

Chapter 24

Topical Application of siRNA Targeting Cutaneous Dendritic Cells in Allergic Skin Disease

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Abstract

RNA interference is a promising method for silencing specific genes and has great potential for therapeutic applications. However, the major hurdle for therapeutic application is the limited stability of double-strand RNA (dsRNA) and the absence of a reliable delivery method to target cells. Skin appears to be a favorable target for small interfering RNA (siRNA) therapy. Dendritic cells (DCs) exist in the skin and mucosae on the front lines of defense; these cells capture antigens and play a crucial role in inducing immunity and tolerance.

In our recent work, we have shown a successful treatment using CD86 siRNA targeting cutaneous DCs. A costimulatory molecule, CD86, is induced on DCs *in situ* after antigen uptake, and CD86-expressing DCs migrate to the regional lymph nodes to present antigens to T cells. Topical application of cream-emulsified CD86 siRNA ameliorated the clinical manifestations in murine contact hypersensitivity (CH) and atopic dermatitis (AD)-like disease. Our method may be advantageous for the treatment of allergic skin diseases.

Key words: Dendritic cells, siRNA, Topical application, CD86, Costimulatory molecules, Skin allergic disease

1. Introduction

Engagement of the B7 family of molecules on antigen-presenting cells (APCs) with their T cell-associated cosignal receptors, such as CD28 and CD152 (CTLA-4), provides pivotal positive and negative costimulatory signals for T-cell activation and tolerance (1). The lack of costimulation after the engagement of the T-cell receptor by antigen, results in a state of antigen-specific unresponsiveness, termed anergy. Manipulation of the CD28/B7 pathway becomes a potential strategy for achieving therapeutic immunosuppression or tolerance. CTLA4-Ig (abatacept)

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—a recombinant protein fused with the extracellular region of the CTLA-4 and the constant region of the heavy chain of IgG1 that can bind to CD28 ligand with a higher affinity than CD28 — and LEA29Y (belatacept) — a two amino acid, substituted CTLA4-Ig to improve the binding avidity for CD80 and CD86 — have already been shown to have beneficial therapeutic effects in patients with rheumatoid arthritis and renal transplantation (2). These new immunosuppressive agents have been systemically administered and are thought to bind both CD80 and CD86 efficiently, competing against the CD28 expressed on T cells.

Dendritic cells (DCs) are a highly specialized population of immune cells involved in both the process of inducing immunity and tolerance as a conductor of the immune system (3). DCs, located at body surfaces, capture pathogens (antigens) at the front lines of defense, and migrate to regional lymph nodes to interact with T cells. Manipulation of the various functional molecules targeting DCs, greatly affects their immunoregulatory capacity (3, 4). CD86 is one of the crucial costimulatory ligands induced on DCs, and blockade of the CD86 pathway efficiently interferes with antigen-specific T-cell responses in murine Th2-mediated allergic disease models (5). Preferential induction and functional contribution of CD86 induced on DCs have been reported in skin inflammatory allergic diseases in both humans (6, 7) and mice (8–10).

We have successfully developed a small interfering RNA (siRNA)-based therapeutic application for murine allergic skin disease that targets the CD86 costimulatory molecule expressed on cutaneous DCs (11). The cream lotion, mixed with siRNA against CD86, has been topically applied to healthy or atopic skin. The treatment with CD86 siRNA cream lotion provided efficient amelioration in contact hypersensitivity reactions and skin allergic atopic dermatitis lesions (11). For siRNA-based clinical application, the targeting of cutaneous DCs may be a promising strategy in the treatment of allergic skin disease. This strategy can be expanded to include the genes for additional costimulatory molecules or cytokines as well as such target tissues as the nasal, oral, alveolar, and gastrointestinal mucosae.

2.2. Selection of Efficient siRNA

2.3. Preparation of Cream-Emulsified siRNA

2.4. Murine Contact Hypersensitivity (C. Model

2.5. Murine Atopic Dermatitis (AD) Mo

2. Materials

2.1. Dilution of siRNA Duplex

1. Lyophilized siRNA (20 nmol) (designed and synthesized by Qiagen 2-for-silencing siRNA duplexes, Qiagen, Valencia, CA).
2. siRNA suspension buffer: 100 mM potassium acetate, 30 mM HEPES-KOH (PH 7.4), and 2 mM magnesium acetate.

**2.2. Selection
of Efficient siRNA**

1. Cultured 293T cells.
2. Complete Dulbecco's modified Eagle's medium (DMEM): DMEM High Glucose with L-Glutamine and Phenol Red (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS, Invitrogen, St.Louis, MO) and gentamycin (Sigma, Steinheim, Germany).
3. A set of 24-well flat-bottom plates (Iwaki, Tokyo, Japan).
4. The full-length mouse CD86 expression vector (pcDNA3/mCD86).
5. Lipofectamine 2000 (Invitrogen).
6. Opti-MEM (Invitrogen).
7. Appropriate fluorochrome-conjugated anti-mouse CD86 mAb or isotype control Ig (eBioscience, San Diego, CA).
8. FACSCalibur and CellQuest software (BD Biosciences, San Jose, CA).

**2.3. Preparation
of Cream-Emulsified
siRNA**

1. 20 μ M siRNA duplex stock solutions. (After thawing, the tubes are spun briefly to bring the contents to the bottom of the tubes).
2. Cream-based ointment (Johnson's baby lotion, no fragrance; Johnson & Johnson, New Brunswick, NJ) is sterilized by autoclave to avoid RNase contamination (see Note 1).
3. End-cut tips (The ends from 200 μ L pipette tips cut at a length of 1 cm. All the end-cut pipette tips should be sterilized by autoclave.) (see Note 2).
4. A set of 1.5 mL microtubes sterilized by autoclave.

**2.4. Murine Contact
Hypersensitivity (CH)
Model**

1. Female 6-week-old BALB/c specific pathogen-free mice (Japan SLC, Hamamatsu, Japan).
2. An electric shaver (Hitachi, Japan).
3. (a) Haptens, 2,4-dinitro-1-fluorobenzene (DNFB, Sigma) or fluorescein isothiocyanate (FITC, Wako, Osaka, Japan) (see Note 3).
(b) DNFB-induced CH: 0.5% DNFB dissolved in acetone:olive oil (4:1 v/v) for sensitization and 0.2% DNFB dissolved in acetone:olive oil (4:1 v/v) for challenge.
(c) FITC-induced CH: 0.5% FITC dissolved in dibutyl phthalate:acetone (1:1 v/v) for sensitization and challenge.
4. A dial thickness gauge (Peacock, Ozaki, Tokyo, Japan).

**2.5. Murine Atopic
Dermatitis (AD) Model**

1. Female 6-week-old NC/Nga mice (12, 13) (Japan SLC, Hamamatsu, Japan). The mice should be maintained under conventional conditions (see Note 4).

2. An electric shaver (Hitachi, Japan).
3. A cream-based hair remover (Kracie, Tokyo, Japan) (see Note 5).
4. Sterile cotton.
5. Sterilized MilliQ water.
6. Mite antigen (Mite Extract *Dermatophagoides farinae* (Df); LSL, Tokyo, Japan) dissolved in 1× PBS.

3.3. Preparation of Cream-Emulsified siRNA

3. Methods

At first, several sequences of siRNA duplexes should be designed, and the silencing effect of each siRNA should be tested by cotransfection with each respective siRNA and expression vector into suitable cells that provide high transfection efficiency. In this protocol, a 293T cell line (human embryonic kidney cell line) is used. For suitable evaluation of the silencing effect by siRNA, the transfected cells should provide more than 70% expression of the targeted gene. The efficiency of siRNA delivery into the cells can be evaluated using fluorochrome (e.g. Alexa 488)-conjugated siRNA by flow cytometry. After confirmation of the silencing effects of siRNA *in vitro*, siRNA should be applied to *in vivo* treatment.

3.4. Induction of CI and Treatment with siRNA

3.1. Dilution and Stock of Lyophilized siRNA (see Note 6)

1. Add 1 mL of the siRNA suspension buffer to the lyophilized siRNA (20 nmol) to obtain a 20 μ M solution.
2. Heat the tubes to 90°C for 1 min.
3. Incubate at 37°C for 60 min.
4. Perform your experiment or store aliquots at -20°C.

3.2. Selection of Efficient siRNA by the Evaluation of *In Vitro* Silencing Effects Using a 293T Transfection System

1. The 293T cells, cultured in complete DMEM (high-glucose DMEM supplemented with 10% fetal bovine serum and gentamycin), are plated into 24-well plates (2×10^5 /well) 1 day before transfection.
2. The cells are co-transfected with 0.2 μ g of the pcDNA3/CD86 expression vector and 40 pmol of either nonsilencing control or CD86 siRNA duplexes with Lipofectamine 2000 diluted in Opti-MEM, according to the manufacturer's instructions. To verify the transfection efficiency of the CD86 gene, make additional wells with or without pcDNA3/CD86 vector alone, in the absence of any siRNA.
3. If required, change the medium at 24 h and culture another 24 h with DMEM. Beware of cells that detach from the culture plate.

3.5. Induction of AI and Treatment with siRNA

4. Harvest cells from each well at 24 or 48 h after transfection.
5. Stain cells with fluorochrome-conjugated anti-CD86 mAbs or appropriate control reagents, and analyze by flow cytometry.
6. Select the best siRNA sequence that provides the greatest silencing effect for the *in vivo* treatment.

3.3. Preparation of Cream-Emulsified siRNA

1. Add sterile cream and either nonsilencing or CD86 siRNA duplex into an autoclaved 1.5 ml microtube, and mix homogeneously. If required, vortex for 30 s. For the control with the cream alone, prepare the cream-based ointment mixed with autoclaved water instead of the siRNA duplex. For CH treatment, mix 7.5 μ L of cream and 12.5 μ L of 20 μ M siRNA duplex (0.25 nmol). For AD treatment, mix 15 μ L of cream and 25 μ L of 20 μ M siRNA duplex (0.5 nmol) (see Note 7).
2. Keep all prepared solutions at 4°C or on ice until use.

3.4. Induction of CH and Treatment with siRNA

1. Maintain BALB/c mice under specific pathogen-free conditions for at least 1 week in your animal facility after transfer.
2. Shave the abdominal area using an electric shaver.
3. For DNFB sensitization, paint 20 μ L of 0.5% DNFB onto the shaved abdominal skin using a 20 μ L-tip on days 0 and 1. For FITC sensitization, paint 400 μ L of 0.5% FITC onto the shaved abdominal skin using a 200 μ L-tip on day 0 once.
4. On day 5, 1 h before challenge, apply 10 μ L each (total 20 μ L) of the siRNA-cream mixture prepared in Subheading 3.3 onto the ventral and dorsal sides of ear skin (see Note 8).
5. For challenge, apply 10 μ L each (total 20 μ L) of either 0.2% DNFB or 0.5% FITC onto the ventral and dorsal sides of the ear on day 5.
6. Measure the ear thickness before challenge and 24, 48, and 72 h after challenge using a dial thickness gauge. The ear swelling value is shown as the mean difference between the pre- and post-challenge values.

3.5. Induction of AD and Treatment with siRNA

1. Maintain NC/Nga mice under conventional conditions (This is important!) (see Note 4).
2. Shave the upper back area of NC/Nga mice roughly using an electric shaver.
3. Apply the cream-based hair remover onto the shaved area for 5 min, and remove the cream using sterile cotton swabs.

4. Notes

4. Wipe out the residual cream with wet cotton swabs or tissue paper, and let the skin dry naturally.
5. Apply 20 μ g of mite antigen, diluted in 100 μ L PBS, onto the shaved upper back skin and dorsal ears of the mice three times per week (14) (see Note 9).
6. Have two persons who are unaware of each mouse's treatment status score the skin lesions weekly, according to the following criteria (Table 1).
7. To see therapeutic effects, start treatment after all the mice develop the skin lesions. In this scoring method, we start the treatment 2 weeks after the initial application of mite antigen when the skin scores in all mice reach over five. Apply a total volume of 40 μ L of the cream-emulsified siRNA onto the upper back skin and dorsal ears every day (see Notes 8 and 10).
8. Monitor the skin score of individuals weekly and, if required, collect blood sera every 2 weeks for the measurement of Df-specific and/or total immunoglobulin (e.g., IgG1, IgE).

Table 1
Scoring of skin lesions

Features	None (0)	Mild (1)	Moderate (2)	Severe (3)
Dorsal Ear Skin				
Erythema/ Hemorrhage		Ery. over half in one side or Hem. point	Ery. over half in one side or Hem. over half in both side	Hem. over half in both side
Excoriation/ Erosion		Exo. one side or Ero. point	Exo. both side or Ero. one side	Ero. both side
Scaling/Dryness		Less than half in one side	Less than half in both side or over half in one side	Over half in both side
Upper back Skin				
Erythema/ Hemorrhage		Ery. ≤ 5 mm ² and Hem. (-)	Ery. < 5 mm ² and Hem. point or Ery. > 5 mm ² and Hem. (-)	Ery. > 5 mm ² and Hem. (+)
Excoriation/ Erosion		Exo. ≤ 3 mm ² and Ero. (-)	Exo. ≤ 3 mm ² and Ero. (+) or Exo. > 3 mm ² and Ero. (-)	Exo. > 3 mm ² and Ero. (+)
Scaling/Dryness		$\leq 1/3$	1/3-2/3	$\geq 2/3$

The areas of dorsal ear and upper back skin are separately scored. The lesions are observed by three categories. The sum of scores is defined as total skin score (range from 0 to 18). *Ery* Erythema, *Hem* Hemorrhage, *Exo* Excoriation, *Ero* Erosion

4. Notes

1. You may use the following formula instead of Johnson's baby lotion:
Low-substituted hydroxypropylcellulose (L-HPC) 1%
High-substituted hydroxypropylcellulose (H-HPC) 1%
Methylparapen 0.2%
Propylene glycol 5% (This is essential!)
Water 92.8%
2. Use the end-cut pipette tips to transfer the cream into the tubes because the cream has high viscosity. If required, measure the weight of the cream in advance and adjust the correct volume of the cream by balance.
3. Hapten solution should be prepared just before use. For the DNFB solution, prepare the olive oil and acetone mixture in a glass tube first. Measure the approximate amount of DNFB in a glass tube or polypropylene centrifuge tube. Then, add the suitable volume of the olive oil and acetone mixture and mix well. For the FITC solution, measure the approximate amount of FITC in a glass or polypropylene centrifuge tube, add the suitable volume of dibutyl phthalate and vortex well. Then add the same volume of acetone into the tube.
4. NC/Nga mice need to be maintained in an air-uncontrolled conventional room. The incidence and severity of dermatitis are greatly affected by the housing conditions (the humidity, temperature, and cleanliness in the room).
5. The selection of cream-based hair remover is important. Because you need to treat with hair remover often, after the development of dermatitis, the treatment itself sometimes induces an exacerbation of the dermatitis.
6. The incubation steps only need to be carried out the first time you use the siRNA. The procedure will disrupt aggregates, which may have formed during the lyophilization process, and is necessary to maximize the siRNA silencing potential. Repeated freeze-thaw cycles will not interfere with the siRNA sample as long as RNase-free conditions are strictly maintained.
7. It has been shown that dsRNA can induce the production of type I interferon by triggering TLR signaling (15). To avoid the IFN effects of siRNA treatment in vivo, the lowest amount of siRNA that can provide the sufficient effect should be considered (16). In our experiments, 0.25 nmol (2.5 μ g) per ear and 0.5 nmol (5 μ g) per mouse were the optimal doses for CH and AD treatments, respectively.

8. The pipette tips for topical painting should be changed individually.
9. The amount of mite antigen for the induction of AD may need to be adjusted, depending on the housing conditions.
10. The induction of AD accelerates the hair growth due to the inflammation. The growing hair may interfere with the absorption of antigen as well as cream-emulsified siRNA. Thus, hair removal is often required. The treatment with hair remover induces bleeding after the development of AD. The scoring should be performed at least two days after the hair treatment.

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