

Role of Eosinophil Chemotactic Factor by T Lymphocytes on Airway Hyperresponsiveness in a Murine Model of Allergic Asthma

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Airway hyperresponsiveness (AHR) is an important feature of bronchial asthma. Although the incidence of AHR has genetic and environmental components, the mechanism of AHR in asthma remains unclear. The identification of genes that are preferentially expressed in a murine model of AHR could help elucidate the molecular mechanisms of this pulmonary pathology. Suppressive subtractive hybridization analysis revealed that eosinophil chemotactic factor by T lymphocytes (ECF-L), a mouse chitinase family protein, was selectively expressed in the lungs of mice with AHR. Induction of ECF-L expression was observed soon after allergen exposure but before the onset of airway inflammation. Cell-specific ECF-L expression was examined by *in situ* hybridization using digoxigenin-labeled antisense RNA probes and immunofluorescence staining. The assay revealed that the ECF-L-expressing cells in the lungs of the AHR-model mice are alveolar macrophages. Intratracheal administration of an adenoviral vector that expressed antisense ECF-L RNA (Ad-ECF-L-AS) suppressed AHR and eosinophil infiltration. These results indicate that ECF-L may play a critical role in allergic inflammation and bronchial asthma.

Keywords: eosinophil chemotactic factor by T lymphocytes (ECF-L); airway hyperresponsiveness (AHR); eosinophil infiltration

Bronchial asthma is a serious chronic illness characterized by one or more of a number of nonspecific symptoms, including episodic shortness of breath, wheezing, coughing, and chest tightness (1). Hallmarks of bronchial inflammation include leukocyte infiltration of the bronchial tissue, excessive mucus production, epithelial damage, basement membrane thickening, and smooth muscle hypertrophy (2, 3). The nonspecific symptoms of bronchial asthma are associated with reversible airway obstruction and airway hyperresponsiveness (AHR), two conditions that are believed to result from chronic inflammation of the bronchial mucosa.

In this study, AHR was induced in a mouse model of allergic lung inflammation (AHR-model mice), first by sensitization and then challenge with ovalbumin (OVA). These mice are widely used to model human allergic inflammation in the lung because the observed pathology shares many features with the human disease (e.g., increased IL-4, IL-5, and IL-13 levels; eosinophil infiltration; IgE response; goblet cell metaplasia; mucus hyperproduction; and AHR) (4). Therefore, this experimental murine model of asthma was chosen as a tool to advance our understanding of human allergic inflammation and to identify potential therapeutic targets.

Although many studies have focused on cytokine production, chemical mediator secretion, and eosinophil infiltration, comparatively little research has been conducted on changes in gene expression in the asthmatic airway. The objective of this study was to assess differential gene expression during the epithelial inflammation process to aid in the clarification of the molecular mechanisms of AHR. Expression analysis was performed using a suppression subtractive hybridization (SSH) technique that has been shown to be highly effective in the identification of disease-related, developmental, tissue-specific, and other differentially expressed genes (5). Comparison of gene expression in AHR-model mice with that of normal mice revealed that ECF-L, which shows eosinophil chemotaxis (6), was specifically induced in the murine asthmatic lung. Furthermore, *in vivo* adenoviral gene transfer with antisense ECF-L (Ad-ECF-L-AS) suppressed the pathology of murine allergic inflammation through the prevention of AHR.

MATERIALS AND METHODS

Murine AHR Models

AHR-model mice have been described previously (7). Male BALB/c mice were immunized by intraperitoneal injection of 20 μ g OVA (Sigma, St. Louis, MO) with 2 mg aluminum hydroxide and were further sensitized by intraperitoneal injection of 10 μ g OVA with 1 mg aluminum hydroxide after 1 wk. Two weeks after the first immunization, mice were challenged for 25 min with an aerosol of 5% (wt/vol) OVA in 0.5 $\times$ PBS each day for six consecutive days. Sham-challenged mice inhaled an aerosol of 0.5 $\times$ PBS alone using the same protocol. Airway responsiveness was measured as previously described (7–9). Twenty-four hours after the final inhalation, mice were anesthetized with 50 mg/kg sodium pentobarbital (Abbot Laboratories, North Chicago, IL) and intubated with PE90 polyethylene tubing equipped with a port for airway pressure measurements (Model 687; Harvard Apparatus, Holliston, MA). Mice were ventilated at a rate of 120 breaths/min with a constant tidal volume of air (0.15 ml). Muscle paralysis was provided by intravenous administration of 10 mg/kg gallamine hydrochloride (Sigma). After establishing a stable baseline airway pressure (6–7 cm H₂O), acetylcholine chloride (ACh) (Tokyo Kasei, Tokyo, Japan) was injected intravenously (62.5–2000 μ g/kg), and the changes in airway pressure were recorded. Airway responsiveness was defined as a change in the peak airway pressure from the baseline reading. Changes in airway pressure were analyzed by ANOVA. The ANOVA was performed using an analysis of repeated measures in which all of the doses were used in the calculation of each group. Bronchoalveolar lavage (BAL) was performed as previously described (10) for the measurement of infiltrating cells. Cytospin preparations of BAL cells were stained with Diff-Quik (Baxter Healthcare, Miami, FL). Cell identification was based on the morphology and staining characteristics of at least 400 cells.

Subtractive Hybridization and Differential Screening

Total RNA from lung tissues was obtained using ISOGEN (Wako Pure Chemicals, Osaka, Japan) according to the manufacturer's instructions. Poly(A)⁺ RNA was isolated using the mRNA Purification Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK). Lung poly(A)⁺ RNA (2- μ g aliquots) from normal and AHR-model mice was used to make driver and tester cDNA, respectively. Suppression subtractive hybridization was performed using the PCR-select cDNA Subtraction Kit (Clontech, Palo Alto, CA) according to the manufacturer's protocol.

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Subtracted PCR products were ligated into the pT7 Blue-T vector (Novagen, Madison, WI), and ligation mixtures were transformed into *Escherichia coli* DH5 α (Toyobo, Osaka, Japan). Differentially expressed products were selected using the PCR-select Differential Screening Kit (Clontech) according to the manufacturer's protocol. Cloned products were sequenced using an ABI PRISM 377 DNA Sequencer (Perkin-Elmer, Norwalk, CT), and sequencing results were compared with GenBank database sequences using the Blast Homology Search Program (NIH, Bethesda, MD) (11). The full-length ECF-L gene sequence was PCR amplified from AHR-model mouse lung poly(A)⁺ RNA after reverse transcription with the cDNA synthesis kit (Takara, Kyoto, Japan). Primer sequences were 5'-AAGACACCATG GCCAAGCTC-3' (upstream) and 5'-ACAAGCATGGTGGTTTTA CAGGAA-3' (downstream). PCR products were cloned into pT7 Blue-T vector to produce the pT7-ECF-L plasmid.

Northern Blot Analysis

Expression and distribution patterns of ECF-L were determined by Northern blot analysis of poly(A)⁺ RNA extracted at the indicated time points from samples from brain, heart, liver, lung, kidney, thymus, spleen, stomach, small intestine, and large intestine from normal and AHR-model mice. Aliquots of 0.5 μ g poly(A)⁺ RNA were electrophoresed on 1.1% agarose gels containing 2.2 M formaldehyde and blotted onto Hybond-N⁺ membranes (Amersham Pharmacia Biotech). ECF-L cDNA was labeled with ³²P using the BcaBEST labeling kit (Takara) and used to probe the membranes. β -actin probes (Clontech) were used as controls for normalization of RNA loading. Filters were hybridized with the probes in Express Hyb hybridization solution (Clontech). ECF-L mRNA expression was quantified with a Bio-Rad GS-800 Calibrated Densitometer (Bio-Rad Laboratories, Hercules, CA) and normalized to that of β -actin.

In Situ Hybridization

Digoxigenin (DIG)-labeled RNA probes (0.6 kb in length) were transcribed *in vitro* from PCR products amplified from ECF-L cDNA using a DIG labeling kit (Roche Diagnostics, Mannheim, Germany). Primer sequences were 5'-TGGTGAAGGAAATGCGTA-3' (upstream) and 5'-GACCACGGCACCTCTCTAAAT-3' (downstream). PCR products were cloned into the pCRII-TOPO vector containing the T7 and SP6 RNA polymerase promoter sequences at either end of the multiple cloning site (Invitrogen, Groningen, The Netherlands). *In vitro* transcription was performed using T7 and SP6 RNA polymerases to generate sense (T7) and antisense (SP6) RNA probes. *In situ* hybridization was performed on paraformaldehyde-fixed murine lung sections using an ISHR starting kit (Nippon Gene, Tokyo, Japan). Bound probes were detected by alkaline phosphatase-conjugated, antidigoxigenin antibodies (Roche Diagnostics) with Nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics) used as substrate. Hybridizations of several sections with sense and antisense ECF-L probes were used as negative and positive controls, respectively.

Immunohistochemistry

Paraformaldehyde-fixed lung sections were used for immunohistochemical analysis. The frozen sections (5 μ m) were air dried at room temperature and washed with PBS/0.1% Triton X-100. Sections were incubated with 5% BSA for 1 h and again washed with PBS/0.1% Triton X-100. Sections were subjected to single or double immunofluorescent staining with antibodies against ECF-L (R&D Systems, Minneapolis, MN) and MOMA-2 (Chemicon, Temecula, CA), which recognizes an intracellular antigen of mouse macrophages. The primary antibodies were followed by staining with secondary anti-goat IgG-specific Alexa Fluor 488 (Molecular Probes, Eugene, OR) or anti-rat IgG-specific Alexa Fluor 568 (Molecular Probes). After three washes with PBS/0.1% Triton X-100, sections were mounted with VECTASHIELD with DAPI (Vector Laboratories, Burlingame, CA) and visualized by fluorescence microscopy. The number of ECF-L expressing cells in each lung section was counted in six different microscopic fields around airways. The numbers from four mice were collected and compared.

Construction and Administration of Recombinant Adenovirus Vectors

ECF-L cDNA fragments were isolated from pT7-ECF-L by digestion with *Hinc* II and *Sma* I restriction enzymes and ligated into *Swa* I-

digested pAxCawt (Takara). Orientation of the ECF-L cDNA was confirmed by digestion with *Xho* I. The resulting plasmid (pAxCACF-L-AS) was cotransfected into HEK-293 cells (ATCC, CRL-1573) following the protocol of the Adenovirus Expression Kit (Takara). Recombinant replication-deficient adenovirus (Ad-ECF-L-AS) was rescued by homologous recombination, and the presence of ECF-L cDNA was verified by analysis of viral genomic DNA fragments after *Xho* I digestion. Control adenovirus vectors, pAxCawt-derived Ad-wt, and pAxCACiLacZ-derived Ad-LacZ were constructed and characterized in the same manner. Viruses were expanded, purified, and plaque-titred in HEK-293 cells according to manufacturer's protocols. A total of 1×10^8 or 5×10^8 plaque-forming units (PFU) of Ad-ECF-L-AS, Ad-wt, or Ad-LacZ in 40 μ l distilled water were instilled intratracheally into the lungs of anesthetized BALB/c mice 1 d before the start of OVA inhalation. Airway reactivity was measured 24 h after the final OVA inhalation, given for six consecutive days as described previously.

Eosinophil Peroxidase Staining of Lung Sections

Lung eosinophil infiltration was studied by microscopic examination after staining for eosinophil peroxidase (EPO) with 3,3-diaminobenzidine tetrahydrochloride (DAB) (12). The sections were initially incubated with 0.3% H₂O₂ in methanol for 3 min and rinsed in PBS, followed by incubation with DAB for 10 min at room temperature. After being rinsed with PBS, the sections were counterstained with hematoxylin and examined under a light microscope. The number of EPO-positive cells in each lung section was counted in six different microscopic fields around the airways. The numbers from four mice were collected and compared.

Statistical Analysis

Statistical analyses were performed using SAS software (SAS Institute Inc., Cary, NC), and all data were represented as means $\pm$ SE. Comparison between groups was performed using the paired or unpaired Student's *t* test. Values of *P* $\leq$ 0.05 were considered statistically significant.

RESULTS

Identification of ECF-L as an AHR-Related Gene

To identify genes that are preferentially expressed in asthmatic lungs, gene expression in the lungs of OVA-sensitized and OVA-challenged mice (AHR-model mice) was compared with that in normal mouse lungs. Using SSH, a pool of differentially expressed genes was isolated as subtracted PCR products. Of the 864 clones screened, 90 produced stronger signals with probes from AHR-model mice than with probes from normal mice. Some of the clones converged at three identical sequences, which indicated that those clones may be more important for AHR. One clone matched the partial sequence of ECF-L in the GenBank database (accession number D87757); the others were partial sequences of gob-5 (accession number AF017156) and pendrin (accession number AF167411). ECF-L was chosen for further analysis because it is a mammalian member of the chitinase protein family. The fact that this family exhibits eosinophil chemotactic activity (6) is suggestive of a relationship with allergic inflammation in the lung.

Tissue distribution of ECF-L in normal and AHR-model mice was examined by Northern blot analysis (Figure 1A). The expression level of ECF-L was increased by $\sim$ 7-fold in the lungs of AHR-model mice compared with that of normal mice (Figure 1B). ECF-L mRNA was also detected in the stomach of normal and AHR-model mice, but the expression level did not differ significantly between the two mouse strains. Thus, ECF-L was specifically induced in association with the development of AHR.

To elucidate the relationship between ECF-L expression and the development of airway inflammation, airway responsiveness was measured after intravenous injection of 1,000 μ g/kg of ACh. The number of infiltrating cells was counted by performing BAL during OVA inhalation. Changes in airway pressure were greater

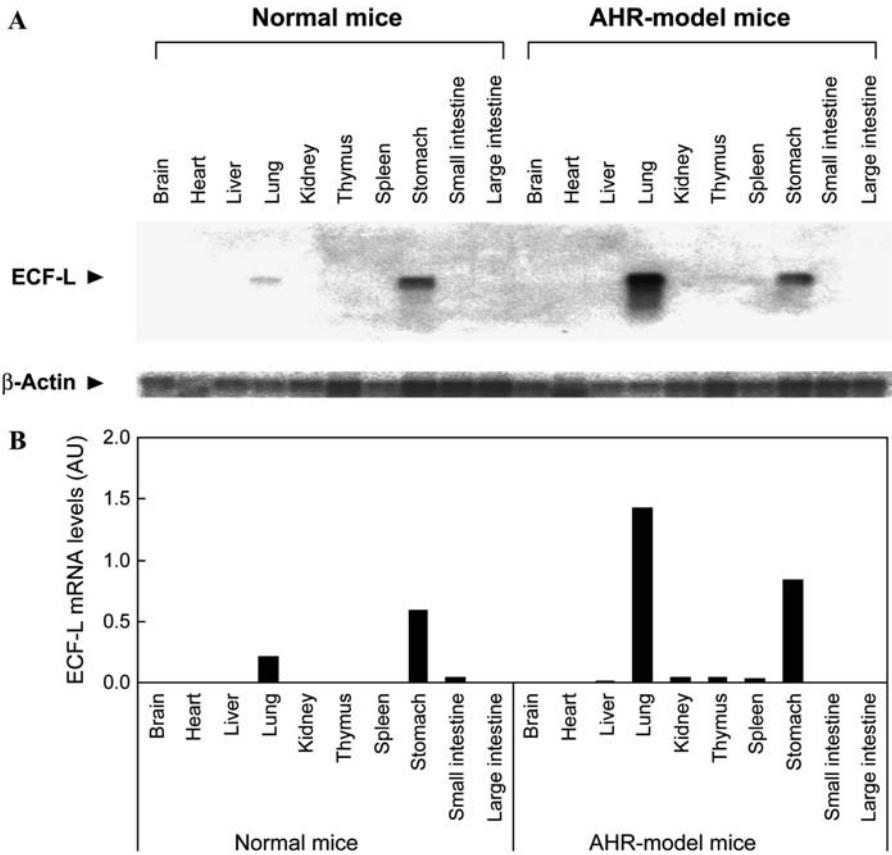


Figure 1. Selective expression of ECF-L mRNA in the lungs from AHR-model mice. Tissue distribution of ECF-L was determined by Northern blot analysis in normal and AHR-model mice. (A) The upper figure shows the results obtained by hybridization with an ECF-L cDNA probe. The murine tissues from which the poly(A)⁺ RNA was prepared are indicated at the top. The same filter was rehybridized with a β-actin probe to control for equal RNA loading (lower figure). (B) ECF-L mRNA expression was quantified with a GS-800 Calibrated Densitometer, using β-actin as a reference. The normalized value of β-actin mRNA was designated as one arbitrary unit (AU).

in mice given OVA by inhalation than in mice given PBS by inhalation, with levels significantly induced from Day 4 after the start of inhalation treatment (Figure 2A). The total number of cells collected by BAL, including eosinophils and other infiltrating cells, was also significantly increased from Day 4 (Figure 2C).

Evaluation by Northern blot analysis of ECF-L mRNA expression levels over time after OVA inhalation is shown in Figure 2B. The data demonstrate that although AHR development and eosinophil infiltration were observed from Day 4, a marked increase in ECF-L expression was observed from Day 2 that

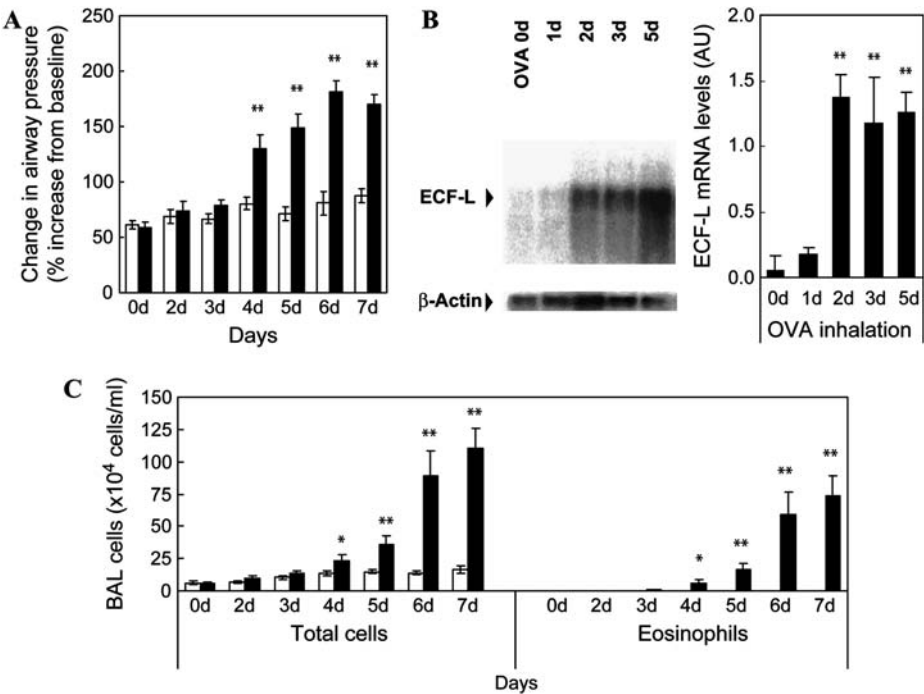


Figure 2. Induction of ECF-L mRNA occurred before the onset of AHR and eosinophilic infiltration. (A) Time-related changes in airways measured after PBS (open bars, $n = 6-7$) or OVA (filled bars, $n = 8-11$) inhalation. After establishing a stable baseline airway pressure, ACh (1,000 μ g/kg) was injected intravenously, and the changes in airway pressure were recorded. $**P \leq 0.01$ compared with PBS inhalation groups. (B) Time-dependent expression of ECF-L was ascertained by Northern blot analysis in lung tissue after OVA inhalation. ECF-L mRNA expression was quantified with a GS-800 Calibrated Densitometer, using β-actin as a reference. $**P \leq 0.01$ compared with OVA 0 d. (C) Time-related changes in the BAL fluid cell population after PBS (open bars, $n = 5-7$) or OVA (filled bars, $n = 7$) inhalation. BAL was performed 24 h after inhalation of aerosolized OVA. Total cells include eosinophils, macrophages, neutrophils, and lymphocytes. $*P \leq 0.05$; $**P \leq 0.01$ compared with the PBS inhalation groups.

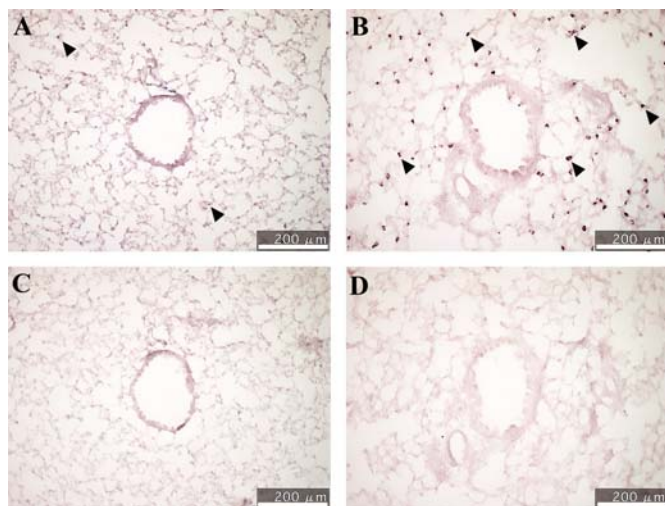


Figure 3. ECF-L is expressed in the AMs from AHR-model mice. Lung sections from normal (A and C) and AHR-model (B and D) mice were hybridized *in situ* with DIG-labeled, single-stranded antisense (A and B) and sense (C and D) RNA probes. Arrowheads point to ECF-L-expressing cells. Scale bars = 200 μ m.

remained level during OVA inhalation. In addition, there was little induction of ECF-L expression in the lung tissues of OVA-sensitized/PBS-inhalation mice and nonsensitized/OVA-inhalation mice after 7 d of inhalation (see online supplement). Thus, ECF-L mRNA expression was induced before the onset of AHR and eosinophil infiltration. These results indicate that ECF-L might play a critical role in allergic inflammation.

Expression of ECF-L in Alveolar Macrophages

To identify cell-specific ECF-L expression, *in situ* hybridizations were performed using DIG-labeled antisense RNA probes. Many stained spots were observed around the alveolar spaces in lungs from AHR-model mice, but only a few spots were found in normal mouse tissues (Figures 3A and 3B). Hybridization with a sense RNA probe produced no staining, which confirmed the specificity of the assay (Figures 3C and 3D). Moreover, no signals were detected in epithelial or endothelial cells from AHR-model mice, an indication that these cells are negative for ECF-L expression during airway inflammation. Based on the size, shape, and ratio of the stained cells, ECF-L expressing cells were deduced to be alveolar macrophages (AMs).

The identification of the ECF-L expressing cells was accomplished by double-staining lung sections from AHR-model mice with antibodies against ECF-L and macrophages (MOMA-2). Consistent with the *in situ* results, cells expressing ECF-L protein were observed around the alveolar spaces in AHR-model mice (Figures 3B and 4B). In the merged picture, ECF-L expressing cells (green) colocalized with AMs (red), resulting in yellow signals (Figure 4). This finding of ECF-L expression in macrophages was consistent with a previous study by Chang and colleagues that used activated peritoneal macrophages (13).

Effect of Intratracheal Administration of Adenovirus Expressing Antisense ECF-L

In vivo adenovirus-mediated gene delivery is an efficient strategy for the study of gene function in the lung. To investigate the role of ECF-L during AHR development, adenoviral vectors that expressed antisense ECF-L RNA (Ad-ECF-L-AS) were intratracheally administered to AHR-model mice. To assess the

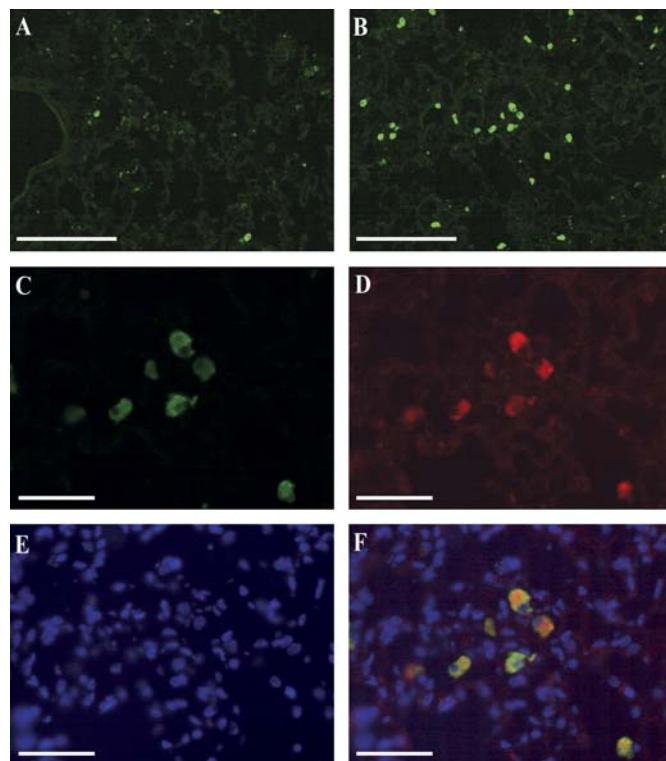


Figure 4. Immunofluorescence staining of lung sections from AHR-model mice. Lung sections from normal (A) and AHR-model (B) mice were stained with ECF-L antibodies. ECF-L expressing cells are represented by green spots. Scale bars = 200 μ m. (C–F) Magnified figure. Images of lung sections from AHR-model mice stained for ECF-L (C), AMs (red) (D), and nuclei (DAPI, blue) (E), were merged (F). ECF-L-expressing cells colocalized with AMs (merged yellow). Scale bars = 50 μ m.

efficacy of intratracheal delivery of the recombinant adenovirus, BALB/c mice were administered 1×10^8 or 5×10^8 PFU of adenovirus containing the *LacZ* (β -galactosidase) gene. Lungs were harvested 24 h later and stained for β -galactosidase activity. Strong dose-dependent staining was observed in lung tissues, especially bronchial epithelial cells and alveolar cells around airways, and confirmed the efficacy of the recombinant adenovirus-mediated gene delivery system (see online supplement). Long-term (7 d) expression of *LacZ* was also documented after administration of 5×10^8 PFU of Ad-*LacZ*. The continuous expression for more than 1 wk after instillation coincided with the report by Sime and colleagues (15, 16) and matched the developmental period of bronchial hyper-reactivity in our AHR-model mice. Thus, this gene delivery system was used to clarify the functional role of ECF-L in the development of AHR.

Either control adenovirus (Ad-wt) or Ad-ECF-L-AS was intratracheally administered to mice at a dose of 5×10^8 PFU 1 d before OVA inhalation. OVA inhalation was performed for six consecutive days; airway responsiveness was measured 24 h after the final inhalation. To elucidate the effect of Ad-ECF-L-AS on the expression of ECF-L protein, lung tissues were harvested for immunohistochemical studies. Immunofluorescent staining with anti-ECF-L antibody showed strong expression of ECF-L protein around alveolar spaces in AHR-model mice but not in tissues from PBS-inhalation mice (Figures 5A and 5B). Lung sections from mice administered Ad-ECF-L-AS showed a significant decrease in the number of ECF-L-expressing cells

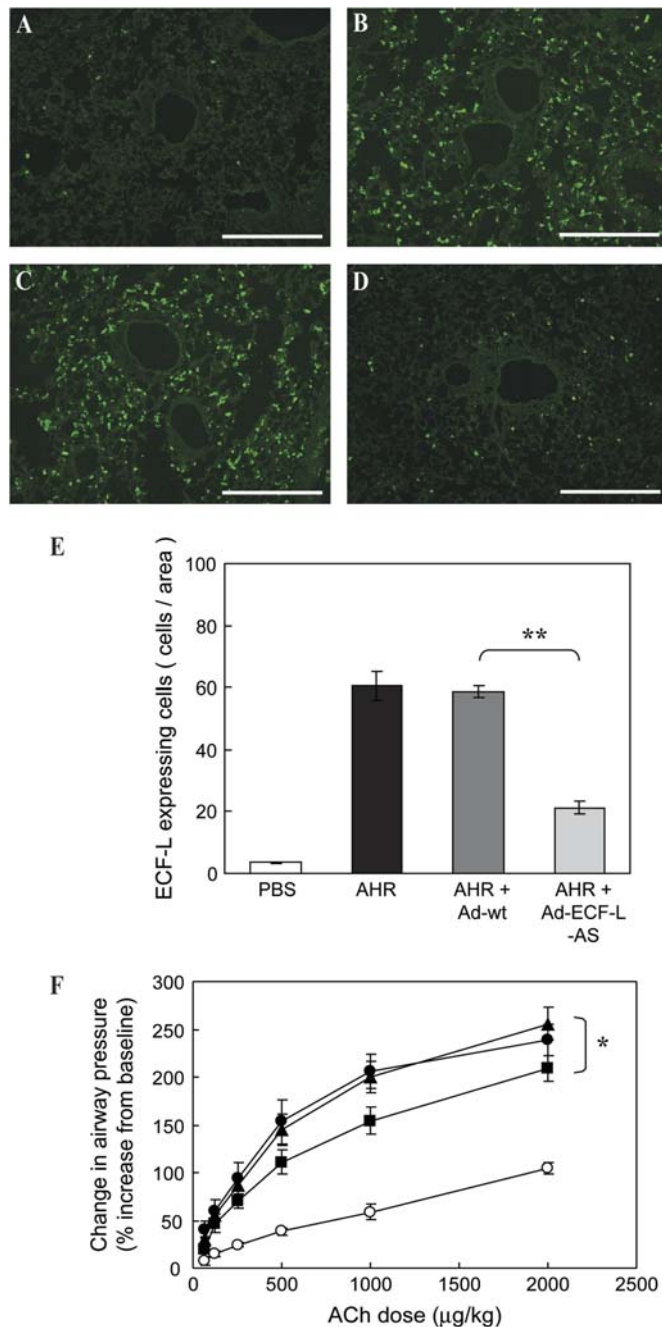


Figure 5. Reduction in ECF-L expression and airway responsiveness after administration of adenovirus expressing ECF-L antisense RNA. Recombinant adenoviruses expressing ECF-L antisense RNA transcripts (Ad-ECF-L-AS) or control adenovirus (Ad-wt) were administered intratracheally 1 d before the start of OVA inhalation. The inhalation was performed for six consecutive days. (A–D) Immunofluorescent staining of cells expressing ECF-L. The lungs from PBS inhalation mice (A), AHR-model mice (B), and AHR-model mice administered Ad-wt (C) or Ad-ECF-L-AS (D) were stained with anti-ECF-L antibody. Scale bars = 400 μm. (E) Quantitative enumeration of ECF-L-expressing cells around airways. The bars represent mean cell counts in microscopic fields from four mice in each group. $**P \leq 0.01$, Ad-wt-administered mice versus Ad-ECF-L-AS-administered mice. (F) Reduction in airway responsiveness after administration of Ad-ECF-L-AS. Airway responsiveness was measured 24 h after the final inhalation, and the results for PBS-inhalation mice (open circles, $n = 5$), AHR-model mice (closed circles, $n = 8$), Ad-wt-administered mice (closed triangles, $n = 8$), and Ad-ECF-L-AS-administered mice (closed squares, $n = 8$) are shown. Three independent experiments showed similar results. $*P \leq 0.05$, Ad-wt-administered mice versus Ad-ECF-L-AS-administered mice.

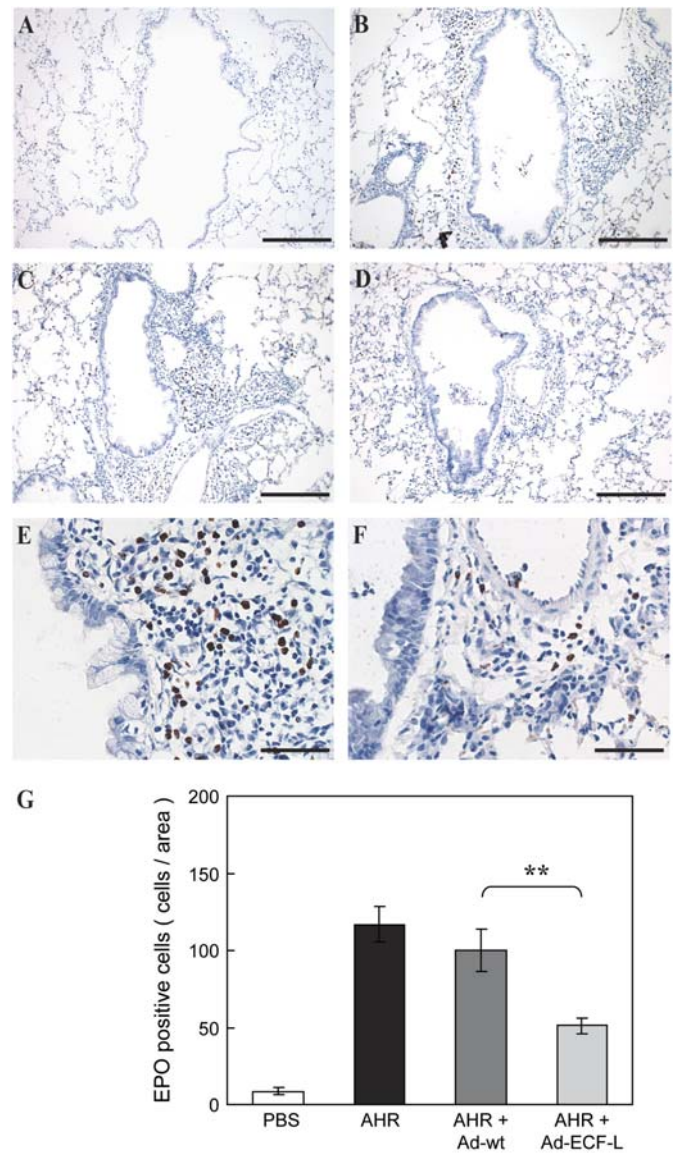


Figure 6. Reduction of eosinophil infiltration after administration of adenovirus expressing ECF-L antisense RNA. (A–F) Eosinophils in lung sections from PBS inhalation mice (A), AHR-model mice (B), and AHR-model mice administered Ad-wt (C and E) or Ad-ECF-L-AS (D and F). Eosinophils were stained by EPO staining. All sections were counterstained with hematoxylin. Scale bars = 400 μm (A–D) or 100 μm (E and F). (G) Quantitative enumeration of EPO-positive eosinophils around airways. The bars represent mean cell counts in microscopic fields from four mice in each group. $**P \leq 0.01$, Ad-wt-administered mice versus Ad-ECF-L-AS-administered mice.

around airways when compared with those from AHR-model mice and mice administered Ad-wt (Figures 5B–5D). After quantitative analysis, the number of ECF-L expressing cells around airways from mice administered Ad-ECF-L-AS showed a decrease of ~60% over that of mice administered Ad-wt (Figure 5E). On the other hand, no difference was observed between AHR-model mice and mice administered Ad-wt.

Using this transgenic approach, the relationship between ECF-L expression and AHR was examined. Administration of Ad-ECF-L-AS significantly suppressed AHR at all ACh dose points, whereas no inhibitory effect on airway reactivity was

observed after administration of Ad-wt (Figure 5F). These results indicate that suppression of ECF-L by adenoviral antisense gene transfer ameliorated the asthmatic phenotype in mice.

Eosinophil Infiltration and Expression of ECF-L

Because ECF-L was reported to be a chemokine family protein due to its ability to attract eosinophils (6), the effect of ECF-L on eosinophil infiltration was examined *in vivo*. After six consecutive days of OVA inhalation, frozen sections were examined for eosinophil infiltration in the lung by EPO staining with DAB. A significant increase in eosinophil numbers was observed in AHR-model mice compared with PBS-inhalation mice (Figures 6A and 6B). This eosinophil infiltration was not influenced by the administration of Ad-wt, and many eosinophils were observed around bronchioles and vessels (Figures 6C and 6E). On the other hand, the number of eosinophils around airways significantly decreased in the lung sections from mice administered Ad-ECF-L-AS (Figures 6D and 6F). The number of eosinophils around airways from Ad-ECF-L-AS mice was calculated to be ~50% less than in mice administered Ad-wt (Figure 6G). These results were positively correlated with a reduction in ECF-L-expressing cells around airways, which may indicate a significant relationship between ECF-L expression and eosinophil infiltration (Figures 5E and 6G).

DISCUSSION

The purpose of this study was to begin to elucidate the mechanism of AHR via an examination of differential gene expression in a murine model of AHR. SSH identified a marked induction of ECF-L expression in the lungs of AHR-model mice. ECF-L is a mammalian member of the chitinase protein family that exhibits eosinophil chemotactic activity (6). Recently, Zimmermann and colleagues reported on a microarray analysis of mouse models of asthma (OVA-treated and *Aspergillus fumigatus* antigen-treated mice models) (14). ECF-L (identified as chitinase 3-like 3) was included on their list, with a 6-fold increase in expression after OVA inhalation. Similarly, a 7-fold increase of ECF-L expression was found in the AHR-model mice in this study. On the other hand, acidic chitinase was not detected using SSH, whereas it was included on their list. The exclusion may denote the influence of experimental conditions on the expression level of chitinase genes.

Expression of ECF-L was found only in the lung and stomach, which differs from a previous report by Owhashi and colleagues, where expression was seen in the spleen, bone marrow, lung, and heart (6). One factor that might explain this discrepancy is the difference in murine models in the two studies. Owhashi and colleagues used *Mesocricetus auratus*-infected mice (the model of parasitic infective eosinophilia) and examined tissue distribution using parasite-infected mice, whereas we used a model of allergic inflammatory eosinophilia. The parasite infection model shows a stronger effect for eosinophil infiltration than the OVA-induced model and may influence some immunologic reactions that induce expression of ECF-L in the spleen and bone marrow.

The findings reported in this article clarify that ECF-L is induced in AMs before the onset of AHR and eosinophil infiltration. AMs enhance inflammation in lungs and produce a wide variety of chemokines, cytokines, and secretory proteins. An *in vivo* transgene approach with recombinant adenovirus was used to examine the relationship between ECF-L and allergic inflammation. Because the airway has a large absorption surface, adenovirus particles can be applied with relative specificity. Furthermore, recombinant adenoviral vectors (Ad-LacZ) were expressed in bronchial epithelial cells and in some of the alveolar cells around airways by this gene delivery system (*see online*

supplement), not in alveolar cells located far from airways (data not shown). This localized transgene approach was suitable for confirming the participation of ECF-L on AHR and eosinophil infiltration because the physiologic change around airways is more important than that in the peripheral alveolar region on AHR and eosinophil infiltration. Thus, we selected intratracheal adenoviral gene transfer for analysis of ECF-L function.

After the efficiency of intratracheal adenovirus administration using a *LacZ* gene was confirmed, adenovirus carrying the ECF-L antisense sequence (Ad-ECF-L-AS) was administered. AHR and eosinophil infiltration in lung tissues were suppressed as ECF-L expression around airways decreased. The suppression level of ECF-L protein was almost the same as that of eosinophil infiltration but was higher than that of AHR. ECF-L has the CXC consensus sequence near the N-terminal that is typical of CXC chemokines and was first reported to be a chemokine family protein due to the property of eosinophil attraction (6). *In vivo*, ECF-L may play a more important role in eosinophil infiltration than in AHR.

The mechanism of ECF-L involvement in eosinophil migration and AHR is unclear. Previous studies have suggested that Ym1, which is highly homologous to ECF-L, has heparin/heparan sulfate-binding properties (17). Heparin/heparan sulfate is a sulfated, negatively charged glycosaminoglycan that is abundant on the cell surface and in the extracellular matrix. ECF-L, which is secreted around the alveolar spaces, may mediate cell-cell and cell-matrix interactions in a manner similar to that of selectins and could promote eosinophil infiltration by increasing the interaction between eosinophils and the airway wall. As a result, airway reactivity may be promoted. Another possibility for a role of ECF-L in AHR is an association with tissue remodeling. The physiologic roles of heparin/heparan sulfate are highly diversified and include cell adhesion, motility, proliferation, differentiation, and tissue morphogenesis. Gp39k, a protein with homology to ECF-L, is expressed only during the differentiation of vascular smooth muscle in culture, and an association with tissue remodeling has been suggested (18). ECF-L may also function in or affect tissue remodeling and could be an important connection to the airway wall thickening that is observed with pathologic change in allergic lung disease (19, 20).

On the other hand, Webb and colleagues reported that Ym1 expression is regulated by IL-4 and IL-13 signaling via the IL-4R α subunit (19). IL-4 and IL-13 play pivotal roles in regulating mucus hypersecretion and AHR, key pathophysiologic processes in asthma. Welch and colleagues also reported that Ym1 mRNA expression is induced in response to stimulation with Th2 cytokines such as IL-4 and IL-13 via the STAT6 signal transduction pathway in multiple macrophage populations (21). Furthermore, Welch and colleagues characterized the Ym1 promoter sequence and identified three new functional STAT6 binding sites and demonstrated the requirement of STAT6 activity for IL-4-stimulated induction. AHR-model mice are widely used as a model for allergic inflammation and exhibit many of the characteristic features of allergy, such as Th2-cell activation; IL-4, IL-5, and IL-13 production; and IgE-mediated responses (4). Thus, ECF-L may be induced by Th2 cytokines during allergic inflammation and may play an important role in Th2-biased immune responses.

Because murine ECF-L seems to function in the regulation of airway inflammation in an allergic mouse model, the human counterpart of ECF-L is a potential target for the treatment of asthma and other allergic diseases. To identify the human counterpart of ECF-L, human cDNA libraries were screened with an ECF-L cDNA probe under low-stringency hybridization conditions. Human acidic mammalian chitinase (hAMCase) was identified as a possible human counterpart of ECF-L (data not shown). Although hAMCase showed high sequence similarity

to ECF-L (81% at the amino acid level), unlike ECF-L, the hAMCase protein has retained all of the essential acidic residues in the active center that are required for chitinase activity and the C-terminal chitin binding domain, which includes six cysteines and three aromatic amino acids (22, 23). Recently, Zhu and colleagues reported that hAMCase was expressed by epithelial cells and macrophages in lung tissue samples taken from patients with asthma (24). Also, hAMCase expression was found to be significantly higher in biopsies from asthmatic subjects than in those from subjects without asthma. They also showed that mouse AMCase played an important role in the development of OVA-induced AHR and emphasized the potential importance of chitinases in allergic diseases such as asthma. In the present study, the suppression of ECF-L transcript levels using recombinant adenoviral vectors ameliorated the asthmatic phenotype in AHR-model mice, even though ECF-L has no active chitinase domain and does not possess chitinase activity (6, 25). Thus, it is possible that ECF-L and hAMCase share an unknown function outside of chitinase activity that is involved in airway reactivity. Further experiments are required to determine such functions.

In conclusion, ECF-L was identified as a selectively expressed protein in the AHR mouse lung. Inhibition of ECF-L expression by administration of Ad-ECF-L-AS significantly suppressed AHR and eosinophil infiltration. These results indicate that ECF-L may play a critical role in allergic inflammation and that the human counterpart of ECF-L may be a potential target for asthma therapy.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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