), Tissue Mount (Siraimatsu, Osaka, Japan), Dako fluorescence measuring medium (Agilent Technologies), and a confocal laser microscope (FV1000D IX81; Olympus) were used to evaluate liposome permeability. In addition, 1-fluoro-2,4-dinitrobenzene (DNFB; Wako Pure Chemical Industries, Ltd., Osaka, Japan) and an enzyme-linked immunosorbent assay (ELISA) kit for RelA (Fivephoton Biochemicals, San Diego, CA, U.S.A.), tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) (R&D Systems Inc., Minneapolis, MN, U.S.A.) were used to induce AD-like skin in the mouse auricle and to measure cytokine levels. Rinderon-V lotion (betamethasone valerate; Shionogi & Co., Ltd., Osaka, Japan) was used as a positive control for AD therapy.

Six-week-old male C57BL/6 and NC/Nga mice were purchased from SLC (Shizuoka, Japan). All animal experiments were carried out in accordance with protocols (#P17-79, #P18-06) approved by the Animal Care and Ethics Committee of Tokyo University of Pharmacy and Life Sciences. The mice were housed under standard conditions at 23.5 ± 1°C, 55 ± 5% relative humidity, and a 12 h/12 h light/dark-cycle. Food and water were supplied ad libitum.

Preparation of siRNA-Encapsulated DOPE/CHEMS Liposomes The siRNA-encapsulated DOPE/CHEMS liposomes were prepared by the small unilamellar vesicle (SUV) fusion method.35) DOPE, DSPE-PEG2000, and CHEMS powders were dissolved in chloroform (5 mg/mL) as stock solutions and stored in glass vials at −20°C before use. They were then mixed in molar ratios of DOPE:CHEMS = 3:2 or DOPE:CHEMS:DSPE-PEG2000 = 3:2:0.5 in a round glass test tube. The solution was dried with a rotary evaporator to form a thin phospholipid film, which was then hydrated with 10 mM HEPES buffer (pH 8.0). The mixture was vortexed and sequentially sonicated in a water bath (Branson 5510; Thermo Fisher Scientific) for 5 min. The hydrated mixture was then sonicated with a probe (30 W; 1 min) to form SUVs.

Next, the siRNA/STR-CH2R4H2C complex (N/P 10) was prepared. The siRNA (11.3 μg in Ultrapure™ DNase- and RNase-free distilled water) and STR-CH2R4H2C [74.4 μg in MES buffer (pH 5.5)] solutions were mixed in equal volumes and incubated for 30 min at 37°C. The siRNA/STR-CH2R4H2C was then added to the SUV solution. The SUVs were negatively charged because of the CHEMS and thus surrounded the positively charged siRNA/STR-CH2R4H2C complex, which fused with the DOPE lipid membranes to yield liposome-encapsulated siRNA.

Measurement of Physical Properties of siRNA-Encapsulated DOPE/CHEMS Liposomes The particle size, zeta potential, and entrapment efficiency of the siRNA-DOPE/CHEMS liposomes were measured. The siRNA/STR-CH2R4H2C and siRNA-DOPE/CHEMS liposome particle sizes were measured with a DLS-700 unit (Otsuka Electronics Co., Ltd., Osaka, Japan), determined by Marquand analysis, and presented as a histogram showing mean particle diameter per unit weight. The zeta potential was measured with a NICOMP 380LS unit (Particle Sizing Systems, Shanghai, China).

The siRNA encapsulation efficiency of the liposomes was evaluated by a SYBR Green assay.30) The siRNA-loaded liposomes (50 μL) and SYBR Green dilution [1:1000 in 50 μL phosphate-buffered saline (PBS)] were added to a 96-well plate; SYBR Green forms a fluorescent complex with free siRNA. Fluorescence was measured with microplate reader (Varioskan Flash 2.4; Thermo Fisher Scientific) at excitation and emission wavelengths of 494 nm and 521 nm, respectively. Then, 0.5 mM dextran sulfate solution (50 μL) was added to the wells and the fluorescence was measured again. Dextran sulfate binds to cationic STR-CH2R4H2C more strongly than siRNA and replaces the liposomal surface-bound siRNA in the fluorescence detection. Subsequently, 10% Triton-X solution (50 μL) was added to the wells and the fluorescence was measured for the last time. The 10% Triton-X breaks the lipid bilayer and releases the siRNA from the liposomes. The percentage of siRNA inside the liposomes was calculated by subtracting the fluorescence intensity of the free- and surface-bound siRNA from the total fluorescence intensity.

Evaluation of siRNA Cellular Uptake Ability RAW264.7 murine macrophage cells (2 × 10⁴) in DMEM with FBS (10%) were precultured in 12-well culture plates at 37°C in a humidified 5% CO₂ atmosphere. After 24-h incubation, the cells were washed with PBS and transfected with naked FAMsiRNA, FAMsiRNA/Lipotrust complex (positive control), or FAMsiRNA-DOPE/CHEMS liposome. The FAM-siRNA concentration in all samples was 200 nM. Four hours after transfection, the RAW264.7 cells were washed with PBS and their uptake capacity was measured with a flow cytometer (BD FACS Canto; BD Biosciences, Franklin Lakes, NJ, U.S.A.). The cell population is gated on the plot of the side scattered light with respect to the forward scattered light. In the plot of the cell number relative to the fluorescence intensity in the control of the target cell group, the range including 95% of the cells is defined as the P1 region (10000 cells) and the range in which the fluorescence intensity is stronger is defined as the P2 region. Thus, the percentage of cells in the P2 region was used as an indicator of siRNA uptake into cells. Additionally, the fluorescence intensity per cell was calculated by the average value of fluorescence intensity in the P2 region as the cellular uptake efficiency.
was measured with a microplate reader (Varioskan Flash 2.4; Thermo Fisher Scientific, Waltham, MA, U.S.A.) at 450 nm. The absorbance of the control cells was set as 100% viability. The viability of all cells was expressed as a percentage relative to the absorbance of the control cells.

Observation of FAMsiRNA Localization in Tape-Stripped Mouse Skin Six-week-old male C57BL/6 mice were anesthetized, and their abdominal hair was removed with hair clippers and depilatory cream. The denuded skin was then washed with water. The next day, the site where the hair was removed was stripped 20 times with surgical tape to remove the stratum corneum. Naked FAMsiRNA, FAMsiRNA/STR-CH2R4H2C, and FAMsiRNA-DOPE/CHEMS liposomes, and liposomes with AT1002 (FAMsiRNA: 5 µg/25 µL; AT1002: 100 µg/10 µL) were applied to a 64-mm² area of the skin surface. Patch testers were set on the treated areas, and samples were uniformly spread using the tip of a micropipette. After 10 h, the mice were sacrificed, their skin tissues were washed with PBS, and skin samples were excised at the application sites. The excised tissues were soaked in tissue mount at 4°C overnight in the dark and then stored at −80°C before use. To confirm the permeability of the skin to siRNA, frozen tissue sections were sliced into 10-µm-thick sections with the HM 550 cryostat, washed with PBS, incubated, and mounted with fluorescence mounting medium. FAMsiRNA localization in the skin was observed under a confocal laser microscope.

Observation of FAMsiRNA Colocalization and Immunohisto-Stained ZO-1 in the Mouse Skin Each FAMsiRNA sample (5 µg) was applied to tape-stripped dorsal skin from C57BL/6 mice. Ten hours after this treatment, the mice were sacrificed and their dorsal skin was removed and washed with saline. The tissues were incubated in mounting solution at 4°C overnight in the dark and then mounted in dry ice with acetone. The tissues were stored at −80°C until use. Frozen 10-µm-thick sections were prepared with the HM550 cryostat to confirm the permeability of the skin to FAMsiRNA or the localization of expressed ZO-1. The slides were washed with PBS and distilled water, blocked with blocking solution, incubated with rabbit IgG anti-mouse ZO-1 for 30 min, washed with PBS, and examined with a vectastain ABC-AP rabbit kit. After 30 min, the sections were incubated with AMCA (350/450 nm) conjugated with avidin D for 30 min and mounted with fluorescence mounting medium. FAMsiRNA and immune-stained AMCA-ZO-1 in the skin were observed under a fluorescence microscope at a depth of 100 µm below the skin surface.

Preparation of AD-Induced NC/Nga Mice Six-week-old male NC/Nga mice were subjected to repeated topical DNFB treatments to prepare the mouse AD model according to previous studies. In brief, a 0.15% DNFB solution in acetone (3:1) was applied to the dorsal skin (100 µL) and the left ear auricle (25 µL) on days first and 4. Then, 0.2% DNFB solution (25 µL) was applied to the left ear auricle again on days 7 and 10. Olive oil was applied to the left ear auricle after every DNFB treatment.

Observation of FAMsiRNA Localization in AD Mouse Auricle Skin AD model NC/Nga mice were used for this experiment 15 d after their first sensitization. Naked FAMsiRNA, FAMsiRNA/STR-CH2R4H2C, and FAMsiRNA-DOPE/CHEMS-liposome, and FAMsiRNA-DOPE/CHEMS-liposome with AT1002 (FAMsiRNA: 5 µg/25 µL; AT1002: 100 µg/10 µL) were applied to the left mouse ear auricles. Ten hours after administration, the auricles were excised, washed with saline, and resected. The tissues were soaked in tissue mount solution at 4°C overnight in the dark and mounted with tissue mount in dry ice with acetone. To confirm FAMsiRNA permeability, frozen sections (10 µm thick) were prepared with the HM550 cryostat. The slides were washed with distilled water and mounted in mounting medium. FAMsiRNA in the left ear auricle sections was observed under a confocal laser microscope.

Therapeutic Effects of siRelA Treatment in AD-Induced Mice Three days after the first sensitization, siRelA agents were applied to the left ear auricles of AD-induced mice every third day for two weeks. The treatments were as follows: normal, untreated, steroid (betamethasone valerate), naked siRelA, siRelA/STR-CH2R4H2C (N/P 10), siControl-DP/CHEMS liposome, siRelA-DP/CHEMS liposome, and siRelA-DP/CHEMS liposome with AT1002 (siRelA: 5 µg; AT1002: 100 µg). Betamethasone valerate (a strong topical steroid) was used as a positive control. Ear thickness and clinical score were chronologically evaluated. Left ear thickness was measured with a digital thickness gauge (Minutolco, Co., Kanagawa, Japan). The clinical score was determined by rating individual deformation, redness, hyperplasia, bleeding, dryness, and swelling severities on a scale of 1–3 (1: mild; 2: moderate; 3: severe) according to a previous report. A total of 15 points indicated maximum symptom severity. On day 15, frozen 10-µm-thick sections from the left ear auricles of each group were prepared, stained with hematoxylin-eosin (HE) or toluidine blue (TB), and observed under an optical microscope. The concentrations of RelA and inflammatory cytokines (TNF-α or IL-6) in the ear tissue were measured by ELISA. After this treatment, each left ear was excised, homogenized with lysis buffer for 10 min on ice, and centrifuged at 15000 rpm for 10 min at 4°C. The supernatant was used as a sample solution for the ELISA according to the kit manufacturer’s instructions.

Statistical Analysis All experimental data are presented as means plus standard divisions. Comparisons between multiple treatments were made with ANOVA followed by Dunnett’s test. Statistical significance was defined as p < 0.05.

RESULTS AND DISCUSSION

Physical Properties of siRNA-Encapsulated DOPE/CHEMS Liposomes The zeta potential of siRNA-encapsulated DOPE/CHEMS liposomes was approximately −30 mV, and the siRNA encapsulation efficiency ratio (determined by SYBR Green) was approx. 90% (Table 1). These results indicated that nano-sized siRNA-encapsulated DOPE/CHEMS liposomes, which are suitable physical properties for dermal application, can be successfully prepared with high encapsulating efficiency using the SUV fusion method. The general method used to encapsulate siRNA into liposomes is to prepare an siRNA core by aggregating a cationic polymer and siRNA via electrostatic interaction, which is coated with a lipid membrane. Particle size is an extremely important factor to consider for dermal applications. The particle size of our prepared siRNA-DOPE/CHEMS liposomes was approx. 70 nm, which is suitable for dermal application. The dermal drug delivery efficacy was reported to increase with decreas-
In addition, the negative charge derived from CHEMS is better suited for transdermal delivery than positively charged particles. Cellular Uptake Ability and Cytotoxicity of siRNA-Encapsulated DOPE/CHEMS Liposomes in RAW264.7 Cells

RAW264.7 murine macrophages were used in the cell study because they control the allergic and inflammatory reactions which occur in AD. The ability of RAW264.7 cells to absorb FAMsiRNA/DOPE/CHEMS liposomes was determined by flow cytometry. The percentage uptake of the FAMsiRNA/DOPE/CHEMS liposome was significantly higher than that of naked FAMsiRNA. Moreover, this result was comparable to that for the positive-control group (Fig. 1a). The fluorescence intensity per cell was significantly higher for siRNA-DOPE/CHEMS liposomes than that of the naked FAMsiRNA and the positive control (Fig. 1b). By contrast, siControl/STR-CH2R4H2C showed significant toxicity at N/P 10 and above. In general, improving cellular uptake efficiency is expected to increases the siRNA treatment efficacy.

Next, the cytotoxicity in immune-competent RAW264.7 murine macrophage cells was evaluated as shown in Fig. 2. No cytotoxicity was evident in the siRNA-DOPE/CHEMS liposome encapsulating the same formulation of the siRNA/STR-CH2R4H2C complex, whereas an N/P ratio of 10 or more showed high cytotoxicity due to the cationic origin derived from arginine. This demonstrated that the cytotoxicity in immune-competent cells can be reduced by forming liposomes. These indicated that our siRNA-encapsulated DOPE/CHEMS liposomes would show good performance in an in vivo study, because it showed the higher cellular uptake ability without cytotoxicity. STR-CH2R4H2C has been suggested to be suitable for intracellular uptake, but concerns of its safety have hindered further clinical application. Based on these consideration, it suggested that the DOPE/CHEMS liposome with STR-CH2R4H2C is more useful for clinical application.

Permeability of FAMsiRNA-Encapsulated DOPE/CHEMS Liposomes in the Mouse Skin

As shown in Fig. 3, no FAM fluorescence was observed in the tape-stripped skin of naked FAMsiRNA-treated mice 10 h after treatment. However, the fluorescence of FAMsiRNA/STR-CH2R4H2C persisted on the skin surface because of the cationic charge of the formulation. The FAMsiRNA/DOPE/CHEMS liposome, and especially the liposome with AT1002, fluoresced strongly over large areas of both the epidermis and dermis.

Table 1. Physical Properties of siRNA/STR-CH2R4H2C and siRNA-DOPE/CHEMS Liposome

|                      | Mean particle size (nm) | Zeta potential (mV) | siRNA encapsulation (%) |
|----------------------|-------------------------|---------------------|-------------------------|
| siRNA/STR-CH2R4H2C   | 56.6                    | 12.5                | —                       |
| siRNA-DOPE/CHEMS liposome | 69.5                   | −25.3               | 93.2                    |

The values represent the average of three samples measured.
Similar results were obtained using an actual living pathological model of AD. FAMsiRNA and AT1002 were applied to the left ears of each treatment group on the 15th day after the first sensitization. As shown in Fig. 4, essentially no fluorescence was observed for the naked FAMsiRNA groups. In contrast, strong fluorescence was detected on the entire auricles of the liposome with AT1002 group mice. Therefore, liposomes with AT1002 could effectively deliver siRNA in AD model mice.

siRNA-DOPE/CHEMS showed high intradermal penetration in both the tape-stripped skin and AD skin of mice. siRNA-DOPE/CHEMS liposomes may diffuse siRNA over a wide range because of the flexibility of the liposome structure and the ability of AT1002 to open the TJs. We previously reported that AT1002 opened TJs 10 h after treatment and reconstructed them after 17 h; therefore, the modulating effect of AT1002 is reversible. These results suggest that the combination of the TJ-opening function of AT1002 and the highly flexible structure of the liposome enabled the deep dermal penetration of the siRNA in not only tape-stripped mice skin but also in the pathological model.

**Colocalization of FAMsiRNA and ZO-1 Expression in**
the Mouse Skin after Application of siRNA-Encapsulated DOPE/CHEMS Liposomes with AT1002 As shown in Fig. 5, the tape-stripped skin of the Control and naked FAMsiRNA treatment groups had no observable epidermal FAMsiRNA but the fluorescence of ZO-1, the main protein associated with dermal TJs, was evident. By contrast, siRNA fluorescence was detected in the deep epidermis of skin sections treated with FAMsiRNA-encapsulated DOPE/CHEMS liposomes, siRNA-DOPE/CHEMS liposome, or liposome with AT1002 on day 3, 5, 7, 9, 11, and 13. (a) Ear thickness and (b) clinical score were evaluated daily. Each bar represents the mean ± S.D. (n=5). *p<0.05, **p<0.01.

Fig. 6. Ear Thickness and AD Clinical Score after Topical siRelA-DOPE/CHEMS Liposome Application

AD was induced on the left ears of mice treated with DNFB on day 1, 3, 7, and 10. The left ears were treated with naked siRelA, steroid, siRelA/STR-CH2R4H2C, siControl-DOPE/CHEMS liposome, siRelA-DOPE/CHEMS liposome, or liposome with AT1002 on day 3, 5, 7, 9, 11, and 13. (a) Ear thickness and (b) clinical score were evaluated daily. Each bar represents the mean ± S.D. (n=5). *p<0.05, **p<0.01.

Fig. 7. Appearance of the Ear Lobes of AD Mice after Treatment

Ear lobes were visually assessed for normal mice, untreated AD-induced mice, and AD-induced mice receiving naked siRelA, steroid, siRelA/STR-CH2R4H2C, siControl-DOPE/CHEMS liposome, siRelA-DOPE/CHEMS liposome, or liposome + AT1002. All treatments were applied to the left ear of AD-induced mice on day 3, 5, 7, 9, 11, and 13. (a) Appearance of both auricles and (b) only the left auricle. Photographs were taken on day 15. (Color figure can be accessed in the online version.)

reported the development of transfomers in which the passage of flexible lipid carriers through the skin is passively driven by the osmotic strength, which forms a water gradient. sowie 41) The passage of conventional liposomes is generally blocked at the upper layers of the epidermis in the intercellular pathway because of their lack of deformability. Moreover, flexible liposomes can penetrate the skin without being ruptured. 45) Furthermore, permeation between intradermal cells is facilitated in the deeper layers of the skin where the intracutaneous water content is relatively high.
Therapeutic Effects of siRelA Application with Liposomes and AT1002 in AD-Induced Mice

Figure 6 shows the ear thickness and clinical scores for mouse ears treated with each sample on the 1st, 7th, and 15th days after sensitization. From the 7th d, hyperplasia and the clinical scores of the untreated, naked siRelA, and siControl-DOPE/CHEMS liposome groups continued to increase and symptoms worsened. Ear thickness on the 15th day of treatment was approx. 1.5 mm. Conversely, the liposome with AT1002 treatment suppressed ear hyperplasia and significantly hindered any rise in the clinical score relative to that of the untreated group.

Figure 7 shows the appearance of the mouse auricles on the 15th day. Scabbing and bleeding were observed in the untreated and naked siRelA groups, whereas the auricles of the steroid, liposome, and especially the liposome plus AT1002 groups appeared as healthy as those of the normal mice.

HE staining of the left ear auricles indicated that the hyperplasia in the untreated, naked siRelA, and siControl-DOPE/CHEMS liposome groups was substantially more severe than it was in the groups treated with steroid, AT1002-free siRelA-DOPE/CHEMS liposome, and particularly siRelA-DOPE/CHEMS with AT1002 (Fig. 8a). As shown in Fig. 8b, many TB-stained mast cells were observed in the epidermis and dermis of the untreated and naked siRelA groups. However, the siRelA administration decreased the number of mast cells, and this effect was especially obvious in the group treated with siRelA-DOPE/CHEMS liposome plus AT1002.

Suppression of Cytokine Production and Silencing Effect in the Ear Auricle Skin of AD-Induced Mice after siRelA Applications

Figure 9 shows the levels of RelA and inflammatory cytokines (TNF-α, IL-6) in the ear tissue after siRelA application. As shown in Fig. 9a, liposomes with AT1002 significantly inhibited RelA production relative to that of the untreated group, suggesting a silencing effect in the former group. Moreover, Figs. 9b and c clearly show that compared to the untreated group, the siRelA-encapsulated liposome with AT1002 significantly inhibited the production of both inflammatory cytokines that play central roles in the pathogenesis of AD. These results indicated that the liposome with AT1002 was effective because it delivered siRNA deep into the skin tissue and suppressed inflammatory cytokine production. By contrast, the siControl-DOPE/CHEMS liposome treatment had no inhibitory effect on cytokine production. siRNA-DOPE/CHEMS liposomes markedly improved ear thickening and clinical scores in AD mice. These beneficial effects are due to the high intradermal permeability owing to the flexibility of DOPE/CHEMS and the TJ opening of AT1002. Additionally, infiltration of mast cells was suppressed according to the TB staining results. In AD, immunocompetent cells such as macrophages and Langerhans cells incorporate antigens, infiltrate the epidermis, and migrate to nearby lymph nodes for antigen presentation. Antigen-presenting cells in the activated lymph nodes then migrate to the inflammation site where they produce cytokines and chemokines and induce the production of inflammatory cells such as mast cells. Given that mast cell...
invasion is a key feature of AD, suppression of this feature and clinical symptoms by siRelA-DOPE/CHEMS liposome with AT1002 treatment indicate that they are more effective than STR-CH2R4H2C in AD-dermal application.

Finally, cytokines (TNF-α, IL-6) and RelA production was inhibited in the auricles treated with siRNA DOPE/CHEMS liposomes as compared with that of the other groups. These results confirmed the sequence specificity and active state of the siRelA. Regardless of the addition of AT1002, cytokine production was suppressed by siRelA-DOPE/CHEMS liposomes but not by siRelA/STR-CH2R4H2C. This may also be explained by the high intradermal delivery of the flexible and stable DOPE/CHEMS liposomes, along with the potential pH responsiveness, as mentioned in the Introduction.

CONCLUSION

The present results indicate that the combination of siRelA-DOPE/CHEMS liposomes and AT1002 is a very effective topical siRNA application system for AD therapy because of the synergy among the components enabling highly effective intradermal delivery. Since understanding of the detailed delivery and therapeutic mechanisms is still immature, further experiments and consideration of multiple factors are needed, and are currently underway. In addition, the storage stability of DOPE-liposome must be improved in some way for clinical application. This study presents new insights into the design of nanocarrier dermal drug delivery systems. Moreover, our novel RNAi-based, topically applied therapeutic system using AT1002 and DOPE/CHEMS liposomes might also be effective in the treatment of other allergic skin diseases and in the topical dermal application of other biopharmaceuticals.

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Conflict of Interest The authors declare no conflict of interest.

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