Increased CD69 expression on activated eosinophils in eosinophilic chronic rhinosinusitis correlates with clinical findings

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ABSTRACT

Background: Eosinophilic chronic rhinosinusitis (ECRS) is a subtype of chronic rhinosinusitis associated with asthma. CD69 is an important marker of activation for eosinophils. But, whether a correlation exist between the CD69 expression on eosinophils and clinical findings is unclear.

Methods: We performed quantitative PCR and/or flow cytometry using tissue and purified eosinophils from the blood and nasal polyps of 12 patients with ECRS and from 8 patients without ECRS (controls). We assessed clinical findings including nasal polyp (NP) scores, sinus CT findings, and pulmonary function test results, and examined their possible association with the CD69 expression. We also performed CD69 cross-linking experiments in mouse eosinophils to investigate the functional role of CD69.

Results: Levels of cytokine mRNAs (IL-4, -5, -10, and -13) were significantly higher in purified NP eosinophils and tissues from patients with ECRS than the levels of those in controls. The expressions of major basic protein (MBP), eosinophilic cationic protein (ECP), eosinophil-derived neurotoxin (EDN), eosinophil peroxidase (EPX) in cytotoxic granules, and CD69 mRNA were significantly higher in purified eosinophils from NPs than in those from blood. We also found a correlation between expression of CD69 and clinical findings. Moreover, we found EPX release from mouse eosinophils following CD69 cross-linking.

Conclusions: These data suggest that increased CD69 expression by eosinophils is not only a biomarker for nasal obstruction and pulmonary dysfunction, but also a potential therapeutic target for patients with ECRS and asthma.

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Introduction

Chronic rhinosinusitis (CRS) is a heterogeneous disease classified into two types based on the presence (CRSwNP) or absence (CRSsNP) of nasal polyps (NP). Approximately, 80% of patients with CRSwNP have infiltration of eosinophils into the NPs. Patients who exhibit CRS with eosinophilia (or eosinophilic chronic rhinosinusitis, ECRS) are diagnosed based on the Japanese Epidemiological Survey of Refractory Eosinophil Chronic Rhinosinusitis (JESREC) scoring system. ECRS is characterized by ethmoid–predominant sinusitis with eosinophilic infiltration. Kobayashi et al. and Ame-link et al. reported that 74% of patients with asthma have ECRS, and that 53% of those with severe asthma and 26.5% of those with mild-to-moderate asthma have NPs. Consequently, ECRS and asthma share macroscopic pathological and histological characteristics, and require comprehensive care according to the united airway concept.

In patients with ECRS with asthma, eosinophils play a critical role in the pathogenesis of the diseases. Eosinophils must migrate from the bloodstream to the site of inflammation to exert injury as effector cells by releasing cytokines, cytotoxic granules, and chemokines. During this process, eosinophils express activation markers such as CD69, CD44, L-selectin, CD54, CD162, and CD11b. CD69 is a type II transmembrane protein, a member of the C-type lectin family, and a very early activation antigen (VEA). This molecule is expressed on infiltrated leukocytes such as T cells, B cells, NK cells, neutrophils, and eosinophils during chronic airway inflammation. In terms of CD69 regulation in eosinophils, Walsh et al. found that GM-CSF, IL-3, or IL-5 induce CD69 expression on peripheral blood eosinophils, but not in untreated ones. Reports have shown that signaling through CD69 modulates the differentiation of T helper cells and induces platelet degranulation and aggregation. Although several CD69 ligands have been isolated, including galectin-1 and myosin light chain 9 and 12 (My9/12), the ligand on eosinophils has not been identified. Whether CD69 is expressed on eosinophils in the NPs and in peripheral blood (PB) of patients with ECRS patients, and whether a correlation exist between CD69 expression on NP eosinophils and clinical findings of patients with ECRS (such as NP score, sinus CT findings, and pulmonary function) remains unclear.

For this report, we developed an approach for obtaining a high number of purified eosinophils from NPs, because conventional protocols lead to poor numbers. We also measured cytokine production, levels of cytotoxic granules, and CD69 expression on purified eosinophils, and determined correlations between their expression levels and clinical findings. Moreover, we investigated the functional role of CD69 signaling in mouse eosinophils.

Methods

Patients

We recruited patients from the Otorhinolaryngology and Head and Neck surgery of the Takeda General Hospital and Kansai Medical University during a two-year period (2017–2018). The diagnoses of patients with ECRS and non-ECRS (NECRS) patients were based on those in the JESREC study. ECRS was diagnosed by 11 points or higher JESREC as reported by Fujieda et al. Moreover, a final diagnosis of ECRS was made by examination of NP under a microscope; mean number of eosinophils in three fields were >70 cells in the high-power field (400×). By contrast, NECRS were defined as both <11 score by JESREC and <70 eosinophils in the tissue. In all, 12 patients with ECRS and 8 controls (NECRS) participated in the study. We obtained NPs and uncinate process tissues (UT) during endoscopic sinus surgeries. Peripheral blood (PB) samples were collected before surgical operations. A respiratory physician diagnosed asthma based on the patient’s history of wheezing responsive to a bronchodilator. No systemic corticosteroids were administered to the patients at least 4 weeks prior to their surgery. We used NP scores to evaluate the severity of NPs in each patient, and we defined sinus CT findings according to the Lund–Mackay scale. The examination of pulmonary function was performed using SPIRO SIFT SP-770COPD (Fukuda Denshi, Tokyo, Japan) measuring the following parameters: the ratio of peak expiratory flow (%PEF), ratio of predicted forced expiratory volume in 1 s to forced vital capacity (FEV1/FVC), percentage of predicted mean forced expiratory flow between 25% and 75% of forced vital capacity (%PEF25–75). Table 1 and Supplementary Table 1 present patient characteristics.

The local ethics committees at the Takeda General Hospital (No 2016-002) and at the Kansai Medical University (No.1313) approved this study. We obtained informed consents from all participants.

Mice

We used the following mouse strains: IL-5 transgenic (Tg) mice (BALB/c background), obtained from the co-author Dr. D. Dombrowicz (Institut Pasteur de Lille, Lille, France). All mice were housed at 21°C–23°C with 40%–60% humidity in animal facilities with a 12-h light/dark cycle and were provided food and water ad libitum. All animal experiments were performed using protocols approved by the Kansai Medical University Animal Ethics Committee (18-082).

Human and mouse eosinophils preparation

We separated human granulocytes from PB using the Ficoll–Paque technique (GE healthcare, Uppsala, Sweden). After lysing red blood cells, eosinophils were isolated by using a cell sorter, Aria III (BD science, New Jersey, USA). Detailed protocols for the human eosinophil purification and isolation from NP and mouse eosinophils purification from splenocytes of IL-5 Tg mice are shown in the Supplementary Methods and Supplementary Figure 1.

Flow cytometric analysis

We performed flow cytometry experiments (Aria III and CantII, BD Biosciences, New Jersey, USA) using the FlowJo software (BD Biosciences, New Jersey, USA).

We stained dissociated cells from human PB or NP with antibodies against CD3, CD14, CD16b, CD19, CD45, CD68, Siglec8, CD69, and/or control antibodies for 30 min at 4°C. For CD69 mean fluorescence index (MFI) ratio on surface expression was calculated according as described. 

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\text{CD69 MFI ratio} = \frac{\text{MFI of anti CD69 reacted cells on NP eosinophils or T cells}}{\text{MFI of isotype}} - \frac{\text{MFI of anti CD69 reacted cells on PB eosinophils or T cells}}{\text{MFI of isotype}}
\]
The purified mouse eosinophils from mouse spleens were stained with Gr-1, CD45, SiglecF, CD69, and/or control antibodies for 30 min at 4 °C. Eosinophils were identified by gated CD45−SiglecF−Gr-1+ (Supplementary Fig. 2). Supplementary Table 2 lists information regarding the antibodies for cell sorting.

**Immunofluorescence staining and quantitative PCR**

We stained tissue sections with antibodies against siglec8, CD69 and control antibodies overnight at 4 °C. We incubated them with an appropriate secondary antibody for 1 h at room temperature (as listed in the Supplementary Table 2). Tissues were counterstained with Hoechst dye and mounted using Fluorescence Mounting Medium (Dako, CA, USA). We captured images with an appropriate secondary antibody for 1 h at room temperature (as listed in the Supplementary Table 2). Tissues and eosinophils were counterstained with Hoechst dye and mounted using Fluorescence Mounting Medium (Dako, CA, USA) or with 10 μg/ml Armenian Hamster IgG (Jackson ImmunoResearch, PA, USA) and analyzed them using a LSM 700 scope (Carl Zeiss, Jena, Germany).

We performed quantitative PCRs (qPCRs) following the extraction of mRNA from tissues and eosinophils. Relative gene expression was calculated using the comparative Ct method. Supplementary Table 3 lists primer sequences. Detailed protocols for immunofluorescence staining and qPCR are shown in the supplementary data.

**EPX releasing from mouse eosinophils by cross-linking of CD69**

We resuspended eosinophils (0.4 M/200ul per well) purified from mouse spleens in PBS and incubated them with 1–100 μg/ml Armenian Hamster anti-mouse CD69 mAb (Novus Biologicals, CO, USA) or with 10 μg/ml Armenian Hamster IgG (BioLegend, CA, USA) as control antibody for 1 h at 4 °C. We then transferred the eosinophils into a 24-well plate coating with 20 μg/ml Goat anti-Armenian Hamster IgG (Jackson ImmunoResearch, PA, USA) and incubated for 2 h at 37 °C. After centrifuge plate, we transferred the supernatants into a microtiter plate and analyzed them using an EPX detection kit as per the manufacturer’s instructions (Cell Technology, CA, USA). Chemiluminescence was immediately measured with a luminometer, EnSpire (PerkinElmer, Mass, USA).

**Results**

**Expression of cytokine mRNAs in tissue from controls and patients with ECRS**

To investigate the cytokine profiles in the UTs and NPs of patients with ECRS, and in the UTs of controls, we quantified cytokine mRNAs by qPCR. The expressions of IL-4, IL-5, IL-10, and IL-13 mRNA in the UTs of patients with ECRS were significantly higher than those in UTs from controls (Fig. 1). Although the expressions of IFNγ mRNA among UT from patients with ECRS, NP from patients with ECRS, and UT from controls were all similar, the expressions were 4.7- and 9.8-fold higher in the UT and NP of patients with ECRS than in the UT of controls.

**Character of purified eosinophils from PB and NP**

To investigate differences in cytokine production between purified eosinophils from PB and NP, we modified a conventional protocol for NP eosinophil purification. As shown in Supplementary Figure 3, the number and percentage of CD45+ dissociated cells from NP using the tumor dissociation kit was significantly higher...
than the number and percentage of the conventional protocol: while the tumor dissociation kit yielded $1.93 \pm 1.07 \times 10^7$ g total cells with $14\% \pm 9\%$ CD45$^+$ cells, we only obtained $0.76 \pm 0.6 \times 10^7$ g total cells and $7\% \pm 4\%$ CD45$^+$ cells after the conventional protocol. Moreover, the % viability between the two protocols was comparable: $76\% \pm 8\%$ viability using the tumor dissociation kit and $75\% \pm 16\%$ viability using the conventional protocol. After tissues dissociation, $0.79\% \pm 0.2\%$ eosinophils and $1.7\% \pm 0.6\%$ T cells from NPs were obtained by cell sorter. Using qPCR, we found significant differences in the levels of cytokines (IL-4, -5, -10, and -13) and cytotoxic granules (MBP, ECP, EDN, and EPX) between purified eosinophils from PB of controls and NP of patients with ECRS (Fig. 2A, B). Interestingly, we found significant differences in levels of MBP, EDN, and EPX between purified eosinophils from PB and NP of patients with ECRS (Fig. 2B and Supplementary Fig. 4); however, their cytokine expressions were similar (Fig. 2A).

**Expression of CD69 on infiltrating eosinophils**

Next, we examined CD69 expression on eosinophils using qPCR and flow cytometry. As shown in Figure 3A, infiltrating eosinophils isolated from the NP of patients with ECRS had significantly higher expression of CD69 mRNA than the eosinophils from PB. According to our flow cytometry analysis results, the MFI of CD69 was significantly increased on eosinophils from NP compared with that in PB eosinophils from the same patients (Fig. 3B). We found similar results regarding CD69 expression on eosinophils using immunofluorescence (Fig. 3C).

**Correlation between CD69 MFI on eosinophils and clinical findings**

We found that the CD69 MFI ratio on eosinophils was correlated with the number of eosinophils infiltrating NPs (Fig. 4A). We also found similar results in terms of the NP score (Fig. 4B) and sinus CT findings as measured by the Lund–Mackay scale (Fig. 4C). Moreover, we found that the CD69 MFI ratio on eosinophils was negatively correlated with pulmonary functions such as FEV$_{1.0}$% and %FEF$_{25-75}$ but not %PEF (Fig. 4D). By contrast, we found no significant correlations between CD69 MFI ratios on T cells and clinical findings (Supplementary Fig. 5). In addition, we found the number of infiltrating eosinophils to NPs and clinical findings correlated negatively with the %PEF$_{25-75}$ (Supplementary Fig. 6).

**Functional role of CD69 signaling in mouse eosinophils**

To investigate the functional role of CD69 signaling, we analyzed EPX release from mouse eosinophils following cross-linking to CD69, since obtaining sufficient numbers of human eosinophils during the protein assays is difficult. We found CD69 (6.1% ± 0.3%) on mouse eosinophils purified from splenocytes (Fig. 5A), but not on PB eosinophils. Moreover, cross-linking of CD69 to mouse eosinophils from spleen induced EPX release in a dose dependent manner (Fig. 5B).

**Discussion**

In this study, we found significantly higher mRNA expressions of the Treg-associated cytokine (IL-10) and Th2 cytokines (IL-4, IL-5, and IL-13) in both UT and NP tissues from patients with ECRS compared with the same expressions in controls. In addition, we observed increased expression of the Th1 cytokine (IFN-γ) in 4 by 12 patients with ECRS, compared with NECRS. As demonstrated by our results, Tomassen et al. used a cluster analysis of biomarkers to show that CRS is a heterogeneous inflammatory disease, and they suggested that patients tend to have endotypes corresponding to diverse inflammatory profiles containing Th1 and Th2 cytokines. To better characterize eosinophils into the inflamed sites, we also developed a purification protocol using a cell sorter following a density gradient technique. Our protocol resulted in a 4-fold increase in the number of CD45$^+$ cells compared to the number obtained after the conventional protocol, with comparable cell viabilities. We found only one other study demonstrating eosinophil purification from NPs in patients with ECRS: A report by Miyake et al., in which authors directly purified eosinophils from dissociated cells from NPs using a cell sorter without density gradient technique. However, they did not mention the total corrected cell numbers or the cell viability.

Next, we also found high mRNA expression of cytokines in the purified eosinophils from NPs in ECRS patients, supporting the
Fig. 3. Eosinophils infiltrating the nasal polyps of patients with ECRS were activated. (A) Quantitative PCR analysis of CD69 mRNA expression levels from peripheral blood (PB) eosinophils and nasal polyp (NP) eosinophils from patients with ECRS. Relative gene expression levels were calculated by the comparative $C_T$ method with PB of controls as the reference. $P$ values determined by the Mann–Whitney U-test (*$P$ < 0.05, ECRS and NECRS are $n = 5, 8$). (B) Histograms of CD69 expression on eosinophils in NP (red) and PB (blue) from patients with ECRS. Representative data from patient #18. The gray bar is isotype control. MFI of CD69 comparing eosinophils in PB to those in NPs. Result shown as MFI subtracting corresponding to the isotype control. $P$ values determined by the Mann–Whitney U-test (*$P$ < 0.05, $n = 12$). (C) Immunofluorescence images of fixed NP sections stained with antibodies against CD69 and Siglec8, and corresponding isotype controls. Representative data from patient #18. 400x, Scale bar 10 $\mu$m.

Fig. 4. Eosinophil activation correlated with clinical findings and pulmonary function results. (A) Positive correlation between CD69 MFI ratio on eosinophils with numbers of infiltrating eosinophils to NPs of patients with ECRS ($P$ < 0.05, $r = 0.7273$, $n = 12$). (B) Positive correlation between CD69 MFI ratio on eosinophils with nasal polyp scores ($P$ < 0.05, $r = 0.624$, $n = 12$). (C) Positive correlation between CD69 MFI ratio on eosinophils with Lund–Mackay CT scale ($P$ < 0.05, $r = 0.6103$, $n = 12$). (D) Negative correlation between CD69 MFI ratio on eosinophils with FEV$_{1.0}$ and $\%$FEF$_{25-75}$ (reflecting asthma severity), but not with $\%$PEF (FEV$_{1.0}$: $P$ < 0.05, $r = -0.6783$, $n = 12$, $\%$FEF$_{25-75}$: $P$ < 0.05, $r = -0.5874$, $n = 12$). We expressed correlations as Spearman’s rank correlation coefficients. PB Eos: Eosinophils from peripheral blood. NP Eos: eosinophils from nasal polyp.
existence of a difference in cytokine expression in the tissues compared with the expressions in controls. These data suggest that eosinophils may be one of the sources of cytokines in ECRS patients as well as ILC. Moreover, we found a significant difference in mRNA levels of the eosinophil-derived cytotoxic granules (MBP, EDN, and EPX) between eosinophils in the PB and NP from patients with ECRS. Although the cytokine levels between these groups were similar, higher levels of cytotoxic granules indicate eosinophil activation, since eosinophil-specific granules are associated with airway remodeling during the allergic disorders. However, Miyata reported similar granule proteins (EDN, ECP, and EXP) contained in eosinophils from both NPs and PB. This discrepancy between mRNA and protein in eosinophils may result from type of eosinophils in each experiment. Eosinophils come in two different types (hypodense and normodense eosinophils), the former and later reflect released and contained rich granules, respectively. Since the ratio of hypodense eosinophils (reflecting activated eosinophils) exists dominantly during allergic inflammations, it is possible that the protein of granules between these eosinophils be similar. By contrast, our results, showing high mRNA expressions of these cytotoxic granules in the tissue eosinophils, suggests hypodense but not normodense eosinophils, reflecting activation.

Interestingly, we also found over 30-fold increase in CD69 expression on eosinophils between NPs and PB from ECRS. Similarly, we found upregulated CD69 protein expression. Thus, the degree of CD69 expression on eosinophils may be used as a biomarker for eosinophilic inflammation. Then, we also investigated the association between the CD69 MFI ratio on eosinophils and the severity of symptoms among patients with ECRS having asthma, and we found that expression of CD69 (as an activation marker) in eosinophils from NPs was positively correlated with the NP score and sinus CT findings, and negatively correlated with pulmonary function (as measured by FEV10% and %FEF25–75). By contrast, we could not find any correlation between the CD69 MFI ratio on T cells and these clinical findings. In ECRS, FEV10% is a noninvasive marker of airway remodeling during asthma, and %FEF25–75 is associated with bronchial hyperresponsiveness in asthma. Studies reported that the FEV10% and %FEF25–75, but not the %PEF, were lower in patients with ECRS than in controls. Therefore, CD69 expression on the eosinophils (but not in T cells isolated from the NP of patients with ECRS) is a potential biomarker for the severity of patients with ECRS.

Moreover, we found EPX release after cross-linking of CD69 onto mouse eosinophils, suggesting that the signaling contributes to development of remodeling during the eosinophilic airway inflammation. By contrast, Walsh et al. reported that CD69 signaling induced apoptosis, but not EPX release. The discrepancies between their and our results could be due to differing incubation times; they analyzed results after 48 h and we after 2 h. Hosokawa et al. showed that airflow inflammation and airflow hyperresponsiveness (AHR) were diminished in CD69 deficient mice, and systemic treatment with anti-CD69 antibody in an asthma mouse model improved symptoms compared with treatment in naïve animals and treatment with isotype control antibodies. These findings suggest that CD69 plays a role as a marker of activation, and that it regulates airway inflammation.

In conclusion, we found that activated eosinophils from the NPs of patients with ECRS expressed CD69, but not eosinophils from the PB of the same patients. In addition, CD69 expression on eosinophils was significantly correlated with the number of eosinophils infiltrating NPs, the NP score, sinus CT findings, and measurements of pulmonary function such as FEV10% and %FEF25–75. Moreover, we found that CD69-expressing eosinophils released EPX following activation (cross-linking to CD69). Thus, our results suggest that CD69 is a biomarker for the severity of nasal obstructions and for the degree of pulmonary dysfunction as well as a novel therapeutic target for patients with ECRS and asthma.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.alit.2019.11.002.
Conflict of interest
The authors have no conflict of interest to declare.

Authors’ contributions
Conceived and designed the experiments: YY, and AK. Performed the experiments: YY, VK, HO, DVB, MA, HI, SK, and AK. Analyzed the data: YY, HO, and AK. Wrote the paper: YY, and AK. All authors read and approved the final manuscript.

References