mined by the Bradford procedure with a protein assay kit using bovine IgG as the standard (Bio-rad, Richmond, CA).

Culture of ocular conjunctival epithelial cells. Human conjunctival epithelial cells, the Wong Kilborne derivative of Chang epithelial cells, were obtained from American Tissue Type Culture Collection. The conjunctival epithelial cells were cultured in 199 Hanks' medium (Gibco BRL, Carlsbad, CA) supplemented with 10% FCS.

Cells were detached by incubating a monolayer of the cells with a pre-warmed trypsin-EDTA solution for 5 min at 37 °C. Trypsin-neutralizing solution (Cambrex, Walkersville, MD) was added to the cell suspension, and cells were formed into pellets by centrifugation at 210 g for 5 min. The cells were resuspended in culture medium, counted by trypan blue exclusion to determine the viable cell number, and plated at 1.2×10^4 cells/well in 96-well plates, 2.5×10^4 cells/well in 48-well plates, or 1.0×10^5 cells/well in 12-well plates (Corning, Corning, NY). The cultures were incubated in a humidified atmosphere containing 5% CO_2 at 37 °C.

Stimulation of ocular conjunctival epithelial cells. The cells grown to 80% confluence were cultured in FCS-negative medium for 6–12 h, washed, and then stimulated with the WCE (5 or 10 μg/ml). In some experiments, the WCE was pretreated with 0.5 mM E-64 (Peptide Institute, Osaka, Japan) and/or 7.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF; Sigma, St. Louis, MO) for 30 min at 37 °C and diluted, or recombinant human SLPI (R&D systems, Minneapolis, MN) or purified human α1-antitrypsin (Sigma) was added to the medium at a final concentration of 100 nM. Final concentrations of E-64 and AEBSF were 3.9 μM and 58 μM, respectively.

ELISA. Concentrations of IL-6 and IL-8 in the culture supernatant of cells were measured with ELISA kits (DuoSet; R&D Systems).

Real-time quantitative PCR. Total RNA was extracted from cells using RNAspin Mini (GE Healthcare, Buckinghamshire, UK) and treated with DNase I (GE Healthcare) or an RNeasy Plus Micro Kit (QIAGEN, Hilden, Germany). cDNA was synthesized with SuperScriptII reverse transcriptase (Invitrogen, Carlsbad, CA) and random primers. Real-time quantitative PCR was performed with a TaqMan method using ABI 7500 (Applied Biosystems, Piscataway, NJ). The mRNA level was normalized to the gene expression of glyceraldehyde-3-phosphate dehydrogenase or β-actin.

Statistics. The significance of differences in mean responses was determined with one-way ANOVA and Tukey's *post hoc* test or Student's *t*-test (two-tailed). A value of $p < 0.05$ was regarded as statistically significant. Data shown are representative of three independent experiments.

Results

Whole-mite culture extract induced the release of IL-8 and IL-6 from human ocular conjunctival epithelial cells

We examined whether WCE stimulates ocular conjunctival epithelial cells to release IL-8 and IL-6 (Fig. 1). Because WCE is a soluble extract prepared by the mild agitation of a whole-mite culture containing mite fecal pellets, body fragments, and also residual feed for the mites' growth, we used fresh feed for mites without the addition of live mites as a negative control. The 24-h stimulation with WCE upregulated the release of IL-8 and IL-6, whereas the feed alone did not (Fig. 1A and C). Concentrations of IL-8 and IL-6 in the culture supernatant increased rapidly until 6 h after the stimulation and slowly after that (Fig. 1B and D).

Whole-mite culture extract-induced release of IL-8 and IL-6 was dependent on the serine protease activity

As the WCE exhibited major serine protease activity and minor cysteine protease activity in analyses using fluorogenic synthetic peptide substrates and irreversible class-specific inhibitors (data

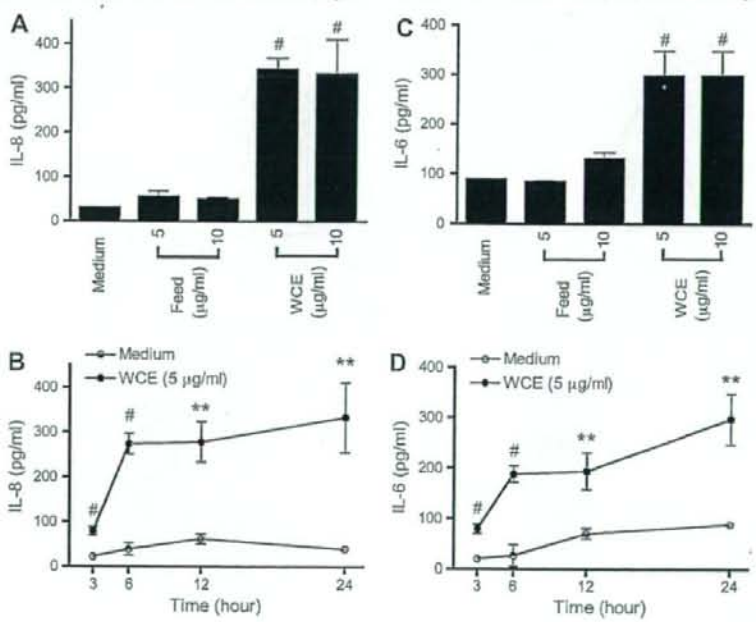

Fig. 1. Whole-mite culture extract induced the release of IL-8 and IL-6 from the human ocular conjunctival epithelial cells. The human ocular conjunctival epithelial cell line Chang was stimulated with whole-mite culture extract (*WCE*), the feed for mites before the addition of live mites as a negative control (*Feed*) at the protein concentrations indicated, or basal medium alone (*Medium*) in 96-well plates (150 μl/well). The culture supernatant was collected at 24 h (**A** and **C**) or at 3, 6, 12, and 24 h (**B** and **D**) and subjected to ELISA. The data indicated are the mean ± SD for three wells and representative of three independent experiments with similar results. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{\#}p < 0.001$ by one-way ANOVA and Tukey's *post hoc* test compared with *Medium* (**A** and **C**) and *t*-test compared with *Medium* (**B** and **D**).

not shown) [16], we next examined whether synthetic irreversible class-specific protease inhibitors can inhibit the WCE-induced release of IL-8 and IL-6. The serine protease-specific inhibitor, AEBSF, inhibited the WCE-induced release of IL-8 and IL-6 significantly (Fig. 2) but the cysteine protease-specific inhibitor, E-64, did not (data not shown).

Whole-mite culture extract induced the expression of IL-8 and IL-6 at the transcriptional level in a serine protease activity-dependent manner

The expression of IL-8 and IL-6 at the transcriptional level was evaluated by real-time quantitative PCR (Fig. 3). WCE significantly upregulated the mRNA expression of IL-8 and IL-6 with a peak at 3 h (Fig. 3A and C). AEBSF significantly inhibited the WCE-upregulated gene expression (Fig. 3B and D).

Innate antiproteases, SLPI and α1-antitrypsin, inhibited the whole-mite culture extract-induced release of IL-8 and IL-6

SLPI and α1-antitrypsin are major innate antiproteases at the ocular mucosa [14]. We examined whether these antiproteases can inhibit the release of cytokines induced by the mite-derived serine protease activity contained in the WCE. SLPI and α1-antitrypsin significantly inhibited the WCE-induced release of IL-8 and IL-6 (Fig. 4).

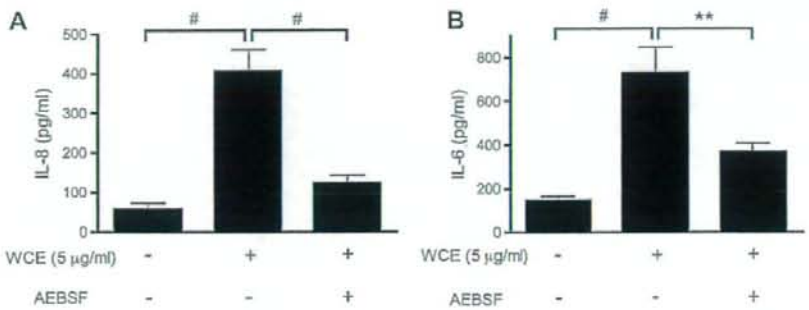

Fig. 2. Whole-mite culture extract-induced release of IL-8 and IL-6 was dependent on the serine protease activity. The human ocular conjunctival epithelial cell line Chang was stimulated with whole-mite culture extract (*WCE*) in the absence or presence of the synthetic irreversible serine protease-specific inhibitor AEBSF, in 48-well plates (250 μl/well). The culture supernatant was collected at 24 h and subjected to ELISA. The data indicated are the mean ± SD for three wells and representative of three independent experiments with similar results. $^{**}p < 0.01$ and $^{\#}p < 0.001$ by one-way ANOVA and Tukey's *post hoc* test.

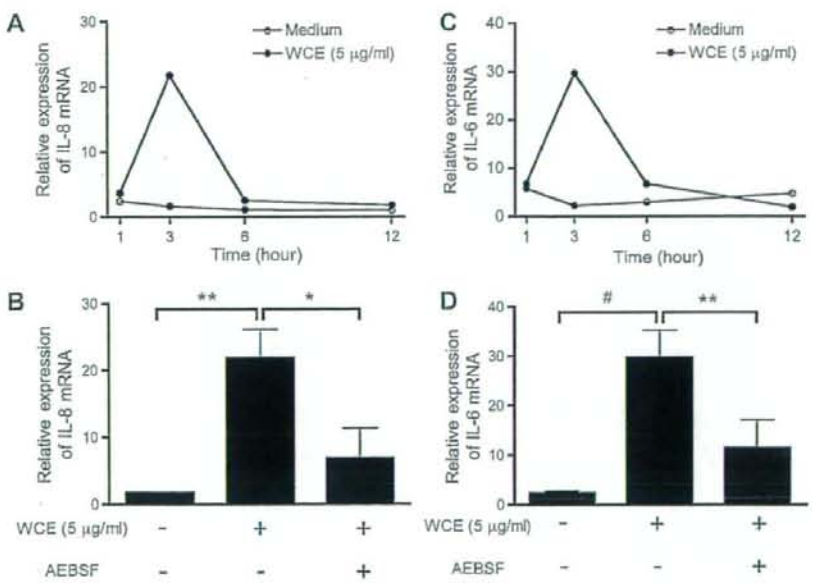

Fig. 3. Whole-mite culture extract induced expression of IL-8 and IL-6 at the transcriptional level in a serine protease activity-dependent manner. The human ocular conjunctival epithelial cell line Chang, was stimulated with whole-mite culture extract (*WCE*) in the absence or presence of the synthetic irreversible serine protease specific inhibitor AEBSF, or basal medium alone (*Medium*), in 12-well plates (1 ml/well). The cells were collected at 1, 3, 6, and 12 h (**A** and **C**) or at 3 h (**B** and **D**). Total RNA was extracted and analyzed by real time quantitative PCR. Changes in normalized gene expression were indicated as fold-increases relative to the mean for three wells just before the stimulation. The data indicated are the values for single wells (**A** and **C**) and the mean ± SD for three wells (**B** and **D**) and are representative of three independent experiments with similar results. $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{\#}p < 0.001$ by one-way ANOVA and Tukey's *post hoc test* (**B** and **D**).

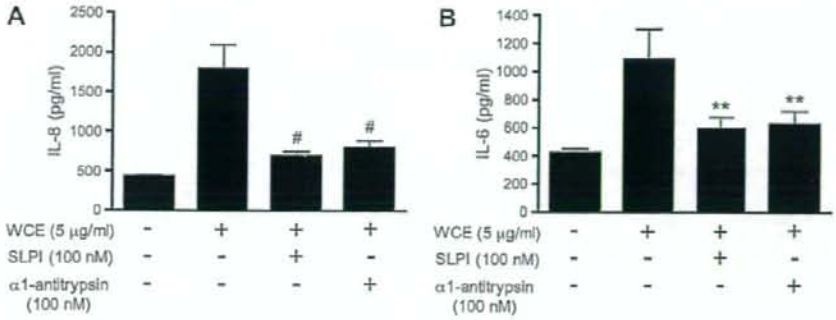

Fig. 4. Innate antiproteases, SLPI and α1-antitrypsin, inhibited the whole-mite culture extract-induced release of IL-8 and IL-6. The human ocular conjunctival epithelial cell line Chang was stimulated with whole-mite culture extract (WCE) in the absence or presence of SLPI and α1-antitrypsin, in 48-well plates (200 μl/well). The culture supernatant was collected at 24 h and subjected to ELISA. The data indicated are the mean ± SD for three wells and representative of three independent experiments with similar results. $^{\#}p < 0.01$ and $^{**}p < 0.001$ by one-way ANOVA and Tukey's *post hoc* test.

Discussion

We demonstrated that cultured ocular conjunctival epithelial cells released IL-6 and IL-8 (Figs. 1 and 2), with an upregulation in their gene expression (Fig. 3), in response to the serine protease activity of WCE, a soluble extract prepared from a whole-mite culture containing mite fecal pellets and body fragments. IL-6 is a pleiotropic cytokine and promotes the proliferation and differentiation of various cell types of a dermal or epidermal origin as well as B cells and may mediate the shift from an acute to chronic phase in inflammatory reactions including allergic diseases. IL-8/CXCL8 is a chemokine, which has an important role in inflammation through its ability to recruit and activate leukocytes. The concentration of WCE used in the present study (5 or 10 μg/ml) is considered relevant to exposure to house dust mite-derived particles because mite fecal pellets are known to contain the major group 1 allergen at relatively high concentrations (10 mg/ml) [17].

The epithelium and epidermis are located at the interface between the body and environment and have critical roles in the response to various stimuli [2,9]. Protease-activated receptor-2 (PAR-2), which can be activated by cleavage with serine proteases, has been implicated in the development of inflammatory reactions including allergies [18–20], suggesting the exogenous proteases contained in allergen sources, such as mite fecal pellets [16] and pollen grains [21,22], to be involved in the sensitization process and effecter reactions. Sun et al. [13] demonstrated that mite serine protease allergens, Der p 3 and Der p 9, induced the release of GM-CSF and eotaxin from the human airway epithelial cell line A549, via the PAR-2 pathway. In the present study, we demonstrated for the first time that mite-derived serine protease activity induces the release of IL-6 and IL-8 in human ocular conjunctival epithelial cells (Figs. 1–3). Recently, primary human corneal epithelial cells [23] and the human conjunctival epithelial cell line used in the present study [24] have been reported to express functional PAR-2 and PAR-1. Whether the mechanism by which the mite-derived serine protease activity acts on the ocular epithelia involves the PAR-2 pathway remains to be addressed.

The IL-6 and IL-8 induction was inhibited not only by AEBSF (Figs. 2 and 3), but also by SLPI and α1-antitrypsin (Fig. 4). SLPI and α1-antitrypsin, major innate antiproteases at the ocular mucosa, are detectable in tear fluid [14]. The concentration of the innate inhibitors used in the present study (100 nM: 1.2 μg/ml SLPI and 4.5 μg/ml α1-antitrypsin) is considered physiologically relevant in the ocular conjunctival mucosa, because even reflex tear samples contained approximately 5–10 μg/ml SLPI and 1–3 μg/ml α1-antitrypsin and closed-eye tear samples contained around 5–20-fold greater concentrations of the inhibitors [14]. Therefore, we consider that SLPI and α1-antitrypsin can inhibit the response of the ocular conjunctival epithelium to mite-derived serine protease activity on low-dose exposure to small numbers of house dust mite-derived particles or on extensive dilution with tear fluid.

The balance between endogenous proteases and innate antiproteases is a major determinant of tissue integrity [14,25,26]. The antiproteases, which serve to neutralize excess protease load and protect host tissues, have been classified as either "systemic" or "alarm" antiproteases [25]. α1-antitrypsin is a "systemic" antiprotease, produced by hepatocytes and reaching the interstitium through diffusion from the circulation. SLPI is an "alarm" antiprotease, produced by cells close to the site of inflammation and providing a local inducible antiprotease defence. The inhibitory effects of SLPI on serine proteases in the granules of plymorphonuclear cells and mast cells suggest therapeutic potential against various diseases [25,26]. In an allergic conjunctivitis model using ovalbumin-sensitized guinea pigs, Murata et al. [27] has demonstrated the therapeutic effect of topical SLPI application. Results in the present study suggest another role for SLPI, preventing the release of proinflammatory cytokines induced by serine protease activity from environmental allergen sources at the ocular conjunctiva. However, exposure to large numbers of house dust mite-derived particles or mite-derived serine protease activity not diluted extensively in individuals with conditions such as dry eyes, could lead to excessive mite serine protease activity against innate inhibitors and be involved in the initiation of sensitization through the ocular conjunctival epithelium and/or exacerbation of allergic conjunctivitis.

In summary, we demonstrated that mite-derived serine protease activity induces the release of proinflammatory cytokines from human ocular conjunctival epithelial cells and that the response can be inhibited by innate antiproteases, SLPI and α1-antitrypsin, at a concentration physiologically relevant at the ocular mucosa. The findings suggest a homeostatic role for SLPI and α1-antitrypsin in the conjunctiva against proteases from environmental allergen sources and that exposure to house dust particles containing mite-derived serine protease activity could be involved in the initiation of sensitization through the ocular conjunctival epithelium and/or exacerbation of allergic conjunctivitis.

Acknowledgements

The authors thank Michiyo Matsumoto for secretarial assistance and Tomoko Tokura, Mutsuko Hara, Takasuke Ogawa, Seiji

Kamijo, Hiroko Ushio, and Keiko Maeda for technical assistance, technical advice, helpful comments, and encouragement.

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研究動向　基礎研究

経皮ワクチン

内藤誠之郎

Skin vaccination

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Abstract

Skin is an immunologically active organ composed of various immunocompetent cells and draining lymph nodes. Especially, Langerhans cells (LCs) are located in the outer layer of the skin and play the role of sentinels. Therefore, LCs could be an ideal targets of vaccination. Recently, many studies have demonstrated that the topical application of antigens on the skin induced both humoral and cellular immune responses. This emerging needle-free vaccination route would have various clinical merits and could be highly promising.

Key words: transcutaneous immunization, needle-free vaccination, Langerhans cell

はじめに

言うまでもないことであるが，ワクチンが働くためには，まず，ワクチンが体内に入らなければならない．ワクチンを体内に入れるためには，注射により直接注入するのが確実である．したがって，現在のほとんどのワクチンは注射により接種されている．一方，注射は，本質的にリスクを伴う．注射局所の腫れや痛み，発熱，アナフィラキシー，脳炎などの副反応の多くは注射に付随するものと考えられる．また，注射をするためには，注射器という特別な器具と訓練を積んだ施術者が必要である．そもそも，注射は痛い．

そこで，長らく，注射によらないワクチンが待ち望まれてきた．注射によらないワクチンとしては，経口および経鼻ルートによる粘膜ワクチンの開発が進んでいる．粘膜に比べて，皮膚は強固な物理的なバリアであり，注射以外の方法でワクチンを体内に送り込むことは難しいと考えられてきた．ところが，近年，無傷の皮膚表面に抗原溶液を塗布するだけで，抗原特異的な免疫応答を誘導しうることが報告された．

本稿では，注射，粘膜に次ぐ，第3の免疫ルートである経皮ワクチンについて概説する．

1. 経皮ワクチンのメカニズム

図1に，現在，一般に考えられている，経皮ワクチンによる免疫誘導の仕組みを示す．

皮膚は，成人では表面積約1.6 m^2，重量約3 kgで，人体でも最大級の臓器である．皮膚は，外側から表皮，真皮，皮下組織の3層よりなる．表皮の厚みは部位により異なるが，平均120 μm程度である．表皮の95％は石垣のように積

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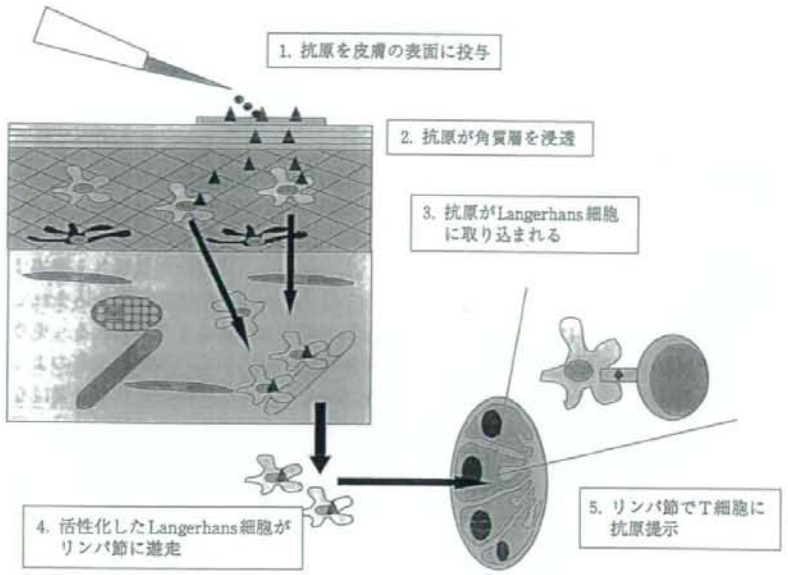

図1 経皮ワクチンのメカニズム

み重なったケラチノサイトであり，残りの5%が樹状細胞の一種であるLangerhans細胞とメラノサイトである．表皮の最外層は，角化したケラチノサイトとその間を埋める細胞間脂質よりなる角質層で覆われている．角質層は，わずか20 μm程度の厚みであるが，外界からの異物の侵入と体内からの水分の蒸散を防ぐ高機能の膜である．真皮は，コラーゲンなどからなる間質組織の中に血管やリンパ管が通り，線維芽細胞，真皮樹状細胞，マスト細胞などが分布している．また，真皮には感覚性の神経終末があり，注射が痛いのは，このためである．

皮膚は，常に外界からの様々な刺激や感染の危険にさらされている．これに対して，皮膚は単なる物理的な防壁ではなく，Langerhans細胞，ケラチノサイト，真皮樹状細胞をはじめとする細胞群と皮膚に分布するリンパ管およびその下流に位置するリンパ節とをあわせて免疫システムを構成している[1]．このシステムはskin-associated lymphoid tissues (SALT) と呼ばれることもある．

経皮ワクチンは，SALTの機能を利用して生体に免疫を与えるワクチンであるということができる．とりわけ，経皮ワクチンに対する免疫応答では，Langerhans細胞が中心的な役割を果たしていると考えられている．皮膚の表面に投与された抗原は，角質層を浸透してLangerhans細胞に取り込まれる．抗原を取り込んだLangerhans細胞は活性型に変化して，真皮のリンパ管から所属リンパ節へと遊走する．同時にLangerhans細胞は，分解した抗原ペプチドを活性化により増加した細胞表面のMHC-class IIとともに$CD4^+$T細胞に提示する．これにより抗原特異的な体液性免疫応答が開始される．更に，一部の抗原ペプチドはクロスプレゼンテーションの機構により，MHC-class Iとともに$CD8^+$T細胞に提示されて，細胞性免疫を誘導するといわれている[2]．

2. 様々な経皮ワクチンの方法

経皮ワクチンは，投与される物質によりタンパク/ペプチドとDNAに分けることができる．

また，特殊なケースとして生のウイルスを投与したケースも報告されている[5]．一方，ワクチンの投与方法についても，下記のように様々な試みがある．共通点は，いずれも注射針を使わないで皮膚の表面から抗原物質を送達する点である．

a. Transcutaneous immunization

1998年，Glennら[6]は，特別な装置や器具を使わずに，タンパク抗原とともにアジュバントとしてコレラトキシン(CT)を皮膚表面に塗布するだけで，マウスの血清中に抗原特異的な抗体産生を誘導できることを報告した．その免疫方法は驚くほど簡単なものであり，バリカンで毛を刈ったマウスの背中の皮膚に，CTを加えた抗原溶液を塗布して2時間放置するというものであった．2時間後には塗布した抗原をマウスが舐めたりしないように，入念に水で洗い流している．また，毛刈りのときの傷口から抗原が体内に入る可能性を否定するために，毛刈りの48時間後に抗原の塗布を行っている．更に，耳への塗布(毛刈りは必要ない)でも免疫が誘導されることを示している．このように入念な実験を行っているのは，当時，皮膚に単に抗原とアジュバントを塗布するだけで免疫応答が誘導できることは常識的には考えられなかったからである．

この方法はGlennらによってtranscutaneous immunization(TCI)と名づけられて，その後，多くの研究者が様々な抗原を用いて有効であることを確認している[5-7]．

その後，CpG-ODN[8]やimiquimod[9]などのTLRリガンドなどもアジュバントとして有効であることが報告されているが，CT(およびその類似物質である大腸菌易熱性毒素(LT))に比べるとアジュバント活性は低い．基本的には，経皮ワクチンにより有効な免疫応答を誘導するためには，アジュバントを使用することが必須であると考えられてきた．しかし，著者ら[10]は，抗原溶液を含ませたガーゼパッチを一昼夜マウスの皮膚に貼り付けておくことにより，CTをアジュバントとして使用した場合と遜色ない免疫応答を誘導できることを示した．

表皮には堅牢なバリア機能があり，一般に500 Daを超える分子量の物質は容易に通過できないと考えられている[11]．したがって，経皮吸収薬のほとんどは500 Da以下である．では，なぜ，500 Daをはるかに超える分子量をもつ抗原タンパクを皮膚の表面に投与して，免疫応答が誘導されるのだろうか．

現在，著者らは以下のように考えている．

まず第一に，抗原は角質層さえ通過すれば，あとはLangerhans細胞に取り込まれて，免疫応答が開始されうると考えられる．免疫を誘導するためには，多くの経皮吸収薬のように真皮まで到達して血中に吸収される必要はない．

第二に，角質層のバリア機能には，意外に脆い面もあるのではないかと考えられることである．例えば，著者らは，抗原パッチを貼り付ける前に，皮膚をアルコール綿で数回スワブするだけでも，血清中の抗体応答が数倍に増強されることを観察している．当然のことながら，テープストリッピングなどにより角質層を破壊すれば，誘導される抗体応答は数十倍から数百倍に増強される．

第三に，免疫を誘導するのに必要な抗原分子は，投与した抗原のうちのほんの一部でよいと考えられることである．著者らのある実験では，マウスの皮膚1 cm^2に卵白アルブミン(OVA)を100 μg投与した．この投与量で十分に有意な免疫応答が誘導される．OVAの分子量は45,000なので，100 μgには約10^{15}個のOVA分子が含まれている．そのうちの0.1%が角質層を通過したとすると，10^{12}個の分子がLangerhans細胞の分布する領域にまで到達することになる．一方，皮膚1 cm^2には約10^5個のLangerhans細胞が分布している．したがって，角質層を通過した分子の，更に0.1%のみがLangerhans細胞に取り込まれたとしても，Langerhans細胞1個あたり10^4個のOVA分子が取り込まれることになる．このように，皮膚の表面に投与した抗原のうちほんのわずかな割合の分子が体内に入るだけで，十分に有意な免疫応答が誘導されうると思われる．

b. その他の経皮ワクチン

TCI法が発見される以前から，経皮吸収薬の研究開発分野では，様々な方法が試みられている．例えば，イオントフォレシス，ソノフォレシス，エレクトロポーレーション，マイクロニードルといった方法である．また，経皮吸収を促進する薬物の研究も進んでいる．これらの方法を経皮ワクチンに応用する試みも行われ始めている[12]．また，ミクロンサイズの金微粒子の表面に固定したDNAを圧縮空気で皮膚に打ち込むジーンガン法は，以前からDNAワクチンの投与法として研究が進められている．近年，この方法をタンパク抗原に応用することも試みられている．そのほかに，抗原をリポソームに封入して投与することにより，経皮免疫応答が増強するとの報告もある．

3. 経皮ワクチンの利点

a. 安全性と簡便性

既に述べたように，経皮ワクチンは，注射によらない方法として，従来の注射によるワクチンと比べて安全性と簡便性が期待できる．

粘膜ワクチンと比べても，経皮ワクチンでは投与したワクチンのうち皮膚というフィルターを通ったごく微量の抗原分子およびアジュバント分子が体内に入って免疫を誘導すると考えられるので，より安全であると期待できる．LTをヒトの皮膚に投与した臨床試験でも，目立った副反応は観察されていない[13]．

簡便性という点でも，経皮ワクチンは大いに期待できる．実現するまでには，まだまだ解決すべき課題は多いと思われるが，バンドエイドを貼るように抗原を含んだパッチを貼るだけでワクチン接種が可能になるかもしれない．このように簡便な方法が実現すれば，医療インフラの不十分な開発途上国でのワクチン接種やパンデミック時の迅速なワクチン接種において極めて有用であろう[14]．

b. 細胞性免疫の誘導

ウイルスやがんに対しては，細胞傷害性T細胞(CTL)による細胞性免疫が有効である．$CD8^+$ T細胞であるCTLを活性化するためには，抗原ペプチドがMHC-class Iとともに提示される必要があるが，このルートで抗原提示されるのは基本的には細胞に内在する抗原である．従来型のワクチン抗原は外来性抗原としてマクロファージなどの抗原提示細胞で処理された後，MHC-class IIとともに$CD4^+$T細胞に抗原提示されるため，CTLの誘導は十分ではない．しかし，近年，外来性の抗原であってもMHC-class Iとともに提示されるルート(クロスプレゼンテーション)があることが明らかにされた．樹状細胞は，特にクロスプレゼンテーションの能力が高いことが知られている．したがって，樹状細胞の一種であるLangerhans細胞が主として抗原提示細胞として働く経皮ワクチンでは，効率的にCTLが活性化されることが期待できる．このことを期待して，経皮ワクチンをがんワクチンに応用する試みも始められている[15]．

c. 粘膜免疫の誘導

興味深いことに，経皮ワクチンは，血清中のIgG抗体産生を誘導するばかりでなく，腸管や気管粘膜でのIgA抗体産生をも誘導することが報告されている．このことは，皮膚免疫系と粘膜免疫系の間に何らかのクロストークの経路が存在することを示唆している．最近，経皮ワクチンによる粘膜免疫の誘導は腸間膜リンパ節の存在に依存するがパイエル板の存在には依存しないこと，経皮ワクチンの投与後に腸間膜リンパ節に$langerin^+CD8\alpha^-$樹状細胞が出現することが報告された[16]．多くの病原体の侵入門戸が粘膜であることを考えると，粘膜免疫を誘導できることはワクチンの投与法として重要である．

4. 経皮ワクチンの課題

かつて注射によらないで経皮的にワクチンを接種することは，事実上，不可能であると考えられていた．現在では，多くの実験的な証拠により，経皮ワクチンが可能であることは疑いない．しかし，経皮ワクチンが可能であることと，これが実用に堪えるものであることとは別である．一部では臨床試験が開始されているが[17]，経皮ワクチンの技術の多くは動物実験の段階にとどまっているものである．今後，ヒトでの有

効性と安全性のチェックを十分に行うことが不可欠だろう.

今後の経皮ワクチンの開発においては,いかにして効率的に皮膚,特に角質層を通して抗原を体内に送り込むかが鍵になると考える.CTおよびLTの経皮アジュバントとしての有効性の発見は,この分野の大きなブレークスルーであったが,必ずしもこれで十分というわけではない.実用に堪える経皮ワクチンの開発には,更にアジュバントや皮膚の透過性亢進法の研究などにより有効性と確実性を高める努力が必要と思われる.本誌カラー図説の親水性ゲルパッチを用いた'貼るワクチン'も,この方向での有力な試みの一つと考える.

経皮ワクチンが免疫応答を誘導する仕組みについては,まだまだ未解明な点が多い.Langerhans細胞の役割についても,いわば'状況証拠'の段階である.また,上皮の95%を占めるケラチノサイトも種々の刺激により様々なサイトカインを産生することが知られており,何らかの役割を果たしていることが予想される.今後,更に,経皮ワクチンによる免疫誘導メカニズムが実証的に解明されることが望まれる.

思えばワクチンの嚆矢,ジェンナーによる種痘法は,一種の,注射によらない経皮的な免疫法である.経皮ワクチンには,他のワクチンにはないユニークな特長がある.実用化に向けて,活発な研究を期待したい.

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Repeated instillations of *Dermatophagoides farinae* into the airways can induce Th2-dependent airway hyperresponsiveness, eosinophilia and remodeling in mice
Effect of intratracheal treatment of fluticasone propionate

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Received 5 December 2006; received in revised form 15 August 2007; accepted 5 September 2007
Available online 20 September 2007

Abstract

Dermatophagoides farinae are known to be a common environmental allergen causing allergic asthma; however, little is known about their pathophysiological effect via the allergenicities in vivo. Therefore, we first established a mouse model of asthma induced by repeated instillations of *D. farinae*. Second, to investigate whether the asthmatic responses are Th2-dependent, we examined the effect of the deficiency of interleukin-4 (IL-4) receptor α chain gene. Finally, we examined the effect of fluticasone propionate on this model. Mice were instilled with *D. farinae* without additional adjuvants into the trachea 8 times. After the final allergen instillation, the airway responsiveness to acetylcholine was measured, and bronchoalveolar lavage and histological examination were carried out. The instillation of the allergen-induced airway hyperresponsiveness, the accumulation of inflammatory cells and increases in the levels of Th2 cytokines and transforming growth factor-β_1 production in the bronchoalveolar lavage fluid dose dependently. The number of goblet cells in the epithelium and the extent of the fibrotic area beneath the basement membrane were also increased in the morphometric study. In contrast, the defect of IL-4/IL-13 signaling through IL-4 receptor α chain completely abrogated all these responses. Furthermore, the simultaneous instillation of fluticasone propionate with the allergen showed significant inhibition or an inhibitory tendency of these changes. These findings demonstrate that the repetitive intratracheal instillations of *D. farinae* can induce airway remodeling through Th2-type inflammation, and that fluticasone propionate inhibits *D. farinae*-induced airway remodeling in mice, and this model would be useful for studying mechanisms involved in the development of allergic asthma.

Keywords: Airway; Asthma; Corticosteroid; *Dermatophagoides farinae*; Eosinophil; Goblet cell; IL-13; Mouse; Subepithelial fibrosis

1. Introduction

Bronchial asthma is one of the most common health problems in the worldwide, especially within industrialized societies, and the prevalence rates have been increasing considerably over the last few decades (Mannino et al., 2002; Robertson et al., 2004; Verlato et al., 2003), for reason that are not yet completely understood. Changes in lifestyle and an increase in indoor allergen exposure caused by higher indoor temperature and humidity have been suggested as potential determinants, and it is reasonable to consider that environmental exposures to allergens are of primary importance for the prevalence and development of asthma, in genetically predisposed individuals, because genes

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doi:10.1016/j.ejphar.2007.09.005

controlling the inflammatory responses, IgE production, cytokine and chemokine production, airway remodeling as well as airway function, has not changed significantly in the last few decades. Among allergens like ragweed, pollens, house dust mite, and cockroach, house dust mites including *Dermatophagoides farinae* and *Dermatophagoides pteronyssinus* are known to be principle allergen for the induction of asthma (Huss et al., 2001; Platts-Mills et al., 1997; Sporik et al., 1990), although the precise molecular mechanisms underlying the allergenicity of the allergens is not fully understood.

In contrast to the increased prevalence of allergic airway diseases, the control of bronchial asthma is becoming easier due to the wide use of inhaled corticosteroid. However, there are still some patients that are resistant to medical treatment. Airway remodeling, which is characterized by goblet cell hyperplasia/hypertrophy, subepithelial fibrosis and smooth muscle hyperplasia/hypertrophy (Aikawa et al., 1992; Heard and Hossain, 1973; Roche et al., 1989), is one of the causes of this problem. Although these structural changes have been considered to be characteristics of chronic and severe asthma, the latest clinical studies, using bronchial biopsy sampling, have demonstrated that they may exist even in patients with mild asthma and early in the disease process. Roche et al. reported that the deposition of collagen beneath the bronchial epithelium was also observed in young patients with mild atopic asthma (Roche et al., 1989), and Boulet et al. demonstrated that the degree of subepithelial collagen deposition in patients with mild asthma recently diagnosed was not significantly different from those of long-standing mild asthma (Boulet et al., 2000). Therefore, the comprehension of the mechanisms underlying airway remodeling is becoming an increasingly important problem, and the development of new anti-remodeling agents is strongly desired.

Animal models have been used for a long time to analyze the pathophysiology of human diseases and to seek new remedies for various diseases. In the case of bronchial asthma, guinea pigs, rats and mice have mainly been used (Pauluhn and Mohr, 2005). Although each animal has some good characteristics, recently, mice have been widely used because this species allows for the application in vivo of a broad range of immunological tools, including gene deletion technology (Kips et al., 2003). To date, in typical experiments using mice, they are systemically immunized to ovalbumin with a T helper type 2 (Th2)-skewing adjuvant, such as aluminum hydroxide gel, and challenged through airway application of the antigen in the form of an aerosol or an intranasal droplet aspiration. In fact, these murine models have been useful in understanding the immune responses, such as Th2 dominant phenotypes underlying allergic sensitization and airway eosinophilic inflammation (Wills-Karp, 2000), but it is still unknown how allergic sensitization via the mucosal surfaces of airways with aeroallergens, including house dust mite, is induced. Several studies have previously investigated aeroallergen-induced airway inflammation in vivo (Johnson et al., 2004; Sadakane et al., 2002; Yu et al., 1999). These studies demonstrated that repeated instillations of *D. farinae* without additional adjuvants can induce airway eosinophilic inflammation, probably through Th2-polarized responses in mice; however, it remains to be determined whether the repeated inoculation of *D. farinae* can induce local Th2 responses and airway remodeling, as well as airway inflammation and airway hyperresponsiveness, and whether the allergic responses induced by this allergen are Th2-dependent. Moreover, the effect of fluticasone propionate on *D. farinae*-induced airway eosinophilic inflammation, hyperresponsiveness to acetylcholine and remodeling in vivo has not been elucidated.

Therefore, to address these unanswered questions, we first established a *D. farinae*-induced airway inflammation using mice, which was locally immunized without any additional adjuvants. Then, we examined the characteristics of this model regarding the local Th1/Th2 balance, the development of airway remodeling and the peculiarity of the allergen by comparing with the characteristics of the ovalbumin-instilled mice. Furthermore, to clarify whether these allergic responses are Th2-dependent, we used interleukin (IL)-4 receptor α chain gene-deficient mice because the IL-4 receptor α chain is a common receptor for IL-4 and IL-13, which are both critical for Th2 polarization and the development. Finally, we examined the effect of fluticasone propionate, one of the strongest anti-inflammatory medicines available at the moment, on this model, especially on airway remodeling.

2. Materials and methods

2.1. Animals

Seven-week-old male BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). IL-4 receptor α chain gene-deficient mice (IL-4Rα; BALB/c background) (Noben-Trauth et al., 1999; Noben-Trauth et al., 1997) were purchased from Immuno-Biological Laboratories, Co. Ltd. (Takasaki, Japan). The animals were housed in plastic cages in an air-conditioned room at 22±1 °C with a relative humidity of 60±5%, fed a standard laboratory diet and given water *ad libitum*. Experiments were undertaken following the guidelines for the care and use of experimental animals of the Japanese Association for Laboratory Animals Science in 1987.

2.2. Agents

The following drugs and chemicals were purchased commercially and used: crude extract of *D. farinae* (LSL Co., Tokyo, Japan), ovalbumin (chicken egg white, grade V, Sigma, St. Louis, MO., USA), dimethyl sulfoxide (DMSO, Nacalai Tesque, Inc., Kyoto, Japan), phosphate-buffered saline (PBS, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), halothane (Takeda Chemical Industries, Ltd, Osaka, Japan), acetylcholine chloride (Nacalai Tesque, Inc.), bovine serum albumin (Seikagaku Kogyo, Tokyo, Japan), Türk solution (Wako Pure Chemical Industries, Ltd., Osaka, Japan), pancuronium bromide (Sigma), sodium pentobarbitone (Abbott Lab., Chicago, IL, USA), disodium ethylenediaminetetraacetic acid (EDTA-2Na; Nacalai Tesque) and Diff-Quick solution (International Reagent Corp., Ltd., Kobe, Japan). Fluticasone propionate was kindly given to us by GlaxoSmithKline, Japan.

2.3. Experimental protocol

Mice were instilled 8 times with *D. farinae* (4 μg or 20 μg of protein in 100 μl of PBS) or PBS alone into the trachea using a polyethylene cannula under halothane anesthesia (Fig. 1A). In the ovalbumin-induced airway inflammation model, mice were instilled with ovalbumin in the same way. Forty-eight hours after the final antigen challenge, the airway responsiveness to acetylcholine was measured, and bronchoalveolar lavage and histological study were performed.

2.4. Treatment with fluticasone propionate

Fluticasone propionate was dissolved in DMSO and diluted in PBS to a final concentration of 0.01%. Fluticasone propionate was instilled with *D. farinae* on day 14, 15, 21, 22, 28 and 29, and without the allergen on days 13, 20 and 27 according to previously described methods (Fig. 1B). The *D. farinae*-instilled group mice, which were the control group mice, were administrated 20 μg of the allergen with 0.01% DMSO.

2.5. Bronchoalveolar lavage

To evaluate airway inflammation, we examined the accumulation of inflammatory cells in bronchoalveolar lavage fluid. Experiments were performed according to previously described methods (Tanaka et al., 2001). Animals were killed with an intraperitoneal injection of sodium pentobarbitone (100 mg/kg). The trachea was cannulated and the left bronchi were tied for histological examination. Then, the right air lumen was washed 4 times with 0.5-ml calcium- and magnesium-free PBS containing 0.1% bovine serum albumin and 0.05 mM EDTA-2Na. This procedure was repeated three times (total volume; 1.3 ml, recovery > 85%). The bronchoalveolar lavage fluid from each animal was pooled in a plastic tube, cooled on ice and centrifuged (150 ×g) at 4 °C for 10 min. Cell pellets were resuspended in the same buffer (0.5 ml). Bronchoalveolar lavage fluid was stained with Türk solution and the number of nucleated cells was counted in a Burker chamber. A differential count was made on a smear prepared with a cytocentrifuge (Cytospin II, Shandon, Cheshire, England) and stained with Diff-Quick solution (based on standard morphological criteria) of at least 300 cells (magnification ×500). The supernatant of the bronchoalveolar lavage fluid was stored at −80 °C for the determination of cytokine production.

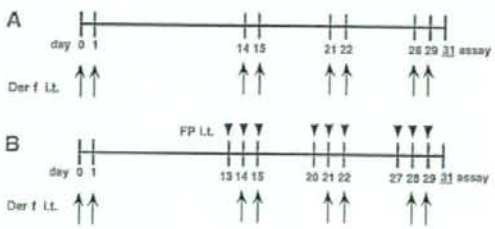

Fig. 1. Experimental protocol for *Dermatophagoides farinae* (Der f)-induced airway inflammation in BALB/c mice. A: *D. farinae* was instilled 8 times into the airways of BALB/c mice. B: Fluticasone propionate (FP) was administrated via the trachea with or without *D. farinae*, starting from the day before the third instillation of the allergen. i.t., intratracheal injection.

2.6. Cytokine levels in the bronchoalveolar lavage fluid

The amount of cytokine in the supernatant of the bronchoalveolar lavage fluid was measured using the enzyme-linked immunosorbent assay (ELISA) (Endogen Inc., Woburn, MA, USA for IL-4, IL-5 and interferon (IFN)-γ; R&D Systems Inc., Minneapolis, MN, USA for IL-13). The transforming growth factor (TGF)-β_1 content in the bronchoalveolar lavage fluid was also measured using ELISA (Genzyme Tecne, Minneapolis, MN, USA), which can detect mouse TGF-β_1 protein, because of the high homology of TGF-β_1 across species. The assay detects only the active form of TGF-β_1. Each sample was activated before measuring, according to the manufacturer's recommendations. The detection limit of each kit was 5 pg/ml for IL-4 and IL-5, 10 pg/ml for IFN- γ, 1.5 pg/ml for IL-13, or 7 pg/ml for TGF-β_1, respectively.

2.7. Measurement of airway function

Measurement of the bronchial responsiveness to intravenous acetylcholine was performed as previously described (Komai et al., 2003; Tanaka et al., 2001). Briefly, to measure the airway responsiveness to acetylcholine, mice were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.) and the jugular vein was cannulated for the intravenous injection of acetylcholine. Mice were injected with pancuronium bromide (0.1 mg/kg, i.v.) to stop spontaneous respiration, and animals were ventilated with a rodent ventilator (New England Medical Instruments Inc., Medway, MA, USA). Bronchoconstriction was measured according to the overflow method, using a bronchospasm transducer (Ugo Basil 7020, Milan, Italy) connected to the tracheal cannula. To measure airway responsiveness to acetylcholine, changes in the respiratory overflow volume were measured using increasing doses of acetylcholine. The increase in respiratory overflow volume induced by acetylcholine was represented as a percentage of the maximal overflow volume (100%) obtained by clamping the tracheal cannula. The area under the curve (AUC) calculated from dose–response curves for acetylcholine is expressed as the magnitude of airway hyperresponsiveness. Briefly, each dose was converted logarithmically, and then AUC was calculated and represented as arbitrary units (Tanaka et al., 2001).

2.8. Histological study

The left lungs were distended by the injection of 10% buffered formalin via the trachea, excised and immersed in the same fixative with the trachea clamped for 24 h. Tissues were sliced and embedded in paraffin, and 6 μm sections were stained with periodic acid-Schiff (PAS) and Masson-trichrome for light microscopy examination.

Examination of goblet cell hyperplasia was carried out with the PAS-stained histological preparations of the left lobe using a Leica image analysis system (Leica, Cambridge, UK). Using a×10 objective, 4 typical areas were chosen from the largest visible airway, which, in these horizontal sections through the hilus, was the left main bronchus. Changing to a ×40 objective, in each chosen area, which corresponded to one microscopic field, the hyperplasia of the goblet cells in the epithelial lining was expressed by a score according to the percentage of the goblet cells in the epithelial cells described below. The length of the epithelial basement membrane of the bronchus of one area was 500 μm and over. To minimize the sampling errors, the 5-point scoring system (grade 0–4) (Tanaka et al., 2001) was adopted: grade 0, no goblet cells; grade 1, <25%; grade 2, 25–50%; grade 3, 50–75%; grade 4, ≥75%. The mean score of the total epithelial cells in the 4 areas of one mouse were counted. The mean scores of hyperplasia of the goblet cells were calculated in 5–9 animals.

Masson-trichrome stained sections were used for the assessment of the thickness of the epithelial layer and the detection of subepithelial fibrosis using a Leica image analysis system (Leica). As described above, using a ×10 objective, 3 representative areas were chosen, avoiding the selection of the furcation of the bronchus and the surrounding blood vessels in the largest airway. Changing a ×40 objective, epithelial basement membrane areas 250 μm and over were selected, and the thickness of the epithelial layer and the fibrotic area (stained in blue) 30 μm beneath the basement membrane of the standardized sampling points were measured. The mean of the thickness of the epithelial layer and the fibrotic area divided by the basement membrane length were calculated in 5–9 animals.

2.9. Statistical analysis

Values are presented as the mean with standard error. Statistical significance between two groups was estimated using the two-tailed Student's *t*-test or the Mann–Whitney's *U*-test after the variances of the data were evaluated with the *F*-test. To define significant differences among PBS-instilled animals and *D. farinae*-instilled animals, and among the control animals and fluticasone propionate-treated animals, the data were subjected to Bartlett's analysis, followed by a parametric or a non-parametric Dunnett's multiple range test. A P value less than 0.05 was considered to be significant.

3. Results

3.1. The effect of repeated instillations of D. farinae into the mouse trachea on airway responsiveness to acetylcholine, Th1/Th2 immune responses and inflammatory infiltrates in the airways

To examine the effect of repeated instillation of *D. farinae* into the mouse trachea on Th1/Th2 immune responses, airway

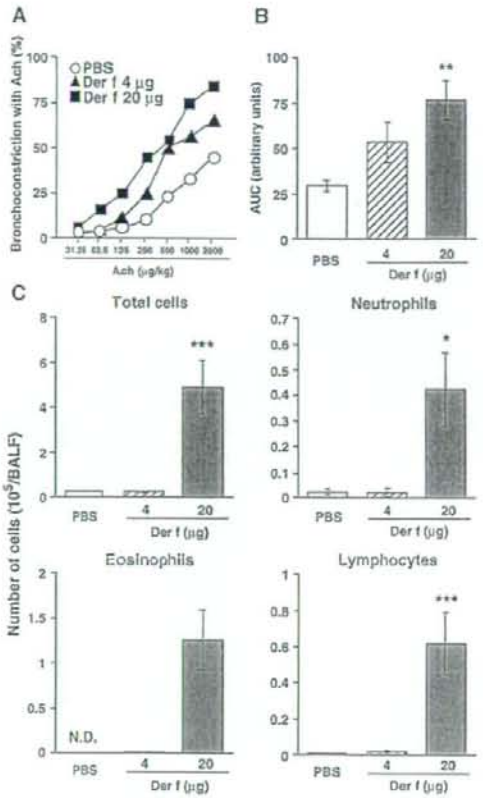

Fig. 2. Effect of repeated allergen instillation on airway responsiveness to acetylcholine and inflammatory infiltrates in the bronchoalveolar lavage fluid in BALB/c mice. A: Airway responsiveness to acetylcholine 48 h after the final allergen challenge. B: Area under the curve (AUC) calculated from the dose–response curves of bronchoconstriction for acetylcholine (range: 31.25–2000 μg/kg). C: Number of leukocytes in the bronchoalveolar lavage fluid 48 h after the final allergen challenge. Values represent the mean ± S.E.M. of seven to eight mice in each group. PBS, phosphate-buffered saline-instilled; Der f, *Dermatophagoides farinae*-instilled; N.D., not detected; $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (vs PBS).

inflammation and airway responsiveness to acetylcholine, we examined the airway responsiveness to acetylcholine, inflammatory infiltrates and cytokine production in the bronchoalveolar lavage fluid, and the histopathological changes in the airways. Repeated allergen instillations induced increases in the airway responsiveness to acetylcholine (Fig. 2A and B) and the numbers of inflammatory cells, including eosinophils, lymphocytes and neutrophils in the bronchoalveolar lavage fluid in a dose-dependent manner (Fig. 2C). Furthermore, as shown in Table 1, the levels of Th2 cytokines, IL-5 and IL-13, in the bronchoalveolar lavage fluid were also significantly increased; whereas, the level of Th1 cytokine, IFN-γ, in the bronchoalveolar lavage fluid was decreased by the allergen instillations in a dose-dependent fashion. However, there were no

Table 1
Cytokine production in bronchoalveolar lavage fluid and structural changes in the airways 48 h after the final allergen challenge in BALB/c mice

Treatment	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-13 (pg/ml)	IFN γ (pg/ml)	Goblet cell hyperplasia (arbitrary unit)	Goblet cell hypertrophy (μm)	Fibrotic area (Area/BM)	TGF-β_1 (pg/ml)
PBS	20.7±6.1	N.D.	0.8±0.4	164.5±10.3	0.08±0.05	32.3±1.7	5.5±0.7	30.6±6.2
Der f 4 μg	20.4±8.4	0.9±0.7	1.6±0.4	149.8±10.7	2.82±0.22	53.5±5.5 b	10.4±1.0 b	42.8±15.5
Der f 20 μg	25.0±5.1	7.1±2.5	10.6±2.5 c	131.9±2.5 a	3.83±0.17 c	77.3±7.9 c	15.6±0.9 c	217.3±38.9 b

Values represent the means±S.E.M. of 7–8 animals in each group. BM, Basement membrane; Der f, *Dermatophagoides farinae*; N.D., not detected.
a $P<0.05$; b $P<0.01$; c $P<0.001$ (vs PBS).

significant differences in the levels of IL-4 in the bronchoalveolar lavage fluid between the PBS group and the allergen group (Table 1).

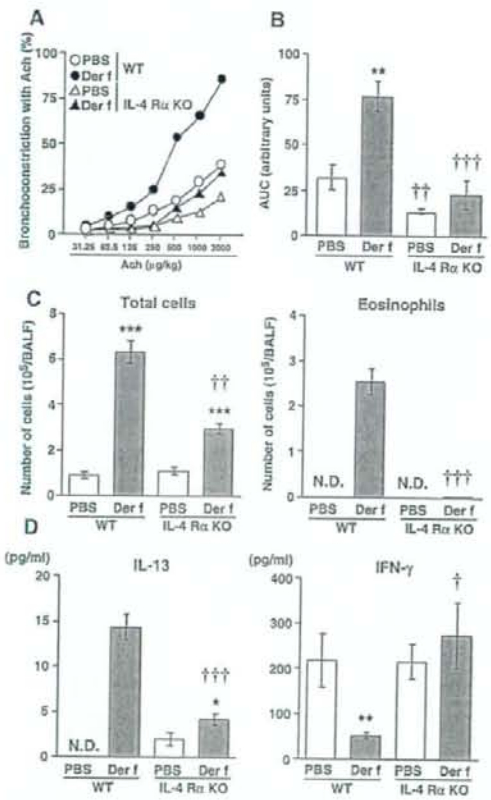

Fig. 3. Effect of IL-4 receptor α chain gene deficiency on the allergen-induced airway hyperresponsiveness to acetylcholine, increases in the numbers of total leukocytes and eosinophils and the production of IL-13 in the bronchoalveolar lavage fluid, and decreases in IFN-γ production in the bronchoalveolar lavage fluid in BALB/c mice. Forty-eight hours after the final allergen instillation, the airway responsiveness to acetylcholine was measured, and bronchoalveolar lavage and histological examination were carried out. Values represent the mean± S.E.M. of seven to nine mice in each group. PBS, phosphate-buffered saline-instilled; Der f, *Dermatophagoides farinae*-instilled; N.D., not detected; *$P<0.05$, **$P<0.01$, ***$P<0.001$ (vs PBS, Student's *t*-test or Mann–Whitney's *U*-test); †$P<0.05$, ††$P<0.01$, †††$P<0.001$ (vs WT, Dunnett's test).

Next, to clarify whether allergic responses induced by *D. farinae* are Th2-dependent or not, we examined the effect of IL-4 receptor α chain gene deficiency using the gene-knockout mice compared with wild-type (BALB/c) mice. As shown in Fig. 3, the defect of IL-4/IL-13 signaling through IL-4 receptor α chain clearly abrogated the airway hyperresponsiveness ($P<0.001$, Fig. 3A and B), airway eosinophilia ($P<0.001$, Fig. 3C), and imbalance in the IL-13/IFN-γ production in the bronchoalveolar lavage fluid ($P<0.001$ for IL-13 or $P<0.05$ for IFN-γ, Fig. 3D) induced by the allergen administration.

Then, to investigate the peculiarity of *D. farinae* in this model, mice were instilled with the same amount of ovalbumin as protein content instead of the allergen in a similar manner. Neither airway hyperresponsiveness, the accumulation of inflammatory cells into the airways or increases in the Th2 cytokine level in the bronchoalveolar lavage fluid was observed in the ovalbumin-instilled group (data not shown).

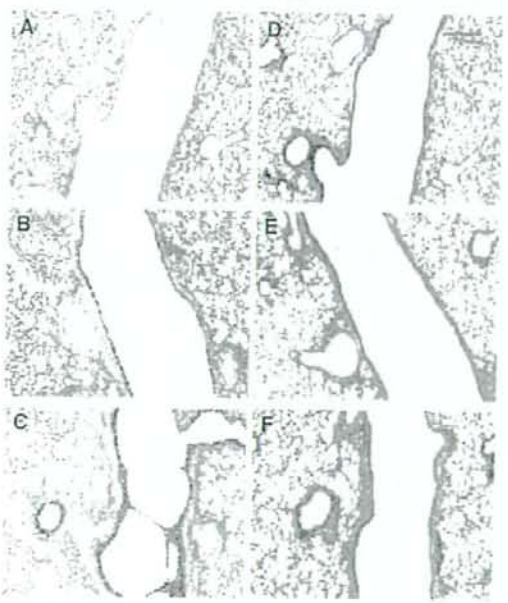

Fig. 4. Histological analysis of lung section with periodic acid-Schiff (A–C) and with Masson-trichrome (D–F) 48 h after the final allergen challenge in BALB/c mice. (A and D) PBS-instilled animal; (B and E) 4 μg of *D. farinae*-instilled animal; (C and F) 20 μg of *D. farinae*-instilled animal. Scale bar 200 μm.

Table 2
Effect of IL-4 receptor α chain gene deficiency on *Dermatophagoides farinae* (Der f)-induced airway remodeling in BALB/c mice

Genotype	Treatment	Goblet cell hyperplasia (arbitrary units)	Goblet cell hypertrophy (μm)	Fibrotic area (Area/BM)	TGF-β_1 (pg/ml)
Wild-type	PBS	0.43±0.12	0.43±0.12	5.41±0.44	55.5±12.4
	Der f 20 μg	3.92±0.04 b	3.92±0.04 b	18.62±2.10 b	334.8±23.9 b
IL-4 α chain gene deficiency	PBS	0.44±0.09	0.44±0.09	5.62±0.52	46.4±3.0
	Der f 20 g	0.57±0.12 c	0.57±0.12 c	5.73±0.36 c	81.8±7.8 a, c

Values represent the means±S.E.M. of 7–9 animals in each group. BM, Basement membrane; TGF, transforming growth factor.
a $P<0.01$; b $P<0.001$ (vs PBS group); c $P<0.001$ (vs Wild-type).

3.2. The effect of repeated instillations of D. farinae into the mouse trachea on airway remodeling

Fig. 4 shows the representative sections of each group stained with PAS for the detection of goblet cells and with Masson-trichrome for the detection of the fibrotic area. Repeated instillations of *D. farinae* caused goblet cell hyperplasia/hypertrophy in the epithelium and enlarged the area of subepithelial fibrosis dose dependently. These structural changes were mainly observed in the central large airways although the peripheral airways were mostly in tact.

To examine these histological changes quantitatively, goblet cell hyperplasia/hypertrophy and subepithelial fibrosis were evaluated by a score of PAS positive cells (stained in purple), the thickness of the epithelial layer and the area of fibrosis (stained in blue). As shown in Table 1, each score was significantly increased by repeated allergen inoculations in a dose-dependent manner. Furthermore, the level of profibrotic cytokine, TGF-β_1, in the bronchoalveolar lavage fluid was also dramatically increased by the instillation of *D. farinae* (Table 1). In contrast, these histopathological changes in the airways and increased TGF-β_1 production in the bronchoalveolar lavage fluid were completely diminished in IL-4 receptor α chain gene-deficient mice ($P<0.001$, Table 2).

3.3. The effect of fluticasone propionate on D. farinae-induced airway hyperresponsiveness, eosinophilic inflammation and Th1/Th2 immune responses

To investigate the effect of fluticasone propionate on the *D. farinae*-induced asthma-like phenotypes, mice were given 100–1000 μg/kg fluticasone propionate via an intratracheal route with the allergen on days 14, 15, 21, 22, 28 and 29, and without the allergen on days 13, 20 and 27 (Fig. 1B). To determine the initial timing of fluticasone propionate treatment, we examined the time course study for repeated allergen instillation in this protocol, and observed increases in the number of eosinophils and the level of IL-13 in the bronchoalveolar lavage fluid on day 17, 48 h after the fourth allergen instillation (data not shown), suggesting that sensitization with *D. farinae* was established and allergic responses in the airways were ongoing at that time.

Fig. 5 shows the effect of fluticasone propionate on allergen-induced airway hyperresponsiveness and leukocyte infiltrates in the bronchoalveolar lavage fluid. Repeated instillations of *D. farinae* significantly increased the airway responsiveness to acetylcholine and the number of inflammatory cells in the bronchoalveolar lavage fluid. Treatment with fluticasone propionate, at doses of 300 and 1000 μg/kg, significantly inhibited the increases in the numbers of neutrophils,

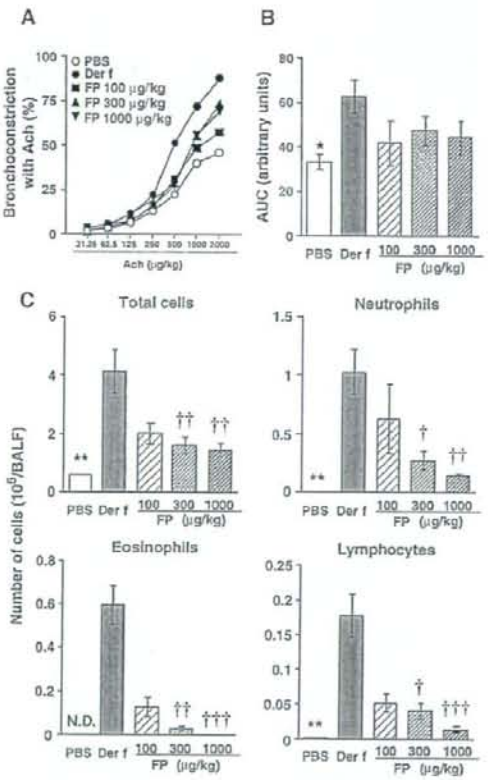

Fig. 5. The effect of fluticasone propionate (FP) on the allergen-induced airway hyperresponsiveness to acetylcholine and on the number of leukocytes in the bronchoalveolar lavage fluid. Forty-eight hours after the final allergen instillation, the airway responsiveness to acetylcholine was measured, and bronchoalveolar lavage was carried out. A: Airway responsiveness to acetylcholine 48 h after the final allergen challenge. B: Area under the curve (AUC) calculated from the dose–response curves of bronchoconstriction for acetylcholine (range: 31.25–2000 g/kg). C: Number of leukocytes in the bronchoalveolar lavage fluid 48 h after the final allergen challenge. Values represent the mean±S.E.M. of five to seven mice in each group. Der f, *Dermatophagoides farinae*; PBS, phosphate-buffered saline-instilled; Der f, 20 μg of *D. farinae*-instilled; FP, Der f and fluticasone propionate-instilled; N.D., not detected; *$P<0.05$, **$P<0.01$, *** $P<0.001$ (vs Der f, Student's *t*-test or Mann–Whitney's *U*-test); †$P<0.05$, ††$P<0.01$, †††$P<0.001$ (vs WT, Dunnett's test).

Table 3
Effect of fluticasone propionate (FP) on *Dermatophagoides farinae* (Der f)-induced Th2-polarized cytokine production and airway remodeling in BALB/c mice

Treatment	IL-4 (pg/ml)	IL-5 (pg/ml)	IL-13 (pg/ml)	IFNγ (pg/ml)	Goblet cell hyperplasia (arbitrary unit)	Goblet cell hypertrophy (μm)	Fibrotic area (Area/BM)	TGF-β_1 (pg/ml)
PBS	6.1±3.2	1.2±0.7a	0.4±0.4 b	168.6±7.1	0.17±0.05 b	36.3±1.6 a	5.1±0.6 b	21.6±3.9 b
Der f	7.2±4.6	13.1±3.6	6.6±1.3	149.8±15.2	3.54±0.07	58.4±5.8	12.6±0.5	253.7±46.3
FP 100 μg/kg	1.2±1.2	10.9±2.3	3.4±1.7	139.5±9.2	3.36±0.20	52.5±1.3	8.7±0.6 e	133.8±35.9
FP 300 μg/kg	8.8±4.4	5.2±2.1	2.3±0.8	171.8±10.1	2.82±0.22	47.9±3.3	7.9±0.3 e	56.7±9.3 d
FP 1000 μg/kg	5.3±3.4	2.5±0.9 d	0.7±0.3 d	173.1±13.7	1.35±0.37 d	41.7±2.7 c	5.3±0.5 e	45.8±8.8 e

Values represent the means±S.E.M. of 5–7 animals in each group. BM, Basement membrane; TGF, transforming growth factor.
a $P<0.05$; b $P<0.01$ (vs Der f group, Mann–Whitney's *U*-test); c $P<0.05$; d $P<0.01$; e $P<0.001$ (vs Der f group, Dunnett's test).

eosinophils and lymphocytes in the bronchoalveolar lavage fluid; whereas, the drug showed a tendency to inhibit airway hyperresponsiveness (Fig. 5). Furthermore, in parallel with the effect on airway eosinophilic inflammation, the increased levels of Th2 cytokines, IL-5 and IL-13, in the bronchoalveolar lavage fluid was almost reversed by fluticasone propionate treatment in a dose-dependent fashion (Table 3).

3.4. The effect of fluticasone propionate on the development of airway structural changes induced by D. farinae

Fig. 6 shows the representative lung specimens of the *D. farinae*-instilled group and the fluticasone propionate-treated group. The quantitative findings of histopathological examination and TGF-β_1 production in each group are shown in Table 3.

As shown in Fig. 6 and Table 3, severe goblet cell hyperplasia/hypertrophy, marked subepithelial fibrosis and the increased production of TGF-β_1 in the bronchoalveolar lavage fluid were all observed in the allergen-instilled group compared with those in the PBS-instilled group. In contrast, the treatment with fluticasone propionate, at a dose of 1000 μg/kg, during allergen challenge significantly inhibited these quantitative changes.

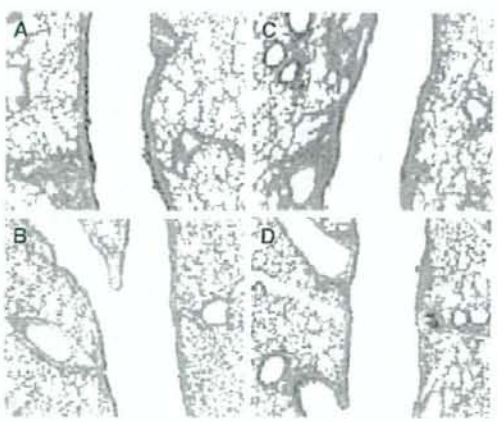

Fig. 6. Histological analysis of a lung section with periodic acid-Schiff (A and B) and with Masson-trichrome (C and D) 48 h after the final allergen challenge in fluticasone propionate-treated mice. (A and C) 20 μg of *Dermatophagoides farinae*-instilled animals; (B and D) fluticasone propionate-treated animals. Scale bar 200 μm.

4. Discussion

Bronchial asthma is a chronic inflammatory airway disease that is characterized by eosinophil inflammation, bronchial hyperresponsiveness and airway remodeling in pathophysiological studies (Cohn et al., 2004). In addition, many clinical and experimental studies suggest that Th2 cells that secrete IL-4, IL-5 and IL-13 have important functions in directing and maintaining the airway inflammatory processes (Robinson et al., 1992; Temann et al., 1997; Tomkinson et al., 2001). To understand the basic cellular and molecular mechanisms of allergic sensitization and airway inflammation, various animal models that possess these characteristics have provided valuable information on several aspects of asthma, and we reported the ovalbumin-induced allergic asthma model in mice by systemic sensitization and repeated allergen exposure (Nagai et al., 1997; Tanaka et al., 2001). Although this model is useful for the analysis of specific IgE-dependent airway eosinophilic inflammation, it is difficult to investigate the onset of the disease (especially local sensitization and challenge) and the specificity of relevant allergens that trigger human asthma. Therefore, we established a mouse model of allergic asthma by the intratracheal instillation of *D. farinae* without additional adjuvants.

In the present study, we showed *D. farinae*-induced airway eosinophilic inflammation in mice by the local instillation. Our mouse model is characterized by eosinophil infiltrates into the airways, increases in the levels of IL-5 and IL-13 in the bronchoalveolar lavage fluid, a decrease in the level of IFN-γ in the bronchoalveolar lavage fluid, and airway hyperresponsiveness to acetylcholine in a dose-dependent manner. Furthermore, these asthma-like phenotypes were completely abrogated in IL-4 receptor α chain gene-deficient mice, suggesting that IL-4 and/or IL-13 play a predominant role in the induction and development of *D. farinae*-induced Th2 responses. In addition, another foreign protein, ovalbumin, could not induce asthmatic responses at all in the same protocol. Indeed, it has been reported that the repeated inoculation of *D. farinae* into the trachea (Yu et al., 1999) or the nose (Johnson et al., 2004) of mice induces airway eosinophilia, but these reports are limited to the increases in Th1/Th2 cytokines in the bronchoalveolar lavage fluid or in Th2 cytokines in the allergen-stimulated splenocytes, respectively. Thus, this is the first report to prove directly that *D. farinae* by itself has the capacity to induce the imbalance of Th2/Th1 responses in the airways and to promote local Th2 type immune responses through IL-4 receptor α chain.

House dust mite is known to be composed of mixed materials, including *D. farinae* and pteronyssinus. Moreover, *D. farinae* contains at least 2 major allergens (*D. farinae* 1 and 2) (King et al., 1994). As described above, it was reported that repeated ovalbumin inhalation to sensitized mice induced tolerance (Holt et al., 1981; Sakai et al., 2001; Swirski et al., 2002); however, in the case of *D. farinae*, the number of eosinophils gradually increased, even after 24 instillations (unpublished data), suggesting that *D. farinae* does not easily induce tolerogenic responses. Thus, there seems to be some different characteristics in the allergen compared to ovalbumin, including their proteolytic activities. More importantly, the molecular mechanisms underlying in mucosal sensitization, airway inflammation and airway remodeling by repeated allergen instillation might be different even though each phenotype like Th2-polarized immune responses is quite similar. Therefore, further experiments are needed to clarify which types of allergen and/or what kinds of components are responsible for these asthmatic phenotypes in this model.

The present data demonstrated that no increase of the IL-4 level was observed 48 h after the final allergen challenge; whereas, there were significant increases in the levels of Th2 cytokines and a decrease in Th1 cytokine production in the bronchoalveolar lavage fluid, respectively. It may be due to the difference of kinetics in the cytokine production after allergen challenge. For instance, Ohkawara et al. demonstrated that the peak level of IL-4 and IL-5 in the fluid occurred 24 h after antigen challenge in the ovalbumin-sensitized mice (Ohkawara et al., 1997). While a remarkable increase in the IL-5 level in the fluid was observed from 3 h after the challenge and continued for 5 days, the kinetics of the IL-4 level in the fluid were much shorter than those of IL-5. In our study, we measured the cytokine levels 48 h after the last antigen challenge; therefore, the peak level of IL-4 in the BALF might have passed.

In contrast to the level of IL-4 in the airways, we detected increased levels of *D. farinae*-specific IgG1 on day 13, and the levels were much increased on day 27 and 31 using an allergen-coated ELISA system (data not shown). Serum allergen-specific IgE was also detected on day 27 by passive cutaneous anaphylaxis in rats (data not shown). Therefore, we provide evidence of Th2-polarized systemic immune responses in the allergen-instilled mice.

Mucus hypersecretion from hyperplastic goblet cells causes airway mucous plugging, especially in the peripheral airways of asthmatics. Mucous plugging has been reported to be an important factor in the mortality rates associated with severe acute asthma (Aikawa et al., 1992; Saetta et al., 1991). Recently, several observations have implicated Th2 cytokines, IL-4, IL-9 and IL-13, in goblet cell metaplasia of mice (Grunig et al., 1998; Louahed et al., 2000; Temann et al., 1997; Wills-Karp et al., 1998). In addition, IL-4 and IL-13 have been shown to be major contributing factors for allergen (ovalbumin)-induced mucus production via IL-4 receptor α chain in an allergic mouse model (Cohn et al., 1999; Kuperman et al., 2005). However, it is not clear whether *D. farinae*-induced mucous production is dependent on IL-4 receptor α chain in vivo. Therefore, we investigated the dependency using IL-4 receptor α chain gene-deficient mice. As a result, goblet cell hyperplasia/hypertrophy caused by the repeated allergen challenge was completely dependent on IL-4 receptor α chain. It is difficult to determine whether the allergen-induced mucous metaplasia is due to a Th2-polarized immune response in the airways, or to the direct effects of *D. farinae*. In the present study, marked increases in goblet cells and goblet cell hypertrophy in the epithelium were observed in both the low dose (4 μg) and high dose (20 μg) *D. farinae*-instilled groups, and their extents were comparable; whereas, Th2 cytokine production and the imbalance of the Th1/Th2 responses in the airways were more prominent in the high dose group. Therefore, it is possible that *D. farinae* directly affected the ciliated epithelial cells to transform/differentiate into goblet cells, probably through their protease activity, and that their protease activity influenced the allergenicity, which can induce Th2-polarized immune responses dependent on IL-4 receptor α chain, although further experiments are needed to clarify whether these protease activities are involved in their transformation/differentiation.

In histological analysis, the fibrotic areas under the basal lamina of the central airways were quantitatively evaluated, and increases in the areas were observed in a dose-dependent fashion. Furthermore, an increase in the TGF-β_1 level in the bronchoalveolar lavage fluid was observed in a dose-dependent fashion, and the increased production after the repeated instillation of *D. farinae* was IL-4/IL-13 dependent through IL-4 receptor α chain. TGF-β_1 plays a central role in the pathogenesis of a variety of fibrotic disorders. It stimulates the production of extracellular matrix proteins and inhibits the formation of extracellular proteases. An increase in the TGF-β_1 levels was observed in the clinical specimens of fibrotic kidney disease, hepatic fibrosis and pulmonary fibrosis (Blobe et al., 2000). In asthmatic patients, the bronchoalveolar lavage study or biopsy specimens indicated an increase in the TGF-β_1 levels, and its expression levels correlated with the severity of the disease and the degree of subepithelial fibrosis (Redington et al., 1997; Vignola et al., 1997). We previously reported that the increased levels of TGF-β_1 in the bronchoalveolar lavage fluid were significantly correlated with fibrotic changes in the morphometric studies following ovalbumin sensitization and challenge in mice (Komai et al., 2003; Tanaka et al., 2001), and that eosinophils and myofibroblasts play a critical role in the development of subepithelial fibrosis in mice (Tanaka et al., 2004). Therefore, it is also possible that these cells that infiltrated around the airways can produce the fibrogenic factor in this model.

Now, inhaled corticosteroid maintains its position as a first-line therapy for bronchial asthma. Several clinical studies have suggested that inhaled corticosteroid is effective for airway inflammation and structural changes, such as the thickness of the subepithelial basement membrane (Olivieri et al., 1997; Sont et al., 1999). However, there are conflicting findings about the effect on airway remodeling (Jeffery et al., 1992; Lundgren et al., 1988). Therefore, we investigated the effect of the corticosteroid, fluticasone propionate, on this model. As a result, it was found that fluticasone propionate dramatically inhibited airway eosinophilic inflammation and increased Th2

cytokines and TGF-β_1 productions in the bronchoalveolar lavage fluid, and that fluticasone propionate showed a tendency to inhibit the airway hyperresponsiveness to acetylcholine. Furthermore, goblet cell hyperplasia in the airways and the fibrotic area beneath the basal lamina of the central airways were significantly improved by fluticasone propionate treatment in a dose-dependent manner. These findings suggest that inhaled corticosteroid can inhibit *D. farinae*-induced airway remodeling, probably through inhibiting Th2-mediated airway eosinophilic inflammation. However, there is a discrepancy in the fluticasone propionate doses between the effects on inflammatory infiltrates and airway remodeling/airway hyperresponsiveness in the present study. Vanacker et al. also demonstrated that fluticasone propionate prevented antigen-induced airway inflammation and structural changes using a rat asthma model, and they also argued that a higher dose of inhaled corticosteroid is needed to prevent allergen-induced structural changes than that required to inhibit eosinophil recruitment (Vanacker et al., 2002). Furthermore, Boulet et al. recently demonstrated that there is no significant difference in airway inflammation and subepithelial collagen deposition in steroid-naïve patients with mild recently diagnosed vs long-standing asthma, and that there was no significant improvement in the airway hyperresponsiveness after high doses of inhaled corticosteroids between these two groups (Boulet et al., 2000). These findings suggest that there are irreversible airway structural and/or physiological changes, despite appropriate and aggressive anti-inflammatory therapies, which may explain the reason why airway remodeling and airway hyperresponsiveness could not be normalized in some patients.

Another question to be addressed is whether fluticasone propionate is effective for airway hyperresponsiveness in this model. We observed that fluticasone propionate showed a tendency to inhibit allergen-induced airway hyperresponsiveness, but it was not significant. Many clinical studies and a few animal studies suggested that inhaled corticosteroid improved airway hyperresponsiveness (Olivieri et al., 1997; Sont et al., 1999); however, these improvements mainly resulted from the strong anti-inflammatory effects of the steroids. The relevance between airway hyperresponsiveness and airway remodeling is always a matter of concern, and undoubtedly, clinical biopsy specimens cannot assess the airways as a whole. Moreover, airway hyperresponsiveness did not return to within normal limits in most long-term studies, although it was improved (van Essen-Zandvliet et al., 1992). Therefore, our result may be due to the remaining airway structural changes, especially goblet cell hyperplasia or other factors; however, little is known about the effect of steroids on airway remodeling, especially in vivo, and further experiments will be needed.

In conclusion, we established a mouse model of atopic asthma, which was locally immunized with a major indoor allergen, *D. farinae*, without additional adjuvants. This model clearly demonstrated the local Th2-dominant inflammation and airway remodeling feature, and those parameters were improved by inhaled corticosteroid. We believe that this model is a useful tool for the investigation of mechanisms and the development of new remedies for airway remodeling in atopic asthma.

Acknowledgements

This work was supported in part by the Ministry of Health, Labor and Welfare (Japan), Health and Labour Science Research Grants, Research on Allergic Disease and Immunology, the Ministry of Education, Culture, Sports, Science and Technology (Japan) (to H.N.), Grants-in-Aid for Scientific Research C 16616005 (to H.T.), and the Takeda Science Foundation (to H. T.). This study was also supported in part by a donation from the late Professor Emeritus, Akihide Koda, for which we are very grateful. The authors thank Mr. Daniel Mrozek for his skillful assistance in the preparation of this manuscript.

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