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Epithelial and Mesenchymal Cell Biology

Controlled Delivery of T-box21 Small Interfering RNA Ameliorates Autoimmune Alopecia (Alopecia Areata) in a C3H/HeJ Mouse Model

Motonobu Nakamura,*† Jun-ichiro Jo,‡ Yasuhiko Tabata,‡ and Osamu Ishikawa*

From the Department of Dermatology, Gunma University, Maebashi; the Department of Dermatology,† Kyoto University, Kyoto; and the Department of Biomaterials,‡ Field of Tissue Engineering, Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan*

Autoimmune alopecia (alopecia areata) is considered to be triggered by a collapse of immune privilege in hair follicles. Here we confirmed that infiltrating CD4 T lymphocytes around hair follicles of patients with alopecia areata were primarily CCR5-positive with few CCR4-positive cells, suggesting a dominant role of Th1 cells in the alopecic lesion. Given this finding, we sought to elucidate the effect of cytokine therapy in C3H/HeJ mice, a mouse model of alopecia areata, by applying recombinant interleukin-4 and neutralizing anti-interferon-γ antibody. We found that local injections of both interleukin-4 and neutralizing anti-interferon-γ antibody effectively treated alopecia in C3H/HeJ mice. Results from immunohistochemistry and semiquantitative reverse transcription-polymerase chain reaction demonstrated that intralesional injection of interleukin-4 suppressed CD8 T cell infiltrates around the hair follicles and repressed enhanced interferon-γ mRNA expression in the affected alopecic skin. Furthermore, Th1 transcription factor T-box21 small interfering RNAs conjugated to cationized gelatin showed mitigating effects on alopecia in C3H/HeJ mice, resulting in the restoration of hair shaft elongation. Taken together, the use of gelatin–small interfering RNA conjugates promises to be a novel, efficient, and safe tool as an alternative gene therapy for the treatment of various human diseases. To our knowledge, this is the first report of effective controlled delivery of small interfering RNA using biodegradable cationized gelatin microspheres in an animal model of disease. ***(Am J Pathol 2008, 172:650–658; DOI: 10.2353/ajpath.2008.061249)***

Alopecia areata is a tissue-restricted autoimmune disease directed at the hair follicle resulting in hair loss.[1,2] It is a common disorder with an estimated prevalence of 1 in 1000 and sometimes extends to the whole scalp area (alopecia totalis). Total loss of scalp and body hair (alopecia universalis) may take place. Patients often feel severe psychological stress from this hair loss; however, conventional therapies such as topical immunotherapy or steroid application often fail to cure the disease.

Since HLA-DR and ICAM1 are ectopically expressed in the outer sheath cells, a collapse of immune privilege is considered to lead to the initiation of alopecia areata. T lymphocytes from the patients could cause alopecia areata, when cultured with hair follicle homogenate along with antigen-presenting cells and injected into the skin explants on severe combined immunodeficiency mice.[3] Purified T cell injection experiments result suggested that both CD4- and CD8-positive T cells have a role in pathogenesis of alopecia areata, CD8-positive cells acting as the main effector cells with the regulatory control by CD4-positive T cells.[4] This study was undertaken to clarify whether Th1 CD4 lymphocytes or Th2 CD4 lymphocytes are dominant in the lesions of patients with alopecia areata and also to elucidate the effect of cytokine therapy applying recombinant interleukin-4 (Il4) and neutralizing anti-interferon-γ (Ifng) antibody into the alopecia areata rodent model C3H/HeJ mice.

Gene therapy is a new therapeutic approach for alopecia areata. Small interfering RNA (siRNA) specifically binds to corresponding mRNA sequence and facilitates its breakage and degradation.[5] Gene therapy using siRNA *in vivo* has been exploited successfully in several mouse disease models; intravenous injections of siRNA limited the rate of metastasis of prostate cancer,[6] and intralesional injections of siRNA decreased the incidence of herpes simplex virus infection.[7] Here we introduced

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Address reprint requests to Motonobu Nakamura, Department of Dermatology, Graduate School of Medicine, Kyoto University, 54 Kawahara-cho, Sakyo-ku, Kyoto, 606-8507, Japan. E-mail: motonaka@kuhp.kyoto-u.ac.jp.

another siRNA drug delivery system using cationized gelatin[8] as a new therapeutic approach, targeting T-box21 gene (*Tbx21*) to repress the expression of interferon-γ gene (*Ifng*). *Tbx21* gene was formerly known as *T-bet* gene and plays an important role in control of the *Ifng* gene expression and Th1 cell differentiation and function.[9,10]

Materials and Methods

Patients

Four patients (three males and one female, mean age 29 years) with multiple alopecia areata of 2 to 8 months' duration in active stages were included in this study. Written informed consents were obtained after the aim, nature, and possible consequences of the study were explained. Protocols were approved by the Ethical Committee of Kyoto University. All four patients satisfied the criteria for alopecia areata proposed by American Academy of Dermatology.[11]. None of the patients had any therapy for 60 days before the scalp skin biopsy.

Skin Biopsies

Two skin specimens, 4 mm in diameter and 6 mm in diameter, were obtained from the peripheral region of the alopecic skin of each patient. The former was fixed in formalin and processed for hematoxylin and eosin staining. The latter was snap-frozen in liquid nitrogen and stored at −80°C until use for immunohistochemistry.

Immunohistochemistry

Fluorescence immunohistochemistry was performed on the serial skin cryosections (6 μm) according to an established immunohistochemistry protocol.[12] In brief, cryostat sections (6 μm) were fixed in acetone (10 minutes at −20°C), washed in phosphate-buffered saline (PBS) three times, blocked with 10% goat serum for 20 minutes at room temperature, and incubated overnight at 4°C with mouse anti-human CD4 antibody (1:100, DAKO Cytomation MT310, Kyoto, Japan), mouse anti-human CCR5 antibody (1:100, DAKO Cytomation RB181), and anti-CCR4 antibody (1:100 MAB 1567, R&D Systems, Minneapolis, MN). After further washing with PBS for 5 minutes three times, fluorescein isothiocyanate-labeled goat anti-mouse IgG (1:200, sc-2010, Santa Cruz Biotechnology, Santa Cruz, CA), phycoerythrin-labeled goat anti-mouse IgG (1:200, sc-3738, Santa Cruz Biotechnology), or fluorescein isothiocyanate-labeled goat anti-rat IgG (1:200, sc-2011, Santa Cruz Biotechnology) was applied for 45 minutes at room temperature. After further washing with PBS three times, sections were counterstained with 4′,6-diamino-2-phenylindole. Sections without primary antibody served as negative controls. Mouse CD8 staining was performed using 100X diluted anti-mouse CD8 antibody (sc-18660, Santa Cruz Biotechnology) and LSAB+ System-HRP (DAKO Cytomation, Glostrup, Denmark) according to the manufacturer's instructions. The number of CD8-positive T cells in each hair follicle was counted for 20 hair follicles in four mice before and after Il4 administration.

Animals

Ten-week-old or older female C3H/HeJ mice having an alopecic region on the back in the waxing phase were purchased from SLC company (Hamamatsu, Japan). Mice were maintained at the Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University, and were fed water and mouse chow ad libitum in compliance with protocols established by the Animal Research Committee of Kyoto University. To avoid the possibility of grooming between the mice, mice were put in separate cages. Whole animal care and experimental procedures were approved by the Graduate School of Medicine, Kyoto University. In total, 78 C3H/HeJ mice with alopecic lesions were used in this study: 10 with Il4 injections, 10 with 0.9% sodium chloride injections, 10 with Il4 and Ifng injections, six with anti-Ifng antibody injections, six with rat IgG injections, six with antisense *Tbx21* oligonucleotide injections, six with non-sense oligonucleotide injections, eight with cationized gelatin-conjugated *Tbx21* siRNA injections, eight with naked *Tbx21* siRNA injections, and eight with cationized gelatin-conjugated non-sense siRNA injections.

Semiquantitative Reverse Transcription-PCR (RT-PCR)

RT-PCR was performed on the published protocol. Total RNA from homogenized full-thickness back skin from C3H/HeJ mice was extracted using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the protocol provided by the company. Subsequently, cDNA was synthesized from 1 μg of total RNA using a first-strand cDNA synthesis kit for RT-PCR (AMV) (Boehringer Mannheim, Germany). The reverse-transcribed cDNA was stored at −80°C for further use. Primers used were formerly described[13] and are listed as follows: *Ifng* forward: 5′-AACGCTACACACTGCATCTTGG-3′; *Ifng* reverse: 5′-GACTTCAAAGAGTCTGAGG-3′; *Il12b* forward: 5′-CGTGCTCATGGCTGGTGCAAAG-3′; *Il12b* reverse: 5′-CTTCATCTGCAAGTTCTTGGGC-3′; *Il4* forward: 5′-GAATGTACCAGGAGCCATATC-3′; *Il4* reverse: 5′-CTCAGTACTACGAGTAATCCA-3′; *Actb* forward: 5′-TGTTACCAACTGGGACGACA-3′; *Actb* reverse: 5′-TCTCAGCTGTGGTGGTGAAG-3′. The PCR was run on an Astec Program Temp Control System (Astec, Fukushima, Japan). The reaction consisted of 1 μl of cDNA, 10 μl of 10X PCR buffer, 8 μl of deoxynucleoside-5′-triphosphate mixture (2.5 mmol/L each), 1 μl of forward primer (50 pmol), 1 μl of reverse primer (50 pmol), 0.5 μl of TaKaRa Ex Taq polymerase (Takara, Otsu, Japan), and 78.5 μl of water. Amplification was performed over 32 cycles for *Actb*, 40 cycles for *Ifng*, 40 cycles for *Il12b,* and 45 cycles for *Il4*. Each cycle consisted of the following steps: denaturation at 94°C, annealing at 55°C, and extension at 72°C. The PCR products were analyzed by agarose gel electro-

phoresis. Densitometry was performed by using Epson Color Imaging ES-2200 and National Institutes of Health Image software for assessing staining results.

Application of Il4 and Anti-Ifng Antibody

Recombinant mouse Il4 and rat anti-mouse Ifng neutralizing antibody (MAB485) were purchased from R&D Systems (Minneapolis, MN). Recombinant mouse Ifng was purchased from Pepro Tech (London, UK). Recombinant mouse Il4 was dissolved in 0.9% sodium chloride and injected subcutaneously at a concentration of 0.1 μg/0.2 ml into 10 alopecic lesions every day for 21 days. Sodium chloride, 0.9%, was applied to 10 alopecic lesions as a negative control. Recombinant mouse Ifng was dissolved in 0.9% sodium chloride and injected subcutaneously at a concentration of 0.01 μg/0.1 ml in conjunction with 0.1 μg/0.1 ml recombinant mouse Il4 into 10 alopecic lesions every day for 21 days. The effect was assessed every week through observation under anesthesia with intraperitoneal injection of 0.15 mg/0.3 ml/20 g body weight pentobarbital (Dainihon Pharmaceutical Company, Osaka, Japan).

Rat anti-mouse Ifng neutralizing antibody was dissolved in PBS and injected subcutaneously into 10 alopecic lesions at a concentration of 0.4 mg/kg in 0.1 ml every day for a week. Rat IgG (DAKO Cytomation) was applied to the 10 alopecic lesions in the same protocol as a negative control. Hair growth was graded every week.

Hair Growth Grading

For each injected site, the percentage of hair surface coverage on alopecic skin was estimated. The quality and density of the hair were assessed and graded on a four-point scale (grade 0, no hair; grade 1, stubble, short broken hairs; grade 2, sparse intermediate length hairs; grade 3, normal length and density hairs) as described[14]. The percentage of hair coverage was multiplied by the quality/density grade for each area. Using this method, the hair growth index may thus range from 0 (no hair) to a maximum of 300 (100% normal hair).

Application of T-box21 Antisense Oligonucleotide

Antisense *Tbx21* oligonucleotide (5′-CTCCACGATGCCCATC-3′) and non-sense oligonucleotide (5′-CTATGTCATCCCGCTCCAC-3′) were purchased from Proligo (Kyoto, Japan). This antisense *Tbx21* oligonucleotide was reported to suppress *Tbx21* expression in the study by Lovett-Racke et al.[9] Antisense Tbx21 oligonucleotide and non-sense oligonucleotide were injected subcutaneously into six alopecic lesions, respectively, at a concentration of 0.5 mg/kg/day in 0.2 ml every 3 days for seven times. The hair growth index was calculated every week.

Preparation of Cationized Gelatin

The gelatin sample with an isoelectric point of 9.0 (molecular weight 100,000) prepared by an acid process of pig skin was kindly supplied by Nitta Gelatin Inc., Osaka, Japan. Ethylenediamine was obtained from Wako Pure Chemical, Ltd., Osaka, Japan. 2,4,6-Trinitrobenzene sulfonic acid, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt and 25 wt % glutaraldehyde aqueous solution were purchased from Nacalai Tesque, Kyoto Japan. The carboxyl groups of gelatin were chemically converted by introducing amino groups for cationization of gelatin.[8] Briefly, ethylenediamine and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt were added to 250 ml of 100 mmol/L PBS containing 5 g of gelatin. The molar ratio of ethylenediamine to the carboxyl groups of gelatin was 50. Immediately after this, the solution pH was adjusted to 5.0 by adding 5 mol/L HCl aqueous solution. The reaction mixture was agitated at 37°C for 18 hours and then dialyzed against double-distilled water for 48 hours at room temperature. The dialyzed solution was freeze-dried to obtain a cationized gelatin. The percentage of amino groups introduced into gelatin was 50.9 mol % per the carboxyl groups of gelatin.

Preparation of Cationized Gelatin Microspheres

Cationized gelatin microspheres were prepared by chemical cross-linking of gelatin in a water-in-oil emulsion state.[8] An aqueous solution of 10 wt % cationized gelatin (10 ml) was preheated at 40°C and then added dropwise into 375 ml of olive oil preheated at 40°C, while an impeller stirring at 420 rpm was used for 10 minutes to yield a water-in-oil emulsion. The emulsion temperature was decreased to 4°C followed by further stirring for 30 minutes for the natural gelation of gelatin aqueous solution. Ice-cold acetone (100 ml) was added to the emulsion and stirring was continued for 10 minutes. The resulting microspheres were washed three times with ice-cold acetone, collected by centrifugation (5000 rpm, 4°C, 5 minutes), fractionated in size by sieves with apertures of 70 and 100 μm, and air-dried at 4°C. The average diameter of microspheres used was 75 μm. The non-cross-linked and dried gelatin microspheres (50 mg) were placed in 25 ml of acetone/0.01 mol/L HCl solution (7:3, v/v) containing 60 μl of 25 wt % glutaraldehyde solution and stirred at 4°C for 24 hours to allow the cationized gelatin to cross-link. After washing by centrifugation (5000 rpm, 4°C, 5 minutes) with double-distilled water, the microspheres were agitated in 25 ml of 100 mmol/L aqueous glycine solution at room temperature for 1 hour to block the residual aldehyde groups of unreacted glutaraldehyde. The resulting microspheres were washed three times with double-distilled water by centrifugation and freeze-dried.

Application of Tbx21 siRNA

Tbx21 siRNA (5′-UGAUCGUCCUGCAGUCUCUdTdT-3′, 3′-dTdTACUAGCAGGACGUCAGAGA-5′) and non-sense

siRNA (5′-CGAACGAGUACCGUACACUdTdT-3′, 3′-dT-dTGCUUGCUCAUGGCAUGUGA-5′) were purchased from Proligo. This Tbx21siRNA suppressed Tbx21expression in the study by Lovett-Racke et al.[9] To impregnate Tbx21 siRNA into cationized gelatin microspheres, 10μl of PBS solution (pH 7.4) containing 10 μg of Tbx21 siRNA was dropped onto 1 mg of the freeze-dried cationized gelatin microspheres, kept overnight at 4°C, and injected subcutaneously into eight alopecic lesions in 0.2 ml of PBS every 7 days for three times. Ten micrograms of naked siRNA and 10 μg of cationized gelatin-conjugated nonsense siRNA were used as negative controls for eight alopecic lesions and eight alopecic lesions, respectively. The hair growth index was calculated every week.

Western Blotting

Skins from mice were washed three times in ice-cold PBS and homogenized in buffer containing 50 mmol/L Tris-HCl, pH 6.8, 20 mg/ml sodium dodecyl sulfate, 60 mg/ml β-mercaptoethanol, 100 mg/ml glycerol at a ratio of 300 μl lysis buffer to 100 mg of tissue. Total cell lysates were centrifuged at 13,000 rpm for 10 minutes. Supernatants were boiled for 5 minutes at 95°C and separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels. Resolved protein was transferred to polyvinylidene difluoride membrane (Bio-Rad Inc., Hercules, CA) and blocked with 5% skim milk (Wako) at 4°C overnight. Blots were incubated with anti-mouse Tbx21 (4B10) antibody (1 μg/ml) (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-Actb (AC-40) antibody (3 μg/ml) for 2 hours at room temperature. After three washes with PBS (10 minutes/wash), blots were incubated with horseradish peroxidase-conjugated goat anti-mouse Ig (P0447) (1:1000) (DAKO, Glostrup, Denmark) for 1 hour at room temperature. After three washes with PBS (10 minutes/wash), blots were incubated with ECL plus solution (GE Health Care, Buckinghamshire, UK) to visualize according to the manufacturer's instructions.

RNA Extraction and Real-Time PCR Assay

Total RNA from homogenized full-thickness back skin from C3H/HeJ mice was extracted using Qiagen RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the protocol provided by the company and was reverse-transcribed into cDNA with a first strand cDNA synthesis kit RT-PCR (Roche Diagnostics, Indianapolis, IN). The expression levels of histocompatibility 2, class II antigen A, β1, actin β (*Actb*) were examined using 20X Assays-on-Demand Gene Expression Assay Mix (Mm00439216_m1, Mm00607939_s1, respectively, Applied Biosystems, Foster City, CA) with a 7300 system (Applied Biosystems) according to the manufacturer's instructions. The same difference was confirmed using glyceraldehyde-3-phosphate dehydrogenase gene (*GAPDH*) expression (Mm_99999915_g1) as a control (data not shown).

Results

CD4-Positive Lymphocytes Infiltrating around the Hair Follicles Are Dominantly CCR5-Positive

In patients with alopecia areata, ectopic major histocompatibility complex (MHC) class I and class II expression in the follicular epithelium leads to a collapse of immunoprivilege of hair follicles.[1] To elucidate the trigger of collapse of immunoprivilege, we examined the profile of infiltrating lymphocytes around the hair bulbs in peripheral region of alopecia areata at the active phase. Hairs surrounding the alopecic region could be plucked without any difficulty and revealed characteristic exclamation mark-like structures with tapering hair shafts in the most proximal region under a light microscope. Skin biopsies were taken from the peripheral region of alopecia areata, because those regions are considered to reveal strongest autoinflammatory reactions.[1]

Hematoxylin and eosin staining disclosed dense, so-called "swarm of bees"-like lymphocyte infiltrates around the hair bulbs (Figure 1A). To elucidate the character of the infiltrating CD4 T lymphocytes, serial frozen sections were stained with anti-CD4, anti-CCR5 (Th1 lymphocyte marker), and anti-CCR4 antibodies (Th2 lymphocyte marker) (Figure 1, B–E).[15] Immunohistochemistry revealed that infiltrative CD4-positive lymphocytes around the hair bulbs are mostly CCR5-positive cells with a few CCR4-positive cells (Figure 1, C–E). More than 80% of infiltrating CD4-positive cells are CCR5-positive, while 10% of CD4-positive cells are CCR4-positive (Figure 1F), suggesting that Th1 reaction is dominant in alopecia areata skin.

Il4 Applications Ameliorated Alopecia in Mouse Model C3H/HeJ Mice

Given the above result that Th1 reactions are dominant in human alopecia areata region, we investigated whether or not Th2 type cytokine recombinant Il4 injection could improve the alopecia in alopecia areata mouse model C3H/HeJ mice. C3H/HeJ mice refers to the rodent alopecia areata model.[16–19] C3H/HeJ mice develop hair loss spontaneously. The hair loss in C3H/HeJ mice and human alopecia areata have the common five characteristics: 1) alopecia occurs spontaneously; 2) both CD4-positive lymphocytes and CD8 lymphocytes infiltrate around the hair follicles; 3) ICAM-1 and MHC class I and MHC class II are ectopically expressed in the hair follicle epithelium; 4) autoreactive circulating antibodies against the hair follicles are existent; 5) topical immunotherapy with squaric acid dibutylester is effective for alopecia both in humans and C3H/HeJ mice.

Since intraperitoneal injections of 0.1 μg of recombinant Il4 suppressed delayed-type hypersensitivity reactions,[20] we used the same amount of Il4 in the present study. Intralesional injections of recombinant Il4 (0.1 μg) every day for 3 weeks significantly restored hair shaft elongations from alopecic skin of C3H/HeJ female mice when compared with the sodium chloride injections. (Fig-

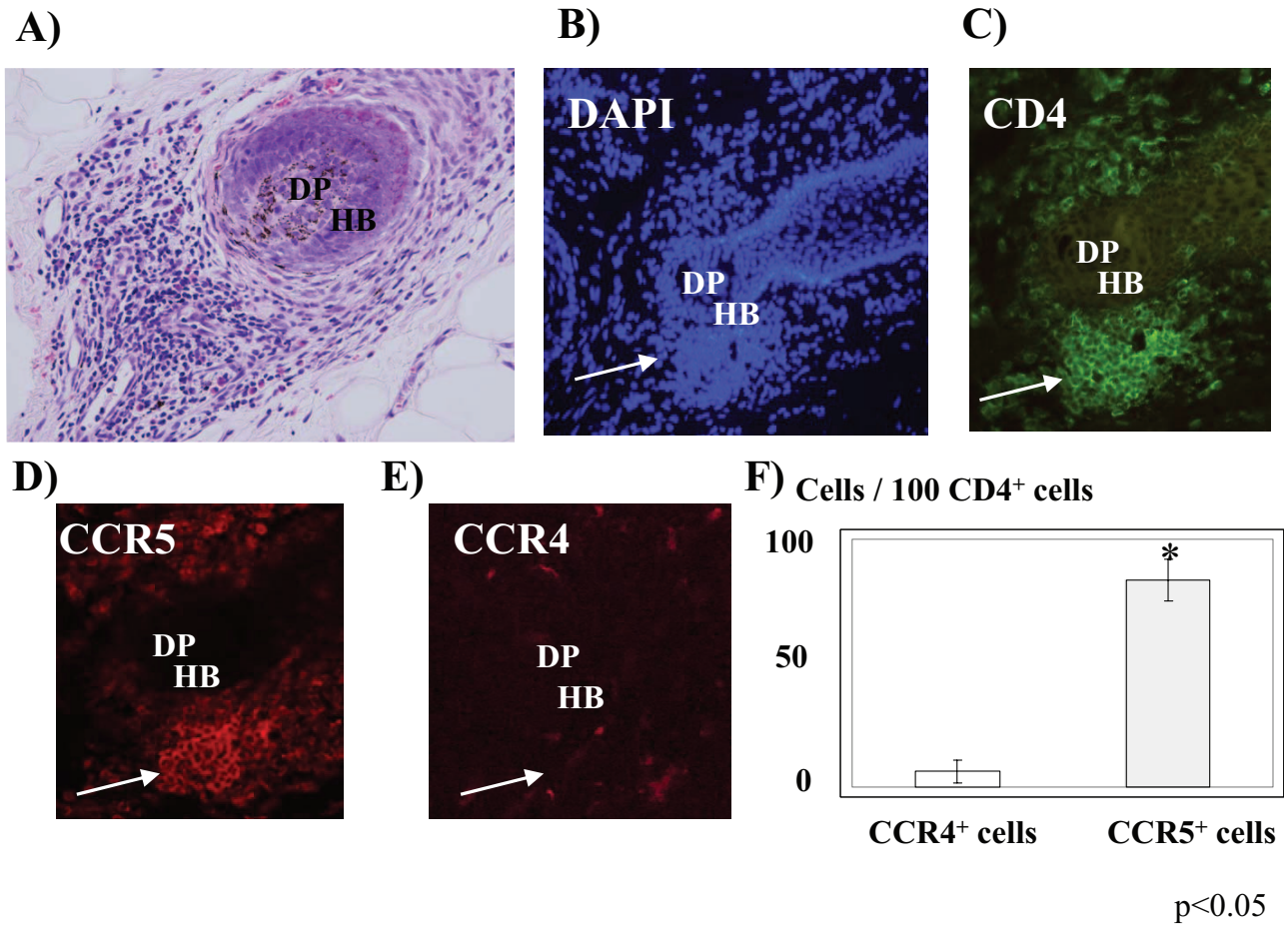

Figure 1. A: Hematoxylin and eosin staining of the section of alopecia areata patient. Dense lymphocytic infiltrates around the hair bulb. Immunohistochemical staining with 4′,6-diamino-2-phenylindole (**B**), anti-CD4 (**C**), anti-CCR5 (**D**), and anti-CCR4 (**E**) antibody. Infiltrating lymphocytes were dominantly CCR5 positive (**arrows**). **F:** Percentage of CCR4-positive and CCR5-positive cells among CD4-positive cells. Predominance of CCR5-positive cells. DP, dermal papilla; HB, hair bulb. $^*P < 0.05$.

ure 2, A–C). There was no recurrence of alopecia from these mice during a 2-month observation period after the cessation of Il4 application. This Il4 effect was suppressed by the simultaneous injection of 0.01 μg of recombinant Ifng (Figure 2C).

To observe whether Il4 suppressed the CD8 T lymphocyte infiltration around the hair follicles, we performed immunohistochemistry with anti-CD8 antibody before and after injections. There were a lot of CD8-positive T lymphocyte infiltrates around the hair follicles as described (Figure 2D).[16] However, after Il4 applications, the number of CD8 T cell infiltrates around the hair follicles statistically significantly decreased (Figure 2, E and F).

Ifng Expression Level Is Elevated in the Alopecic Region in C3H/HeJ Mice and Suppressed by Intralesional Il4 Injections

To explore which cytokines are up-regulated or down-regulated in the alopecic region in C3H/HeJ mice, we performed semiquantitative RT-PCR using skin cDNA with Th1 cytokine, *Ifng* and IL-12b (*Il12b*), and Th2 cytokine *Il4* primers. In the alopecic skin, Th1 cytokine Ifng mRNA was expressed nine times stronger than in unaffected skin compared to β-actin (*Actb*) cytoplasmic expression as a control (Figure 3, A and D). Intralesional Il4 injections into the alopecic skin significantly suppressed the enhanced expression of Ifng mRNA (Figure 3, A and D). The expression level of Ifng mRNA in the Il4-injected skin returned to the same level as that in unaffected skin.

There was no statistically significant difference in the expression level of Il4 or Il12b mRNA between in alopecic skin and in unaffected skin (Figure 3, B and C). Furthermore, intralesional Il4 injections into the alopecic skin had no significant effect on the expression of Il4 or Il12b mRNA.

Intralesional Anti-Ifng Neutralizing Antibody Injections Were Effective Treatment for the Alopecia in Alopecia Areata Mouse Model C3H/HeJ Mice

The above result suggested that higher expression of Ifng mRNA expression in the alopecic skin might play an important rule in the pathogenesis of alopecia areata in

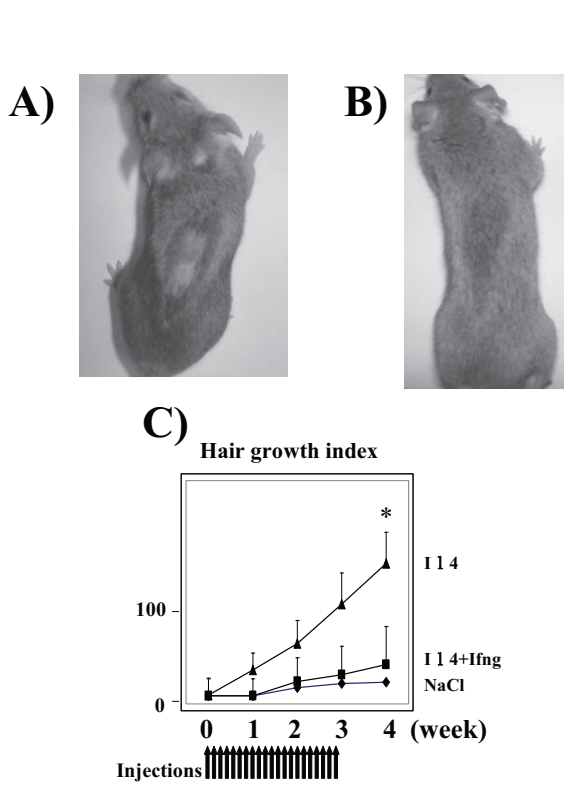

Figure 2. Alopecic skin lesion on the back of the C3H/HeJ mouse before (**A**) and after (**B**) recombinant Il4 injections. **C:** Hair growth index of alopecia with Il4, Il4 + Ifng and control NaCl injections ($n = 10$ each). CD8-positive cell infiltrates into the hair follicles before (**D**) and after (**E**) the Il4 injections. **F:** The number of CD8 T cell infiltrates per hair follicle was diminished after Il4 injections ($n = 4$ each). HS, hair shaft. $^*P < 0.05$.

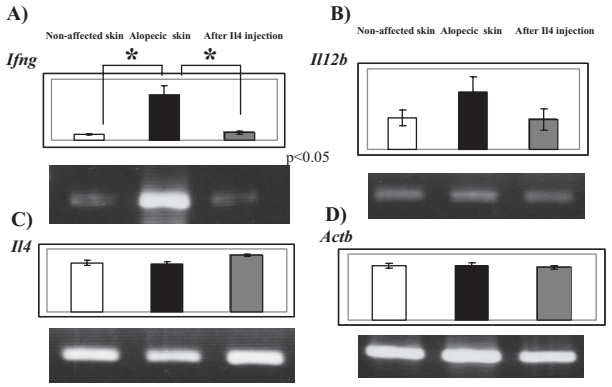

Figure 3. Expression of *Ifng* (**A**), *Il12b* (**B**), *Il4* (**C**), and *Actb* (**D**) mRNA in unaffected skin and alopecic skin. *Ifng* mRNA was suppressed by Il4 injections (**A**) ($n = 3$ each). $^*P < 0.05$.

C3H/HeJ mice. To determine whether blockade of Ifng actions in the skin leads to the restoration of the hair elongation, rat anti-neutralizing antibody against mouse Ifng was injected into the alopecic skin of the female C3H/HeJ mice. Anti-Ifng antibody injections improved the hair growth index more efficiently than the control rat IgG (Figure 4). There was no disappearance of hair shafts from these mice during a 2-month observation period after the cessation of antibody application.

Intralesional Injections of Tbx21 Antisense Oligonucleotide Were Effective Treatment for the Alopecia in Alopecia Areata Mouse Model C3H/HeJ Mice

Tbx21 (formerly known as T-bet) is a T-box containing transcription factor and indispensable for Th1 differentiation. This protein binds to the promoter of *Ifng* gene and augments its expression. To examine a role of *Tbx21* in the pathogenesis of alopecia areata, we subcutaneously injected antisense *Tbx21* oligonucleotide into female C3H/HeJ mice alopecic lesions every other day for seven times. Although the effect of antisense *Tbx21* oligonucleotide took place more slowly than Il4 or neutralizing anti-Ifng antibody (Figures 2, 4, and 5), antisense *Tbx21* oligonucleotide was significantly more effective for alopecia than non-sense oligonucleotide (Figure 5). There was no disappearance of hair shafts from these mice

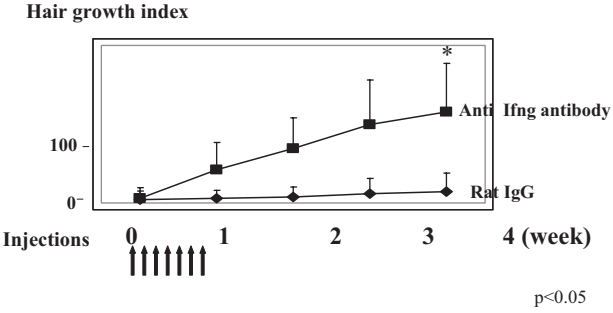

Figure 4. Hair growth index of alopecia with rat neutralizing anti-Ifng antibody and control rat IgG injections ($n = 8$ each). $^*P < 0.05$.

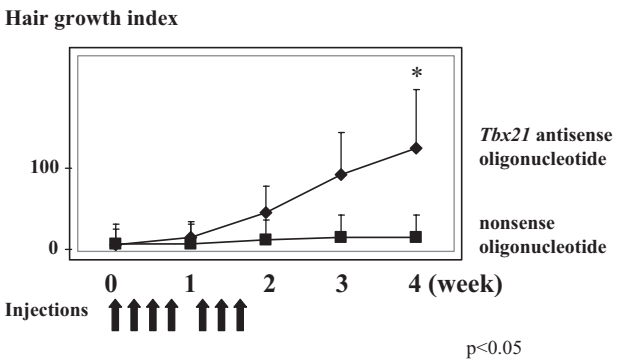

Figure 5. Hair growth index of alopecia with *Tbx21* antisense oligonucleotide and non-sense oligonucleotide injections ($n = 6$ each). $^*P < 0.05$.

during a 2-month observation period after the cessation of antisense oligonucleotide application.

Intralesional Injections of Tbx21 siRNA Conjugated with Cationized Gelatin Were Effective Treatment for the Alopecia in Alopecia Areata Mouse Model C3H/HeJ Mice

To decrease the number of injections, we introduced a new drug delivery system, that is, a controlled release of siRNA conjugated with a biodegradable cationized gelatin. After conjugation of siRNA with the cationized gelatin at 4°C overnight, we subcutaneously injected siRNA conjugated with cationized gelatin once a week three times. Cationized gelatin is gradually processed in the skin, and, in turn, released siRNA binds sequence-specifically to mRNA. Cationized gelatin-conjugated *Tbx21* siRNA injections were more effective than naked *Tbx21* siRNA injections or non-sense siRNA conjugated with cationized gelatin (Figure 6B). There was no recurrence of alopecia in the mice during a 2-month observation period after the cessation of *Tbx21* siRNA application. The result suggests that controlled release of siRNA with a biodegradable cationized gelatin effectively worked *in vivo*.

Controlled Release of Tbx21 siRNA Suppressed Tbx21 Protein Expression in Skin

To investigate whether controlled release of *Tbx21* siRNA in skin really suppressed the Tbx21 protein expression, we checked the Tbx21 expression level by Western blotting. Tbx21 protein expression was indeed suppressed by the controlled release of *Tbx21* siRNA (Figure 6A). The similar intensity of the control Actb band between before and after *Tbx21*siRNA injections indicated that suppression of mRNA expression by *Tbx21* siRNA was gene-specific.

Controlled Release of Tbx21 siRNA Suppressed MHC Class II Expression in Alopecic Skin

Since ectopic expression of MHC class I and MHC class II in the hair follicles may destroy the immune privilege of

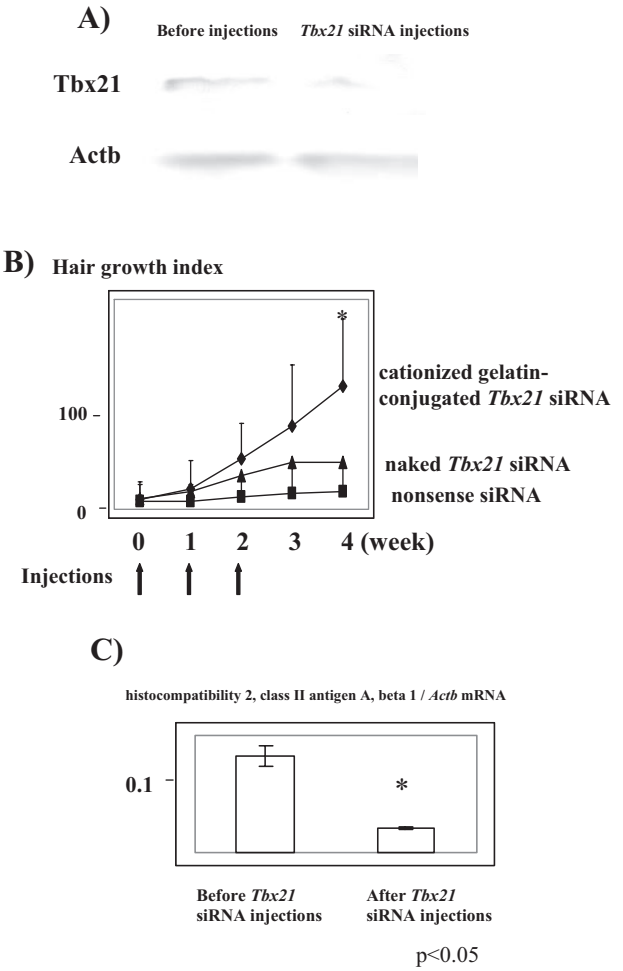

Figure 6. A: Western blotting of skin lysates before and after cationized gelatin-conjugated *Tbx21* siRNA injections. Tbx21 protein expression was suppressed after cationized gelatin-conjugated *Tbx21* siRNA injections, while control Actb protein expression remained at the same level. **B:** Hair growth index of alopecia with cationized gelatin-conjugated *Tbx21* siRNA, naked *Tbx21* siRNA, and control cationized gelatin-conjugated non-sense siRNA injections ($n = 8$ each). $^*P < 0.05$. **C:** Histocompatibility 2, class II antigen A, β1 mRNA expression in alopecic skin measured with real-time PCR was suppressed by cationized gelatin-conjugated *Tbx21* siRNA injections ($n = 4$ each). $^*P < 0.05$.

hair follicles, we explored the expression change of histocompatibility 2, class II antigen A, β1 mRNA after the *Tbx21* siRNA injections. Real-time RT-PCR study revealed that controlled release of *Tbx21* siRNA suppressed histocompatibility 2, class II antigen A, β1 mRNA expression in alopecic skin (Figure 6C). This result suggests a restoration of immune privilege of hair follicles after *Tbx21* siRNA injections.

Discussion

Alopecia areata is an autoimmune disease that sometimes evolves into alopecia totalis affecting the whole scalp.[1] This severe type of alopecia tends to be resistant to conventional therapies such as topical immunotherapy with squaric acid dibutylester or diphenylcyclopropenone, oral steroid intake, psoralen plus ultraviolet A treat-

ment, or topical steroid application. Therefore, these patients wait for a new therapy.

Cytokine therapy is a novel therapeutic approach for alopecia areata resistant to previous therapies, since cytokine therapy has already been proved to be effective for a skin inflammatory disease, psoriasis vulgaris.[21,22] Psoriasis vulgaris is a hyperproliferative skin disease in which Th1 lymphocytes predominantly infiltrate in the dermis. Systemic Il4 injections are effective for amelioration of psoriasis vulgaris.[21] In our study, we demonstrated that infiltrating CD4 lymphocytes around the hair follicles are mostly CCR5-positive cells indicating that these cells are Th1 CD4 lymphocytes (Figure 1). The injection of Th2 cytokine, Il4, intralesionally instead of systematically, resulted in hair shaft elongation through the suppression of CD8 T lymphocyte infiltrates around the hair follicles (Figure 2). Alopecia areata is usually limited to the head and, hence, it serves a good candidate for a local injection. Local administration of cytokines might be better than a systemic administration of cytokines for the following reasons: 1) a required dose might be smaller; 2) systemic side effects might be less; and 3) topical application is easier than systemic administration. Biedermann et al demonstrated that more than three intraperitoneal Il4 injections per day was necessary to improve delayed-type hypersensitivity reactions[20] in contrast to a single subcutaneous injection per day in our study. Drug delivery of Il4 and half-time of Il4 decay may be different due to the means of application.

In our study, intralesional injections of Il4 suppressed an enhanced expression of *Ifng* in alopecic skin (Figure 3). In a previous study, when Ifng was injected into non-alopecic genetically susceptible C3H/HeJ mice, alopecia was induced with an ectopic expression of MHC class I and class II on follicular epithelium.[19] Our study demonstrated that anti-Ifng neutralizing antibody injections ameliorated alopecia in C3H/HeJ mice (Figure 4). Taken together, Ifng plays a pivotal role both in the induction and the maintenance of alopecia in genetically susceptible C3H/HeJ mice.

Gene therapy is a new therapeutic approach for intractable alopecia areata. To establish a gene therapy for a rodent alopecia areata model C3H/HeJ mice, we focused on *Tbx21* gene. Tbx21 plays a key role in Th1 cell development.[9] Tbx21 deficient mice revealed a defect in Th1 immune reaction.[9] Moreover, a recent study showed that Tbx21 binds to a distal conserved sequence element of *Ifng* gene and enhances *Ifng* gene expression.[10] Our study demonstrated that intralesional injections of *Tbx21* antisense oligonucleotide restored the hair shaft elongation in alopecia areata model C3H/HeJ mice (Figure 5). Therefore, local injection of *Tbx21* antisense oligonucleotide may exert similar effects on alopecia by suppressing the expression of *Ifng*.

RNA interference by siRNA is a recently developed powerful technique to suppress the expression of certain genes with a high specificity.[5] The gene delivery system is generally divided into two categories: viral and nonviral vectors. Although viral vectors such as retrovirus, adenovirus, and adeno-associated virus are potentially efficient, nonviral vectors have the advantages of less toxicity, less immunogenicity, and easier preparation.[8] So far, several methods for delivering genes with nonviral carriers have been developed, including naked plasmid DNA injection and complex formation with cationized polymers or cationized liposomes. However, there are several drawbacks with each nonviral vector, including a low efficiency of gene transfection compared with viral vectors and a transient gene expression. In this study, we introduce a system of prolonged release of siRNA from cationized gelatin microspheres. The controlled release of the siRNA may prevent rapid degradation of DNA and facilitate exposure and transduction of siRNA to cells. Moreover, the *in vivo* enzymatic degradation of gelatin microspheres depends on their cross-linking extent, which can be regulated by changing the concentration of glutaraldehyde used for microsphere preparation.

Cationized gelatin used in our study is a very safe molecule for human beings. Comparison between the cationized gelatin-conjugated siRNA and naked siRNA in our study revealed that controlled delivery of siRNA with cationized gelatin was indeed more efficient for alopecia than naked siRNA (Figure 6B). It is likely that naked siRNA is more easily degraded by nuclease than siRNA conjugated with cationized gelatin.

Trial of the treatment with controlled delivery of siRNA for skin diseases has several advantages over usage of siRNA for other organ diseases. First, local applications of siRNA to skin lesions are very easy. Second, the cutaneous changes induced by siRNA application can be observed without any difficulty or expensive machines. Third, pathological changes can be assessed easily by skin biopsies.

The drug delivery system is totally different between human skin and mouse skin. Moreover, the mouse model used in our study shows a number of differences to human alopecia areata. Before application of this siRNA delivery system into therapeutic use for human alopecia areata, these points should be carefully and critically assessed.

In conclusion, we have developed an efficient and safe method for *Tbx21* siRNA delivery to alopecic skin and demonstrated specific inhibition of target gene expression resulting in a restoration of hair shaft elongation. To our knowledge, this is the first report of effective controlled delivery of siRNA using biodegradable cationized gelatin in animal models of diseases.

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