

Amphiregulin Production by Human Eosinophils

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Key Words

Eosinophil · Amphiregulin · Remodeling

Abstract

Background: Amphiregulin (AREG) plays critical roles in mammary gland development, immune responses against nematode infection, and mucous production in the lung. Since remarkable eosinophil infiltration has been shown at all of these tissue sites, we examined AREG production by human eosinophils in vitro. **Methods:** Using Ficoll and antibody-coated immunomagnetic beads, eosinophils and other blood cells were purified from peripheral blood samples obtained from normal or mild allergic patients. Eosinophils were cultured in the presence of 10 ng/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF), 10 ng/ml of IL-5, 100 ng/ml of IFN- γ or immobilized secretory IgA. Total mRNA was extracted after 6 h of culture, and mRNA expression was measured using a microarray and RT-PCR. The AREG concentration in the supernatant and cell lysate after 48 h of culture was measured using ELISA. **Results:** AREG mRNA was constitutively expressed in fresh eosinophils, and the expression level in the eosinophils was higher than that in other types of blood cells. AREG mRNA increased significantly upon stimulation with GM-CSF and IL-5 but not with IFN- γ . AREG mRNA expression in GM-CSF-stimulated eosinophils was enhanced by dexamethasone. Fresh eosinophils

did not contain AREG protein. AREG was significantly released into the supernatant when the eosinophils were stimulated with GM-CSF, but not with IFN- γ or immobilized secretory IgA. **Conclusion:** Our data suggested that eosinophils produce and release AREG, and participate in some physiological and pathological conditions in vivo.

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Introduction

Amphiregulin (AREG) is a member of the epidermal growth factor family [1]. Recent studies have shown that AREG knockout mice exhibit severely impaired mammary gland development [2] as well as impaired immune responses against nematode infection [3]. In addition, the concentration of AREG in the bronchoalveolar lavage fluid of patients with bronchial asthma is strongly correlated with the mucous index in the lungs [4]. Therefore, AREG is considered to play critical roles in several physiological and pathological conditions in vivo.

Interestingly, remarkable eosinophil infiltration has been reported at all of the above-mentioned tissue sites [5–7]. In addition, the neutralization or genetic deletion of IL-5 impairs not only eosinophilopoiesis, but also mammary gland development [8] and host defense against helminth infection [9], and improves allergic in-

flammation [10] in mouse models. These findings strongly suggest that eosinophils accumulate and play some role(s) at tissue sites where AREG has critical activities. However, the precise roles of eosinophils with regard to these physiological and pathological conditions are not fully understood [6, 11].

In the present study, we examined AREG production by human peripheral blood eosinophils *in vitro*.

Materials and Methods

Reagents

Recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-5, and IFN- γ were purchased from PeproTech (Rocky Hill, N.J., USA). Eotaxin was purchased from R&D Systems (Minneapolis, Minn., USA). Platelet-activating factor (PAF: 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine) was purchased from Sigma (St. Louis, Mo., USA). Secretory IgA (sIgA) was purchased from ICN Biomedicals, Inc. (Aurora, Ohio, USA).

Leukocyte Preparation

Eosinophils were purified from the peripheral blood of normal subjects or subjects with mild allergic diseases ($n = 12$) using a Ficoll-sodium diatrizoate density gradient (lymphocyte separation medium; ICN Biomedicals, Inc.) sedimentation and negative selection with anti-CD16 antibody-coated immunomagnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), as previously described [12]. Both the purity and the viability of the eosinophils were confirmed to exceed 97 and 99%, respectively, based on light-microscopic examinations of the cytocentrifuged preparations stained with Diff-Quik (American Scientific Products, McGraw Park, Ill., USA) or trypan blue (Sigma) dye exclusion, respectively. All the allergic donors had been diagnosed as having either allergic rhinitis and/or bronchial asthma but had exhibited no or mild symptoms at the time of blood collection.

CD14+, CD4+, CD8+, CD19+ cells, neutrophils and basophils were purified from the peripheral blood of normal subjects as previously described [13]. Briefly, CD4+, CD8+ and CD14+ cells were purified using density gradient sedimentation and positive selection with anti-CD4, CD8 and CD14 immunomagnetic beads (Miltenyi Biotec), respectively. CD19+ cells were purified using a combination of negative (antibodies to CD3, CD7, CD14, CD42b, and CD56) and positive (antibody to CD19) selection using MicroBeads (Miltenyi Biotec). Neutrophils were purified using density gradient sedimentation and a combination of positive (anti-CD16) and negative (anti-CD81) selection using MicroBeads (Miltenyi Biotec). Basophils were semipurified using Percoll (Pharmacia, Uppsala, Sweden) density gradient centrifugation, and the cells were further purified by negative selection using a MACS Basophil Isolation Kit (Miltenyi Biotec), as previously described [14]. In all cell types, the purity and the viability of the cells exceeded 98 and 99%, respectively.

Written informed consent was obtained from all the subjects, and the study was approved by the Ethical Review Board of the National Research Institute for Child Health and Development, Tokyo, Japan.

Eosinophil Culture

Purified eosinophils were suspended at a cell density of 1×10^6 /ml in Iscove's minimal essential medium (IMDM; Gibco, Grand Island, N.Y., USA) supplemented with 10% FCS (HyClone Laboratories, Inc., Logan, Utah, USA) and cultured in 24-well microtiter plates (Costar Corp., Cambridge, Mass., USA) previously coated with 2-ml aliquots of PBS containing 1% HSA (heat-denatured at 65°C for 1 h) for overnight at 4°C to reduce nonspecific adherence to the plastic. The eosinophils were cultured in the presence or absence of 10 ng/ml GM-CSF, 10 ng/ml IL-5, 100 ng/ml IFN- γ , 100 ng/ml eotaxin, and 10^{-8} M PAF for up to 48 h at 37°C. For activation with sIgA, 24-well microtiter plates were previously coated with 0.5-ml aliquots of 100 ng/ml sIgA overnight at 4°C and then washed and coated with 2-ml aliquots of PBS containing 1% HSA for at least 2 h at room temperature [15]. In some experiments, 10^{-7} M dexamethasone (DEX; Sigma) was added to the wells at the same time as the 10 ng/ml GM-CSF.

Microarray Analysis of Freshly Isolated Leukocytes and Activated Eosinophils

Total RNA samples were extracted from freshly isolated CD14+, CD4+, CD8+, CD19+ cells, neutrophils and basophils using RNeasy (Qiagen, Valencia, Calif., USA) and digested with RNase-free DNase I (Qiagen), according to the manufacturer's instructions. To normalize the results and to obtain 5 μ g of total RNA for each cell type, the same amounts of total RNA from 3 to 8 separate donors were collected and mixed. For eosinophils, freshly isolated or activated eosinophils (6 h after activation) were first lysed in Isogen (Nippon Gene, Toyama, Japan), and the total RNA was extracted using PCI (buffer saturated phenol:chloroform:isoamyl alcohol, 25:24:1, v/v; Wako Pure Chemical Industry, Saitama, Japan) and RNeasy (Qiagen) and digested with RNase-free DNase I (Qiagen), according to the manufacturer's instructions.

5 μ g of total RNA from each type of leukocyte were used to prepare the cRNA. Gene expression was examined using the GeneChip Human Genome U133 plus 2.0 probe arrays (Affymetrix, Santa Clara, Calif., USA), which contain the oligonucleotide probe sets for 54,120 full-length genes and expressed sequence tags, according to the manufacturer's protocol [13]. The data analysis was performed using GeneSpring software version 7.2 (Silicon Genetics, Redwood City, Calif., USA). To normalize the staining intensity variations among the chips, the average difference values for all the genes on a given chip were divided by the median expression value for all the genes on the chip. To eliminate genes whose expressions only represented background noise, genes were selected only if the raw data was >100 , and the gene expression was judged to be 'present' by the GeneChip Analysis Suite 5.0 (Affymetrix). A hierarchical clustering analysis was performed using a minimum distance value of 0.001, a separation ratio of 0.5, and the standard definition of the correlation distance.

RT-PCR and Real-Time Quantitative PCR

The primer sets for AREG (sense, 5'-TGATACTCGGCTCAGGCCATT-3'; antisense, 5'-AAATCCATCAGCACTGTGGTCC-3'), a disintegrin and metalloprotease domain 17 (ADAM17)/TNF α -converting enzyme (TACE) (sense, 5'-GAGAGGGAA-CAGCAGCTGGA-3'; antisense, 5'-GCATCGACATAGGGGCACACA-3'), and β -actin (sense, 5'-CCCAGCCATGTACGTTGC-

TAT-3'; antisense, 5'-TCACCGGAGTCCATCACGAT-3') were synthesized at FASMAC (Kanagawa, Japan). First-strand cDNA was synthesized using an Oligo (dT) (12-18 mers) primer (Invitrogen, Carlsbad, Calif., USA) and I Script™ cDNA synthesis kit (Bio-Rad Laboratories, Hercules, Calif., USA). For the RT-PCR, cDNA generated from 5 ng of total RNA was amplified using rTaq polymerase (Toyobo, Osaka, Japan) and a thermal cycler (GeneAmp PCR System 9700; PE Biosystems, Foster City, Calif., USA) under the following conditions: 94°C/30 s, 60°C/30 s, 72°C/1 min for 34 cycles for ADAM17, or for 30 cycles for β -actin. The PCR products were visualized on a 0.8% agarose gel (BRL Life Technologies Inc., Grand Island, N.Y., USA) containing 0.05 μ g/ml ethidium bromide (Sigma).

Real-time quantitative PCR analyses were performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif., USA) and SYBR Green I PCR reagents (Toyobo), as previously described [16]. To determine the exact copy numbers of the target genes, the quantified concentrations of subcloned PCR fragments of AREG and β -actin were serially diluted and used as standards in each experiment. Aliquots of cDNA equivalent to 5 ng of the total RNA samples were used for each real-time PCR. Data were normalized with the β -actin levels in each sample. The copy number was expressed as the number of transcripts/ng total RNA.

AREG Concentration in the Supernatant and Cell Lysate

Eosinophil culture supernatants were harvested 48 h after stimulation with cytokines, chemokines or immobilized sIgA. To measure the amount of pre-stored AREG in the eosinophils, both fresh and stimulated eosinophils were washed with PBS and re-suspended at a cell density of 1×10^6 /ml in PBS. Cell lysates were obtained by freezing and thawing the cell suspension three times. The concentration of AREG was measured using ELISA kits (R&D Systems, Inc.) according to the manufacturer's instructions with a slight modification, consisting of the addition of serially diluted standard samples to define the minimal detection limits extensively in every assay. The minimal detection limit of each assay was at least 3.91 pg/ml.

Statistical Analysis

All the data were presented as the median and 90th percentiles unless otherwise indicated. Differences between the groups were analyzed using a Mann-Whitney's U test after a Kruskal-Wallis nonparametric one-way analysis of variance (ANOVA) test, and StatView™ SE+Graphics software (Abacus Concepts, Inc., Berkeley, Calif., USA). Differences were considered significant if $p < 0.05$.

Results

AREG mRNA Expression

AREG mRNA expression was determined in various types of freshly isolated blood cells using the GeneChip system (fig. 1). AREG mRNA was constitutively expressed in fresh eosinophils (raw data: 1,267), and the expression level in the eosinophils was higher than the levels in other types of blood cells.

Microarray analyses also revealed that AREG mRNA expression was increased significantly by 3.8- and 2.9-fold upon stimulation with IL-5 and GM-CSF, respectively, but not with IFN- γ (fig. 2). These effects were further confirmed in a separate experiment using eosinophils with similar stimuli and a different microarray system (GeneChip U95A; data not shown).

To determine the effect of exogenous corticosteroid on AREG mRNA expression in eosinophils, eosinophils were stimulated with 10^{-7} M DEX simultaneously with 10 ng/ml GM-CSF for 6 h. As a result, AREG mRNA expression in GM-CSF-stimulated eosinophils was markedly enhanced by DEX (fig. 3).

AREG Protein in the Culture Supernatants and Cell Lysates

The average concentration of AREG in the supernatant of unstimulated eosinophils after 48 h of culture was below the detection limit of the ELISA assays (3.91 pg/ml). However, AREG was significantly released into the supernatant when the eosinophils were stimulated with GM-CSF, but not with IFN- γ or immobilized sIgA for 48 h (fig. 4). IL-5 tended to induce AREG release into the supernatant; however, the concentration of AREG did not reach statistical significance (fig. 4). The amount of AREG protein in the supernatant after stimulation with GM-CSF varied widely (fig. 4). To examine the in vivo priming effect of eosinophils in different donors, the GM-CSF-induced AREG release from normal donors ($n = 3$) was compared with that from mildly allergic donors ($n = 5$); however, no statistical difference in the AREG concentrations was observed between these two groups (data not shown).

On the other hand, fresh eosinophils did not contain AREG protein. In addition, eosinophil lysates did not contain a detectable amount of AREG protein even after stimulation with cytokines for 48 h (data not shown).

Expression of TNF α -Converting Enzyme/ a Disintegrin and Metalloprotease Domain 17 in Eosinophils

Because the release of AREG is known to be regulated by the action of TACE/ADAM17 [17], TACE mRNA expression was determined using RT-PCR (fig. 5). Eosinophils constitutively expressed a high amount of TACE mRNA both before and after various stimuli for 6 h.

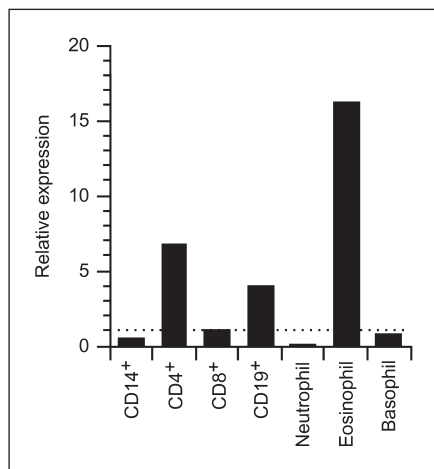


Fig. 1. Microarray analysis of AREG mRNA expression in freshly isolated leukocytes. To normalize the results and to obtain 5 μ g of total RNA for each cell type, the same amounts of total RNA from 3 to 8 separate donors were collected and mixed. AREG mRNA expression in various types of freshly isolated blood cells was determined using the GeneChip system. The relative AREG mRNA expression levels are shown after per chip normalization.

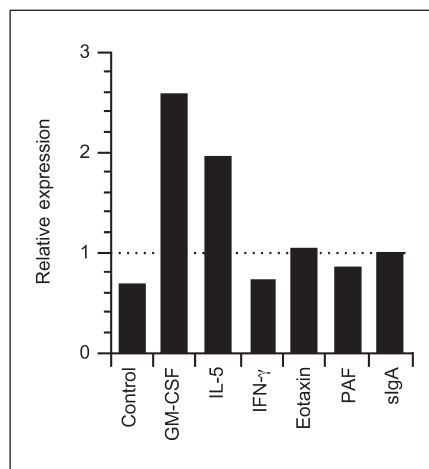


Fig. 2. Microarray analysis of AREG mRNA expression in activated eosinophils. AREG mRNA expression in eosinophils after stimulation with vehicle (control), 10 ng/ml GM-CSF, 10 ng/ml IL-5, 100 ng/ml IFN- γ , 100 ng/ml eotaxin, 10^{-7} M PAF, or 100 μ g/ml immobilized sIgA was determined using the GeneChip system. The relative AREG mRNA expression levels are shown after per chip normalization.

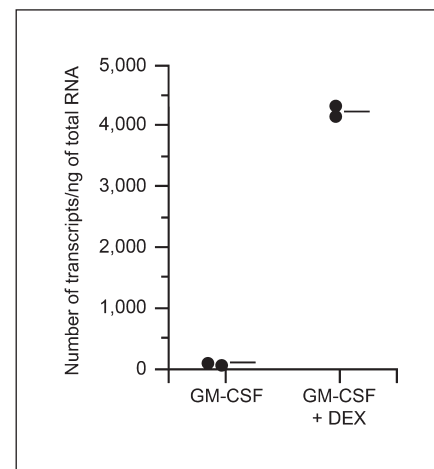


Fig. 3. Real-time quantitative PCR analysis of AREG mRNA expression in DEX-activated eosinophils. AREG mRNA expression in eosinophils after stimulation for 6 h with 10 ng/ml GM-CSF in the presence or absence of 10^{-7} M DEX was determined using real-time PCR. The results are shown as the number of transcripts/ng of total RNA equivalent from 2 separate experiments using eosinophils from 2 different donors. The bars indicate the average of 2 experiments.

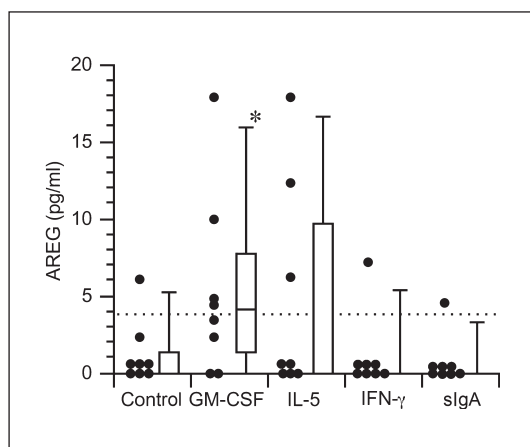


Fig. 4. AREG concentration in the supernatant of eosinophils. AREG concentration in the supernatant 48 h after stimulation with vehicle (control), 10 ng/ml GM-CSF, 10 ng/ml IL-5, 100 ng/ml IFN- γ , or 100 μ g/ml immobilized sIgA was determined using an ELISA. The data shown are the real values, the median and 90th percentile of 8 separate experiments. Dotted line indicates the minimal detection limit (3.91 pg/ml). * $p < 0.05$.

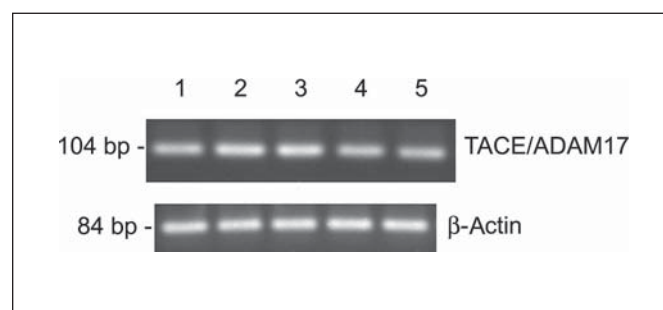


Fig. 5. RT-PCR analysis of TACE/ADAM17 mRNA expression in activated eosinophils. TACE mRNA expression in eosinophils after stimulation for 6 h was determined using RT-PCR. The PCR products were visualized on a 0.8% agarose gel containing 0.05 μ g/ml ethidium bromide. The figure shows data from a single experiment that was representative of 3 separate experiments using samples from different donors. Lanes: (1) vehicle (control); (2) 10 ng/ml GM-CSF; (3) 10 ng/ml IL-5; (4) 100 ng/ml IFN- γ ; (5) 100 μ m/ml immobilized sIgA.

Discussion

Eosinophils produce and release various cytokines and chemokines upon stimulation with cytokines, chemokines or immobilized immunoglobulins [11]. In the present study, we found that human peripheral eosinophils constitutively expressed AREG mRNA and that the expression level was higher in the eosinophils than in other types of blood cells (fig. 1). In addition, AREG mRNA expression in the eosinophils was significantly increased after stimulation with GM-CSF and IL-5, but not after stimulation with IFN- γ (fig. 2). ELISA assays revealed that fresh eosinophils neither contained nor released AREG, even though eosinophils constitutively express AREG mRNA. Eosinophil lysates did not contain AREG protein even after stimulation with cytokines for 48 h. However, after 48 h of stimulation with GM-CSF and IL-5, the eosinophils produced and released AREG into the supernatant.

AREG is synthesized as pre-AREG, and TACE is responsible for the release of AREG into the supernatant through cleavage at a specific cleavage site [17]. Presumably because eosinophils constitutively express TACE (fig. 5), the eosinophils did not store AREG protein even after stimulation with cytokines. However, whether the AREG ELISA kit that we used can detect pre-AREG is uncertain. In addition, whether the freeze-thaw method that we used in this study might affect the results of the ELISA is also unknown. Thus, further examination is required before we can conclude that eosinophils do not store pre-AREG and/or mature AREG.

Human mast cells produce and release AREG upon stimulation via cell surface Fc ϵ RI [4, 18]. Compared with mast cells, eosinophils did not release a large amount of AREG. However, the number of infiltrating eosinophils into tissue sites seems to be much higher than the number of tissue-dwelling mast cells. Thus, AREG released from eosinophils may play some role(s) in allergic inflammatory sites. It is of particular interest that AREG production by mast cells was not inhibited by the addition of DEX [4]. When eosinophils were treated with GM-CSF and 10^{-7} M DEX, AREG mRNA expression was markedly increased (fig. 3). However, corticosteroids simultaneously induce eosinophil apoptosis [19]; thus, whether corticosteroids alter AREG release from eosinophils should be further examined.

Chronic allergic inflammation results in several characteristic changes in tissue structure, which is now referred to as airway remodeling [20, 21]. Recent studies have indicated that eosinophils play important roles in

airway remodeling [22], at least in part, through the release of vascular endothelial growth factors [23], transforming growth factor- β [24] and matrix metalloproteinase 9 [25]. However, the mechanisms through which eosinophils are involved in airway remodeling are not yet fully understood. The fact that AREG induces the proliferation of epithelial cells [26], fibroblasts [27], and mucous production [4] strongly suggests that AREG is another candidate cytokine through which eosinophils are involved in airway remodeling.

In the present study, we demonstrated that eosinophils produce and release AREG after stimulation with physiological concentrations of GM-CSF and IL-5. However, AREG is also expressed in epithelial cells [28]. Understanding the precise role and the trigger of AREG production in eosinophils under physiological conditions will require further investigation.

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Disclosure Statement

The authors declare that no financial or other conflict of interest exists in relation to the content of the article.

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