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Eosinophil-derived neurotoxin enhances airway remodeling in eosinophilic chronic rhinosinusitis and correlates with disease severity

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Abstract

Eosinophilic chronic rhinosinusitis (ECRS) is a subtype of chronic rhinosinusitis (CRS) that is characterized by intractable nasal polyp formation. Eosinophil-derived neurotoxin (EDN) is an eosinophil granule protein that is closely related to allergic inflammation, but the pathological implications of EDN in ECRS remain unknown. In this study, we evaluated the function of EDN in ECRS pathogenesis and assessed its potential as a disease activity marker. Serum EDN levels were significantly higher in patients with ECRS than in those with other nasal and paranasal diseases, and were positively correlated with clinical disease activity. Production of EDN from isolated human eosinophils was induced by stimulation with IL-5 *in vitro*. Human nasal epithelial cells were stimulated with EDN, and the resultant changes in gene expression were detected by RNA sequencing. Pathway analysis revealed that the major canonical pathway affected by EDN stimulation was 'regulation of the epithelial–mesenchymal transition pathway'; the only gene in this pathway to be up-regulated was matrix metalloproteinase 9 (MMP-9). Consistent with this, immunostaining analysis revealed intense staining of both EDN and MMP-9 in nasal polyps from patients with ECRS. In conclusion, our data demonstrate that serum EDN level is a useful marker for the evaluation of ECRS severity. Furthermore, EDN induces production of MMP-9 from the nasal epithelium, which may be involved in the pathogenesis of ECRS.

Keywords: ECRS, EDN, matrix metalloproteinase 9

Introduction

Chronic rhinosinusitis (CRS) is clinically classified into CRS with nasal polyps (CRSwNP) or without nasal polyps (CRSsNP). CRSwNP has a variable clinical course because several types of cells, including immune and epithelial cells, play key roles in its pathogenesis. Therefore, identifying the subtypes of CRSwNP based on the underlying molecular mechanisms, which classifies the disease into endotypes, is important for the individualization of therapy (1).

Because the phenotypes of CRSwNP are heterogeneous in East Asia, CRSwNP in this region is classified into eosinophilic chronic rhinosinusitis (ECRS) and non-eosinophilic chronic rhinosinusitis (NECRS). ECRS is based on T-helper

type 2–driven inflammation (T_h2 inflammation) and is associated with strong nasal mucosal eosinophilic infiltration. In ECRS, nasal polyps are treatment resistant and frequently recur. Recurrent nasal polyp formation may be closely related to eosinophil effector functions, but most of the pathological mechanisms have not been clarified.

The eosinophil is a type of granulocyte that contains eosinophilic granule proteins (EGPs). There are four EGPs: major basic protein (MBP), eosinophilic cationic protein (ECP), eosinophil peroxidase (EPO), and eosinophil-derived neurotoxin (EDN). All EGPs have antiparasitic and antibacterial activity. Because EDN promotes an allergic reaction via dendritic cell activation, its levels are closely related with T_h2 inflammation. Many studies have suggested that EDN plays an important role in allergic disease, but only few studies have shown the association between EDN and ECRS.

In this study, we sought to clarify the mechanism of intractable nasal polyposis in ECRS by estimating the effects of EDN on nasal epithelial cells. Furthermore, we investigated whether serum EDN level is a useful marker for the evaluation of disease severity by assessing the relationship between serum EDN levels and clinical features.

Methods

Patients and control group

We included 115 patients with nose and paranasal sinus disease treated between 2014 and 2017 at the Department of Otorhinolaryngology–Head and Neck Surgery, Osaka University Graduate School of Medicine. CRS was diagnosed based on patient symptoms according to the guidelines in the European Position Paper on Rhinosinusitis and Nasal Polyps (2). CRSwNP was classified into two subtypes, ECRS and NECRS, according to the Japanese criteria (3).

Blood samples were obtained from 34 patients with ECRS, 30 with NECRS, 31 with allergic rhinitis, 20 with other sinus diseases (choanal polyp, paranasal benign tumor, organized hematoma and postoperative sinus cyst) and 8 healthy controls. Clinical data and demographics are presented in Table 1. Patients receiving oral steroids were excluded from the study.

Tissue samples were obtained from patients undergoing endoscopic sinus surgery. Nasal polyps were collected from five patients with ECRS and five with NECRS. The study was approved by the ethics committee of Osaka University (14463), and informed consent was obtained from each patient prior to sample collection.

Nasal polyp score

Nasal polyps were evaluated in each patient by endoscopy. Polyps were graded into five stages as described by Meltzer *et al.* (4). Mean values were used as polyp scores.

Enzyme-linked immunosorbent assay and cytometric bead array

Serum interleukin 5 (IL-5) levels were measured by flow cytometric bead assay using the Cytometric Bead Array Human IL-5 Flex Set (BD Bioscience, Tokyo, Japan). Matrix metalloproteinase 9 (MMP-9) levels in serum and cell culture supernatants were measured with the Human MMP-9 Quantikine enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA), and EDN levels in serum and cell culture supernatants were measured with the EDN ELISA kit (MBL, Nagoya, Japan). Serum ECP levels were measured using the human eosinophil cationic protein ELISA kit (MyBioSource, San Diego, CA, USA). Serum EPO levels were measured using the human eosinophil peroxidase ELISA kit (MyBioSource).

Isolation of eosinophils

Human polymorphonuclear cells were separated from heparinized venous blood collected from healthy donors by using Ficoll-Plaque Plus (GE Healthcare Japan, Tokyo, Japan). Eosinophils were isolated from polymorphonuclear cells by negative selection using the Human Eosinophil Isolation kit (Miltenyi Biotec, Tokyo, Japan).

Table 1. Clinical data and demographics of patients and healthy controls

	ECRS	NECRS	AR	Others	Healthy control
No. of subjects	34	30	31	20	8
Age (years)	57.5 ± 13.5	59.4 ± 16.8	54.7 ± 18.1	60.7 ± 13.7	61.5 ± 7.0
Sex (male/female)	18/16	15/15	18/13	10/10	3/5
White blood cell count (μl^{-1})	6841 ± 1485	6866 ± 2141	6434 ± 1529	6462 ± 1729	–
Eosinophil count (μl^{-1})*	613.5 ± 346.1	220.8 ± 184.3	215.1 ± 234.0	177.3 ± 105.8	–

Characteristics of 34 patients with ECRS, 30 with NECRS, 31 with AR, 20 with other paranasal diseases and 8 healthy controls. AR, allergic rhinosinusitis.

*$P < 0.01$. Kruskal–Wallis test.

Eosinophil degranulation

Separated eosinophils were incubated with various cytokines for 4 h at 37°C, and then the supernatant was collected. The medium was RPMI 1640 supplemented with 10% fetal calf serum and 1% penicillin–streptomycin solution (Pen-Strep) (Thermo Fisher Scientific, Yokohama, Japan). Human recombinant IL-4, IL-5, IL-10 and IL-13 (Peprotech, Rocky Hill, NJ, USA) were used for stimulation, at a final concentration of 10, 100 or 1000 ng ml^{-1}.

EDN stimulation of human nasal epithelial cells

Primary human nasal epithelial cells (HNEpCs; PromoCell, Heidelberg, Germany) were cultured at 37°C, 5% CO_2, using an airway epithelial cell growth medium with supplements (PromoCell). After the monolayer reached to 90% confluence in 6-well plate, the cells were treated for 24 or 48 h with rEDN (2 μg ml^{-1}) (Abcam, Tokyo, Japan). Supernatants and cell pellets were collected and preserved at –80°C.

RNA extraction and reverse transcription

Total RNA from HNEpCs stimulated with EDN was extracted using QIAzol (Qiagen, Osaka, Japan) and the RNeasy Mini Kit (Qiagen). The collected RNA was evaluated on a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). Complementary DNA (cDNA) was synthesized using the PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara Bio, Kusatsu, Japan).

Reverse transcription–polymerase chain reaction

Using the TaqMan PCR protocol, quantitative reverse transcription–polymerase chain reaction (qRT–PCR) was performed on a 7900HT Fast Real-time PCR system (Thermo Fisher Scientific). Assay numbers of the TaqMan primers were as follows: tenascin C, Hs01115665; vimentin, Hs00958111; and fibronectin, Hs01549976. The mRNA level of each gene was normalized to that of ACTB (Hs01060665) using the calibration curve method.

RNA sequencing

Total RNA extracted from HNEpCs stimulated with EDN (2 μg ml^{-1}) for 24 h was evaluated by RNA sequencing. Library preparation was performed using the TruSeq stranded mRNA sample prep kit (Illumina, San Diego, CA, USA). Sequencing was performed on an Illumina HiSeq 2500 platform in 75-base single-end mode. The Illumina CASAVA 1.8.2 software was used for base calling. Sequenced reads were mapped to the human reference genome sequence (hg 19) using TopHat ver. 2.0.13 in combination with Bowtie2 ver. 2.2.3 and SAMtools ver. 0.1.19. Fragments per kilobase of exon per million mapped fragments (FPKMs) were calculated using Cuffnorm ver. 2.2.1. Ingenuity Pathway Analysis (IPA) was used to identify canonical pathways from differentially expressed genes. Access to data concerning this study can be found under GEO experiment accession number (GSE111634).

Immunohistochemical staining

After deparaffinizing paraffin-fixed tissue, antigen retrieval was performed by autoclaving for 15 min at 125°C in an ethylenediaminetetraacetate buffer solution (pH 9). Endogenous peroxidase activity was blocked with REAL Peroxidase Blocking Reagent (Dako, Carpinteria, CA, USA). Sections were reacted overnight at 4°C with EDN antibody (1:200 Novus Biologicals, Littleton, CO, USA) or anti-MMP-9 antibody (1:500 Abcam). After incubating with peroxidase-labeled polymer conjugated to secondary anti-rabbit immunoglobulins (Dako EnVision+ System-HRP Labelled Polymer, Dako), slides were developed using 3,3-diaminobenzidine as the chromogen. Rabbit IgG, polyclonal-Isotype Control (Abcam) was used as the primary antibody in the negative control.

Statistical analysis

All statistical analyses were performed with GraphPad Prism ver. 7 (GraphPad Software, San Diego, CA, USA). Data were expressed as means ± SD. The Shapiro–Wilk test was used to examine continuous variables. Comparisons between two groups were performed by nonparametric Mann–Whitney *U*-test or Student's *t*-test. Kruskal–Wallis tests were used to compare several groups. Correlations were expressed as the Spearman's rank correlation coefficient. A P value <0.05 was considered to indicate statistical significance.

Results

Correlation of serum EGP levels with clinical findings

To validate serum EGP levels as a biomarker for disease progression, we measured serum ECP, EPO and EDN levels in patients with nasal and paranasal diseases. We then evaluated the correlations between blood eosinophils (counts and percentages), polyp score, Japanese Epidemiological Survey of Refractory Eosinophilic Chronic Rhinosinusitis (JESREC) score, stage, serum IL-5 levels, serum MMP-9 levels and serum EGP levels in 34 patients with ECRS.

Serum EDN levels were significantly higher in patients with ECRS than those in patients with other sinus diseases or in healthy individuals ($P < 0.01$) (Fig. 1). Serum EDN levels were positively correlated with JESREC score, stage, polyp score and blood eosinophils (counts and percentages) (Fig. 2).

Serum ECP levels were also elevated in patients with ECRS ($P < 0.01$), but were not correlated with any clinical disease marker. Serum EPO levels were not elevated (Supplementary Figures 1 and 2). These data imply that EDN somehow contributes to the ECRS pathogenesis.

Effect of cytokines on eosinophil EDN degranulation

Previous reports suggested that IL-5 induces eosinophil activation and degranulation. Hence, we investigated whether IL-5 enhanced the production of EDN. In addition, we evaluated the capacity of other cytokines involved in T_h2 inflammation to induce EDN production.

After stimulation of human eosinophils with 1000 ng ml^{-1} of IL-5 or IL-13, the levels of EDN in the supernatant were significantly elevated. In particular, the concentrations of EDN from IL-5-treated eosinophils were significantly higher than those from cells stimulated with other cytokines ($P < 0.01$). Even at lower concentrations (10 ng ml^{-1} or 100 ng ml^{-1}), IL-5

could induce EDN degranulation (Supplementary Figure 3). EDN levels were positively correlated with IL-5 levels in patients with ECRS (r = 0.51, P < 0.01) (Fig. 3) and serum IL-5 levels were positively correlated with some markers of clinical disease activity (Supplementary Figure 4). These results show that IL-5 induces eosinophil activation and EDN degranulation.

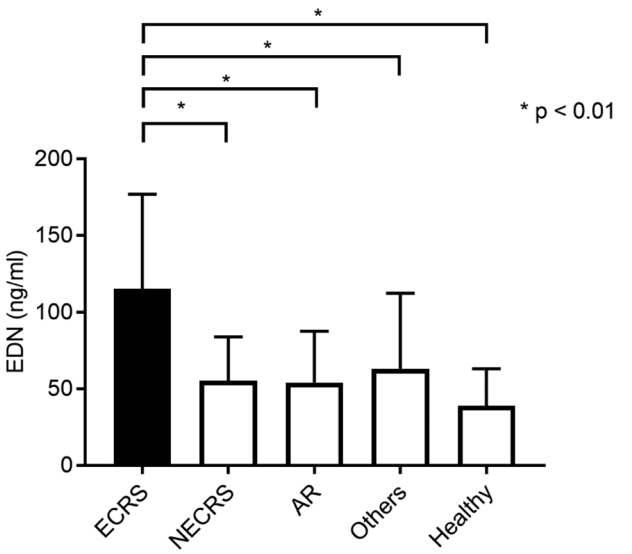


Fig. 1. Serum EDN levels are significantly higher in patients with ECRS than in patients with other sinus diseases or in healthy controls. Serum EDN levels were determined in 34 patients with ECRS, 30 with NECRS, 31 with allergic rhinosinusitis (AR), 20 with other paranasal diseases (choanal polyp, paranasal benign tumor, organized hematoma and postoperative sinus cyst) and 8 healthy controls. *P < 0.01, Kruskal–Wallis test.

EDN pathophysiology in nasal epithelial cells

To determine the pathological role of EDN in ECRS, we used RNA sequencing to analyze gene expression in HNEpCs stimulated with EDN. A total of 87 differentially expressed genes were detected (Supplementary Table S1). Pathway analysis revealed that the major canonical pathway affected by stimulation was 'regulation of the epithelial–mesenchymal transition (EMT) pathway'. Four genes were included in this pathway. *MMP9* was the only gene with a normalized FPKM value >1.0. Consistent with this, the MMP-9 protein concentration in the supernatant was significantly elevated in the EDN-stimulated group at 24 and 48 h (P < 0.05). Serum EDN levels were positively correlated with MMP-9 levels in patients with ECRS (r = 0.34, P < 0.05) (Fig. 4). After 24 or 48 h of EDN stimulation, expression of EMT marker mRNA was higher in HNEpCs stimulated with EDN than in control cells (P < 0.01) (Fig. 5).

Expression of EDN and MMP-9 in nasal polyps

To monitor the expression of EDN and MMP-9 proteins in nasal tissue, we conducted immunohistochemical analysis. In nasal polyp tissue from ECRS patients, tissue-infiltrated eosinophils stained intensely with anti-EDN antibody, while epithelial cells and inflammatory cells stained positively for MMP-9. EDN and MMP-9 were expressed in polyps from NECRS patients; however, the levels were lower than those in ECRS patients (Fig. 6 and Supplementary Figure 5).

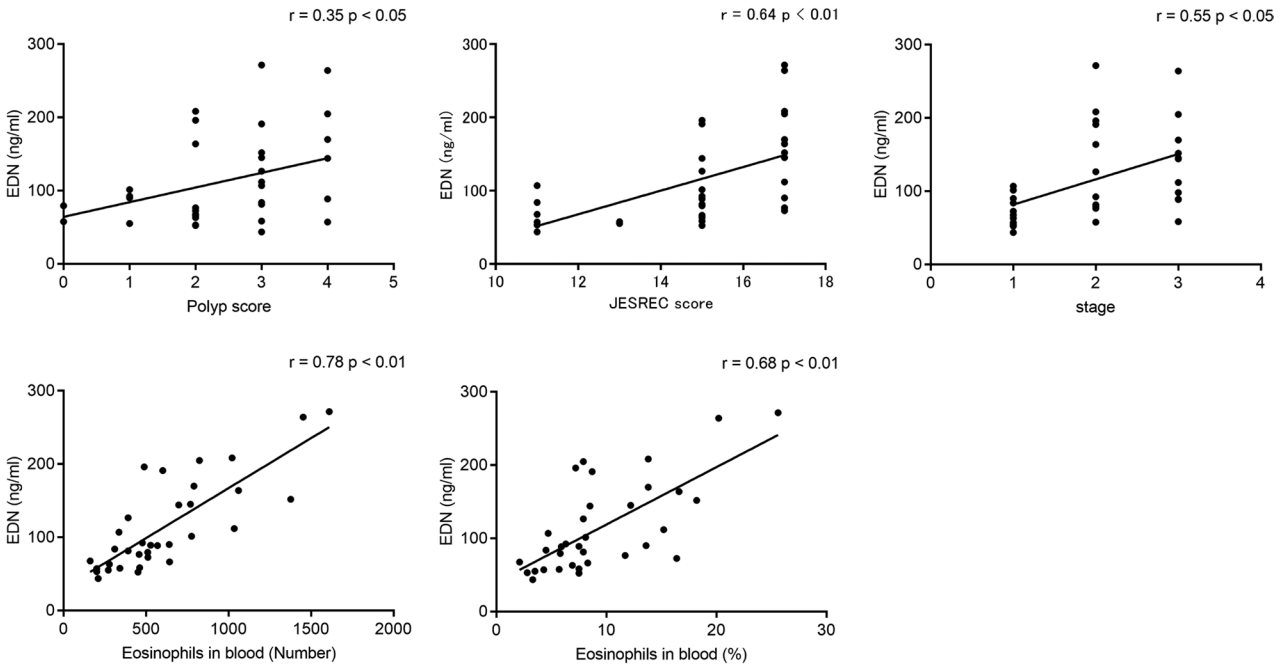


Fig. 2. Serum EDN levels are positively correlated with severity of clinical disease. Correlations of serum levels of EDN with ECRS disease activity. Positive correlations were observed between serum EDN levels and JESREC score, stage, polyp score and blood eosinophils (counts and percentages) in ECRS patients (n = 34). Correlations are expressed as Spearman's rank correlation coefficient.

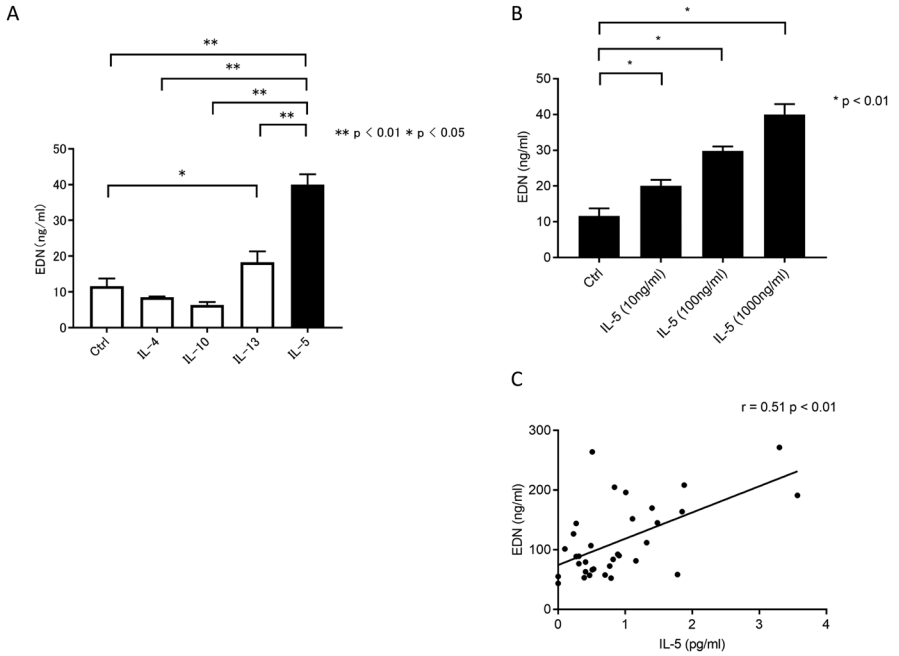
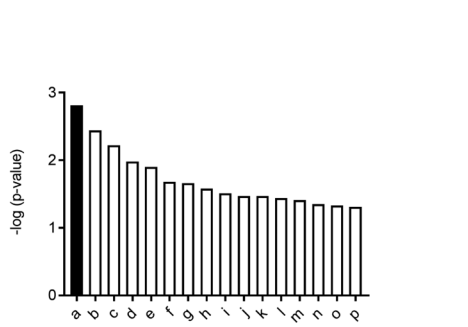


Fig. 3. IL-5 induces eosinophil degranulation. Correlations between serum EDN levels and serum IL-5 levels in patients with ECRS. (A) Isolated eosinophils were stimulated with recombinant IL-4, IL-5, IL-10 or IL-13 (1000 ng ml^{-1}). The EDN concentration was significantly higher in supernatant of cells stimulated with rIL-5 than in supernatants of cells stimulated with other interleukins (n = 3, technical triplicates). *P < 0.01, Kruskal–Wallis test. (B) Recombinant IL-5 stimulation was performed at concentrations of 10 and 100 ng ml^{-1}. EDN concentration increased in a dose-dependent manner (n = 3, technical triplicates). *P < 0.05, **P < 0.01, Student's t-test. (C) A positive correlation was observed between serum EDN levels and serum IL-5 levels in ECRS patients (n = 34, r = 0.51, P < 0.01). Correlation is expressed as Spearman's rank correlation coefficient.



a	Regulation of the Epithelial-Mesenchymal Transition Pathway
b	p38 MAPK Signaling
c	Aryl Hydrocarbon Receptor Signaling
d	Arsenate Detoxification I (Glutaredoxin)
e	Hepatic Fibrosis / Hepatic Stellate Cell Activation
f	Airway Pathology in Chronic Obstructive Pulmonary Disease
g	Bladder Cancer Signaling
h	Glucose and Glucose-1-phosphate Degradation
i	Hematopoiesis from Multipotent Stem Cells
j	Gαs Signaling
k	Guanosine Nucleotides Degradation III
l	Androgen Biosynthesis
m	Pancreatic Adenocarcinoma Signaling
n	Atherosclerosis Signaling
o	Differential Regulation of Cytokine Production in Macrophages and T Helper Cells by IL-17A and IL-17F
p	Histamine Degradation

(B)

Gene Symbol	Fold Change	P - Value
ID2	2.642	0.021
MMP9	2.03	0.002
TGFB3	-2.176	0.005
FGF1	-2.624	0.013

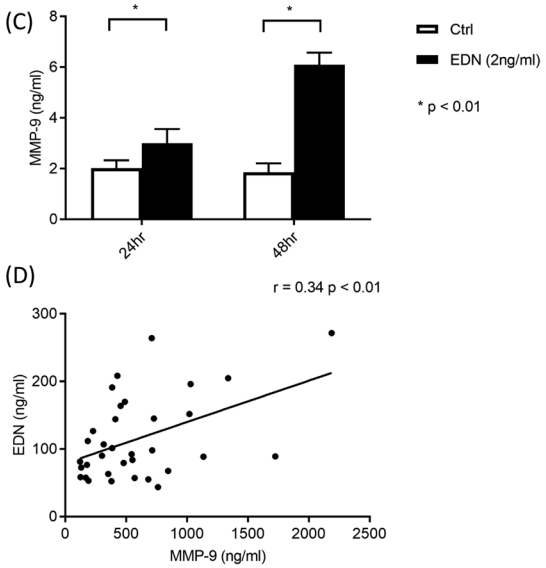


Fig. 4. EDN induces MMP-9 secretion from nasal epithelial cells. HNEpCs were stimulated with EDN, and gene expression was analyzed by RNA sequencing. (A) Pathway analysis of differentially expressed genes revealed that the major canonical pathway affected by treatment was 'regulation of the EMT pathway'. (B) Four genes were included in the 'regulation of the EMT pathway'. *MMP9* (encoding matrix metalloprotease 9) was the only gene with a normalized value of FPKMs >1.0. (C) MMP-9 levels in the supernatant were increased by EDN stimulation (n = 3, technical triplicate). *P < 0.01, Student's t-test. (D) A positive correlation was observed between serum EDN levels and serum MMP-9 levels in patients with ECRS (n = 34, r = 0.34, P < 0.05). Correlation is expressed as Spearman's rank correlation coefficient.

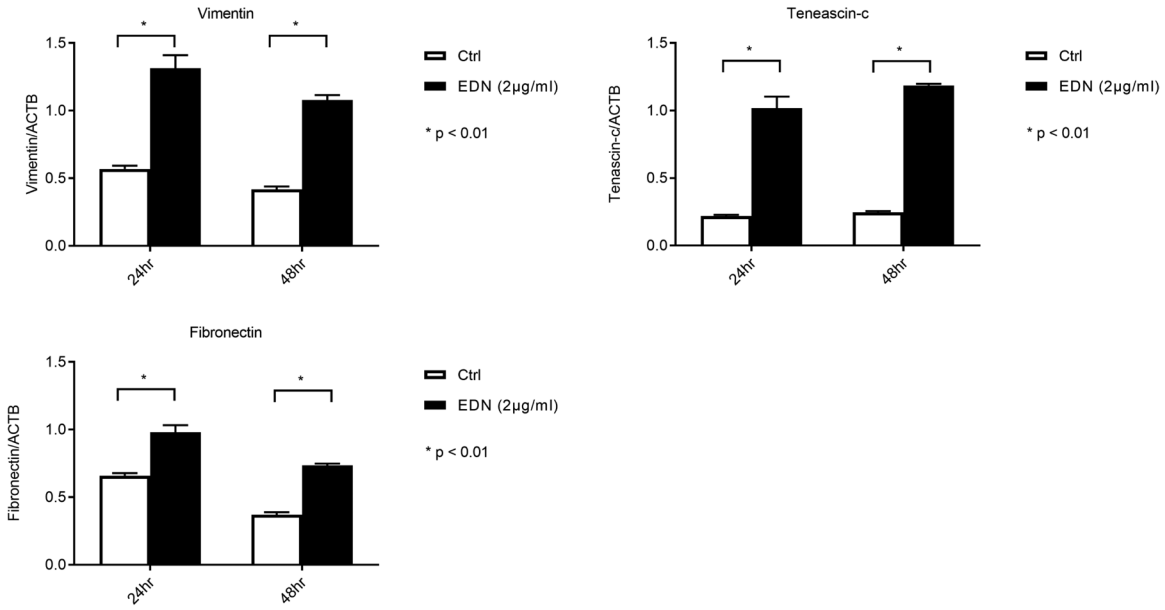


Fig. 5. EDN exacerbates airway remodeling. EDN stimulation increased expression of fibronectin, tenascin C and vimentin in nasal epithelial cells. Results are representative of three independent experiments. *$P < 0.01$, Student's *t*-test.

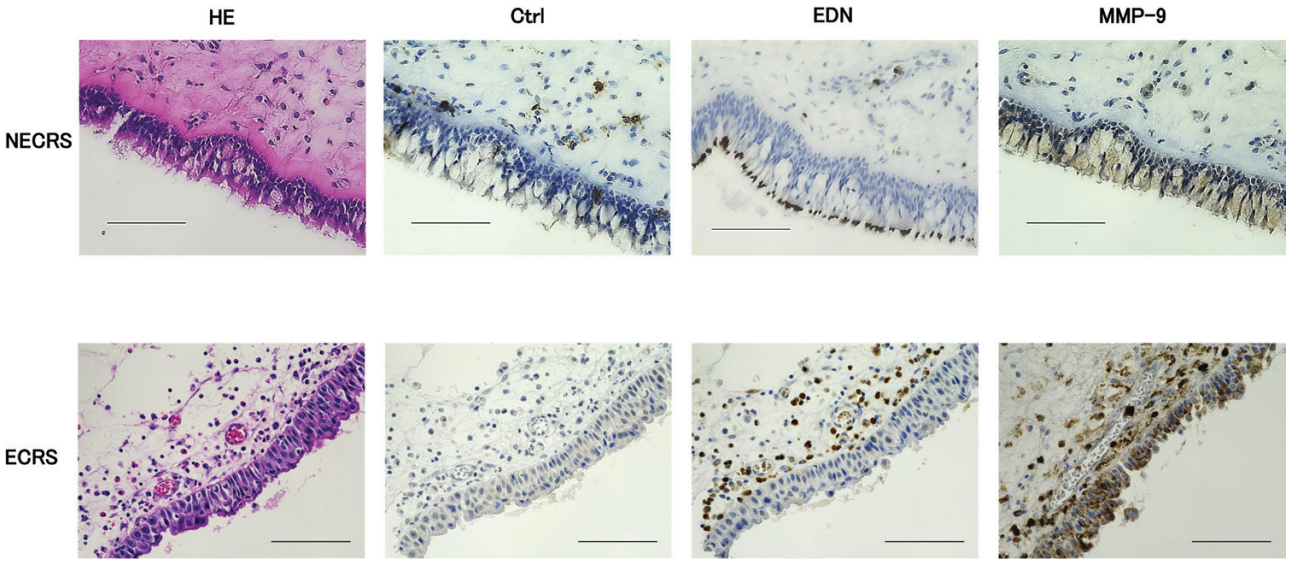


Fig. 6. EDN and MMP-9 are expressed in nasal polyps from ECRS patients. Hematoxylin/eosin and immunohistochemical staining for EDN and MMP-9. Scale bars, 100 um. Images are representative of samples from seven ECRS patients and seven NECRS patients. In nasal polyps from patients with ECRS, EDN expression was observed in eosinophils, and MMP-9 expression was observed in epithelial cells and inflammatory cells.

Discussion

In this study, we examined the clinical implications of EDN in ECRS. The ECRS concept was originally proposed based on the diversity of CRSwNP in East Asia. ECRS is similar to CRSwNP in Europe, although there are slight differences (5). ECRS is characterized by formation of intractable nasal polyps with a thick basal membrane and significant infiltration of eosinophils, similar to the phenotypes of lower airway remodeling in asthma (6, 7). Although eosinophils are closely associated with remodeling, many aspects of the underlying mechanism remain unclear. We revealed the function of EDN, a type of EGP, in this mechanism. EGPs react against foreign antigens and promote inflammation, thereby causing significant damage to surrounding structures (8–11). EDN, which belongs to the ribonuclease A superfamily, is less cytotoxic than other EGPs, but it was originally identified as a factor that causes neurotoxic damage (12).

First, we considered whether serum EGP levels are useful for evaluating the severity of ECRS. Many reports have suggested that serum EDN levels are useful for evaluating

eosinophilic inflammation such as atopic dermatitis, asthma and eosinophilic esophagitis (13–19). However, no reports have assessed the usefulness of EDN levels for determining ECRS disease activity. We found that serum EDN levels were significantly higher in patients with ECRS than in other groups. Moreover, serum EDN levels were positively correlated with clinical disease activity in ECRS patients. Some studies suggested that other EGPs may be relevant ECRS (20); hence, we evaluated serum levels of ECP and EPO in our patients. Serum ECP levels were elevated in ECRS patients, but were not correlated with any clinical disease activity markers, and serum EPO levels were not elevated in ECRS patients. These findings indicate that measurements of serum EDN levels are useful for diagnosing ECRS and assessing its severity.

Next, to identify the EDN degranulation factor, we stimulated eosinophils with various cytokines. Previous reports showed that IL-5 induced EDN degranulation, but other cytokines were not validated (21–23). In this study, we showed that IL-5 and IL-13 induced EDN degranulation from eosinophils; IL-5 induced degranulation of EDN to a greater extent than did other cytokines. Serum IL-5 levels were positively correlated with serum EDN levels in ECRS patients. Some clinical disease activity markers were correlated with serum IL-5 levels, whereas others were not.

Finally, we considered the role of EDN in the pathogenesis of ECRS. EDN increases T_h2 inflammation through dendritic cells, and it is also closely associated with exacerbation of allergic disease (24–26). However, no reports have described the function of EDN in the nasal epithelium or its role in nasal polyp remodeling. RNA sequencing of nasal epithelial cells following EDN stimulation revealed that the treatment affected the EMT pathway and that MMP-9 production from nasal epithelial cells was significantly increased by EDN stimulation. MMP-9 affects epithelial regeneration and can break down the extracellular matrix, and is thus closely associated with tissue remodeling in asthma and CRS (7, 27–30). In this study, we observed that the levels of multiple EMT markers were elevated in nasal epithelial cells following EDN stimulation.

These data imply that EDN degranulation from eosinophils activated by IL-5 promotes secretion of MMP-9 from the nasal epithelium. This mechanism ultimately may cause nasal remodeling followed by intractable nasal polyposis in ECRS.

This study had some limitations: the patients' backgrounds differed between disease groups and the sample sizes were small. However, the study has two novel features: we investigated the usefulness of serum EDN levels for the diagnosis of ECRS, and performed RNA sequencing to examine the role of EDN in recurrent nasal polyposis. EDN induces the formation of intractable nasal polyps, implying that blocking EDN could promote recovery from the disease. To date, however, no studies have examined EDN as a therapeutic target. Therefore, studies with larger sample sizes, as well as clinical tests of EDN-blocking therapies, should be pursued in the future.

In conclusion, our results suggest that serum EDN levels are helpful for diagnosis of ECRS as other allergic diseases, and may be useful for predicting the severity of ECRS. EDN induces MMP-9 production from the nasal epithelium, resulting in epithelial remodeling, which may be involved in formation of intractable nasal polyps. It seems likely that inhibiting production of MMP-9 by neutralizing EDN therapy would be useful for ECRS management. Further studies of EDN-blocking therapy are required.

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A.K. designed the study and supervised the project. T.T. analyzed the data and drafted the manuscript. Y.M., M.N., S.K., H.I. and A.K. assisted in analyzing the data and supervised preparation of the draft. D.O. conducted some experiments and analyzed the data. Y.H., S.N., H.T., Y. Kinehara, Y. Kato, T.N., K.T., M.H., S.O., H.A. and T.S. collected clinical specimens and provided advice on data interpretation. All authors participated in discussion of the results and critically polished and approved the final draft.

Conflicts of interest statement: The authors declared no conflicts of interest.

References

1 Akdis, C. A., Bachert, C., Cingi, C. *et al.* 2013. Endotypes and phenotypes of chronic rhinosinusitis: a PRACTALL document of the European Academy of Allergy and Clinical Immunology and the American Academy of Allergy, Asthma & Immunology. *J. Allergy Clin. Immunol.* 131:1479.
2 Fokkens, W. J., Lund, V. J., Mullol, J. *et al.*; EPOS. 2012. European position paper on rhinosinusitis and nasal polyps 2012. A summary for otorhinolaryngologists. *Rhinology* 50:1.
3 Tokunaga, T., Sakashita, M., Haruna, T. *et al.* 2015. Novel scoring system and algorithm for classifying chronic rhinosinusitis: the JESREC Study. *Allergy* 70:995.
4 Meltzer, E. O., Hamilos, D. L., Hadley, J. A. *et al.*; Rhinosinusitis Initiative. 2006. Rhinosinusitis: developing guidance for clinical trials. *J. Allergy Clin. Immunol.* 118(Suppl. 5):S17.
5 Ishitoya, J., Sakuma, Y. and Tsukuda, M. 2010. Eosinophilic chronic rhinosinusitis in Japan. *Allergol. Int.* 59:239.
6 Ponikau, J. U., Sherris, D. A., Kephart, G. M, Kern, E. B, Gaffey, T. A, Tarara, J. E. and Kita, H. 2003. Features of airway remodeling and eosinophilic inflammation in chronic rhinosinusitis: is the histopathology similar to asthma? *J Allergy Clin. Immunol.* 112:877.
7 Pawankar, R. and Nonaka, M. 2007. Inflammatory mechanisms and remodeling in chronic rhinosinusitis and nasal polyps. *Curr. Allergy Asthma Rep.* 7:202.
8 Spencer, L. A., Szela, C. T., Perez, S. A. *et al.* 2009. Human eosinophils constitutively express multiple T_h1, T_h2, and immunoregulatory cytokines that are secreted rapidly and differentially. *J. Leukoc. Biol.* 85:117.
9 Munitz, A. and Levi-Schaffer, F. 2004. Eosinophils: 'new' roles for 'old' cells. *Allergy* 59:268.
10 Domachowske, J. B., Dyer, K. D., Bonville, C. A. and Rosenberg, H. F. 1998. Recombinant human eosinophil-derived neurotoxin/RNase 2 functions as an effective antiviral agent against respiratory syncytial virus. *J. Infect. Dis.* 177:1458.
11 Pégorier, S., Wagner, L. A., Gleich, G. J. and Pretolani, M. 2006. Eosinophil-derived cationic proteins activate the synthesis of remodeling factors by airway epithelial cells. *J. Immunol.* 177:4861.
12 Gleich, G. J., Loegering, D. A., Bell, M. P., Checkel, J. L., Ackerman, S. J. and McKean, D. J. 1986. Biochemical and functional similarities between human eosinophil-derived neurotoxin

and eosinophil cationic protein: homology with ribonuclease. *Proc. Natl Acad. Sci. USA* 83:3146.
13 Gon, Y., Ito, R., Hattori, T. *et al*. 2015. Serum eosinophil-derived neurotoxin: correlation with persistent airflow limitation in adults with house-dust mite allergic asthma. *Allergy Asthma Proc*. 36:e113.
14 Taniuchi, S., Chihara, J., Kojima, T., Yamamoto, A., Sasai, M. and Kobayash, Y. 2001. Serum eosinophil derived neurotoxin may reflect more strongly disease severity in childhood atopic dermatitis than eosinophil cationic protein. *J. Dermatol. Sci*. 26:79.
15 Konikoff, M. R., Blanchard, C., Kirby, C. *et al*. 2006. Potential of blood eosinophils, eosinophil-derived neurotoxin, and eotaxin-3 as biomarkers of eosinophilic esophagitis. *Clin. Gastroenterol. Hepatol*. 4:1328.
16 Kim, C.-K., Callaway, Z., Park, J.-S. and Kwon, E. 2017. Utility of serum eosinophil-derived neurotoxin (EDN) measurement by ELISA in young children with asthma. *Allergol. Int*. 66:70.
17 Morioka, J., Tomita, M., Yoshizawa, Y., Inamura, H. and Kurosawa, M. 2004. Concentrations of eosinophil-derived neurotoxin in the blood and urine of patients with allergic diseases. *Allergol. Int*. 53:359.
18 Kim, K. W., Lee, K. E., Kim, E. S., Song, T. W., Sohn, M. H. and Kim, K.-E. 2007. Serum eosinophil-derived neurotoxin (EDN) in diagnosis and evaluation of severity and bronchial hyperresponsiveness in childhood asthma. *Lung* 185:97.
19 Kim, H. S., Kim, J. H., Seo, Y. M. *et al*. 2017. Eosinophil-derived neurotoxin as a biomarker for disease severity and relapse in recalcitrant atopic dermatitis. *Ann. Allergy Asthma Immunol*. 119:441.
20 Thompson, C. F., Price, C. P., Huang, J. H. *et al*. 2016. A pilot study of symptom profiles from a polyp vs an eosinophilic-based classification of chronic rhinosinusitis. *Int. Forum Allergy Rhinol*. 6:500.
21 Ohta, K., Nagase, H., Suzukawa, M. and Ohta, S. 2017. Antibody therapy for the management of severe asthma with eosinophilic inflammation. *Int. Immunol*. 29:337.
22 Fujisawa, T., Abu-Ghazaleh, R., Kita, H., Sanderson, C. J. and Gleich, G. J. 1990. Regulatory effect of cytokines on eosinophil degranulation. *J. Immunol*. 144:642.
23 Kita, H., Weiler, D. A., Abu-Ghazaleh, R., Sanderson, C. J. and Gleich, G. J. 1992. Release of granule proteins from eosinophils cultured with IL-5. *J. Immunol*. 149:629.
24 Yang, D., Chen, Q., Rosenberg, H. F. *et al*. 2004. Human ribonuclease A superfamily members, eosinophil-derived neurotoxin and pancreatic ribonuclease, induce dendritic cell maturation and activation. *J. Immunol*. 173:6134.
25 Yang, D., Rosenberg, H. F., Chen, Q., Dyer, K. D., Kurosaka, K. and Oppenheim, J. J. 2003. Eosinophil-derived neurotoxin (EDN), an antimicrobial protein with chemotactic activities for dendritic cells. *Blood* 102:3396.
26 Yang, D., Chen, Q., Su, S. B. *et al*. 2008. Eosinophil-derived neurotoxin acts as an alarmin to activate the TLR2-MyD88 signal pathway in dendritic cells and enhances T_h2 immune responses. *J. Exp. Med*. 205:79.
27 Mohan, R., Chintala, S. K., Jung, J. C. *et al*. 2002. Matrix metalloproteinase gelatinase B (MMP-9) coordinates and effects epithelial regeneration. *J. Biol. Chem*. 277:2065.
28 Han, Z., Junxu, and Zhong, N. 2003. Expression of matrix metalloproteinases MMP-9 within the airways in asthma. *Respir. Med*. 97:563.
29 Chen, Y. S., Langhammer, T., Westhofen, M. and Lorenzen, J. 2007. Relationship between matrix metalloproteinases MMP-2, MMP-9, tissue inhibitor of matrix metalloproteinases-1 and IL-5, IL-8 in nasal polyps. *Allergy* 62:66.
30 Watelet, J. B., Bachert, C., Claeys, C. and Van Cauwenberge, P. 2004. Matrix metalloproteinases MMP-7, MMP-9 and their tissue inhibitor TIMP-1: expression in chronic sinusitis vs nasal polyposis. *Allergy* 59:54.