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Increased exhaled nitric oxide and its oxidation metabolism in eosinophilic chronic rhinosinusitis

Sachio Takeno *, Takayuki Taruya, Tsutomu Ueda, Noriaki Noda, Katsuhiro Hirakawa

Department of Otolaryngology, Head and Neck Surgery, Division of Clinical Medical Science, Programs for Applied Biomedicine, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

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ABSTRACT

Objective: Monitoring of fractional concentrations of exhaled nitric oxide (FeNO) has become a reliable marker of inflammation in human nose and paranasal sinuses. However, it is still unknown to what extent nasal NO levels contribute to the pathology of chronic rhinosinusitis (CRS). In the present study, we aimed to examine FeNO levels and the underlying mechanism of NO production and metabolism in patients with eosinophilic chronic rhinosinusitis (ECRS) and non-ECRS.

Methods: Thirty-three untreated ECRS patients, 16 non-ECRS patients, and 38 normal subjects were enrolled in this cross-sectional study of FeNO levels. Oral and nasal FeNO levels were measured before treatment using an electrochemical NO analyzer (NObreath®) with a nose adaptor. The mRNA expression of three nitric oxide synthase (NOS) isoforms, interleukin-5 (IL-5), and transforming growth factor-beta (TGF-β) in the ethmoid sinus mucosa and nasal polyps were analyzed by real-time PCR. Immunohistological localization of inducible NOS (iNOS) and nitrotyrosine (NT), a marker for oxidized NO metabolites, was also examined.

Results: ECRS patients showed significantly higher oral FeNO levels compared to non-ECRS patients and normal subjects (mean values, 47.6, 13.5, and 15.3 ppb, respectively). Nasal FeNO levels of the non-ECRS patients (30.5 ppb) were significantly lower than those of the ECRS patients (53.9 ppb) and normal subjects (45.5 ppb). Positive correlations existed between the blood eosinophil percentage and FeNO levels in ECRS patients. Histologically, ECRS patients showed higher eosinophil accumulation in the ethmoid mucosa than non-ECRS patients (103.1 vs. 16.3 cells/HPF). Real-time PCR analysis showed significant upregulation of iNOS and IL-5 mRNA expression in the ethmoid mucosa of the ECRS patients compared to those of non-ECRS patients. Positive iNOS immunoreactivity was observed in ciliated epithelial cells, submucosal glands and associated inflammatory cells in both groups. NT immunoreactivity was detected in the epithelium and around inflammatory cells. Intense NT staining was co-localized with eosinophil accumulation and ECRS patients showed significantly higher rates of NT-positive cells than non-ECRS patients.

Conclusion: A combination of oral and nasal FeNO measurement is a valid marker for the classification and definition of different CRS subtypes in Japan. Higher levels of oral and nasal FeNO in ECRS patients may reflect the persistence of eosinophilic inflammation in sinus mucosa with concomitant iNOS upregulation and accompanying deposition of oxidized NO metabolites.

1. Introduction

Nitric oxide (NO) has been proposed to have a variety of roles in the human nose and paranasal sinuses relevant to airway defense mechanisms, as well as being an inflammatory mediator [1,2]. The standardization of measuring techniques by the American Thoracic Society/European Respiratory Society has opened the way for the collection of comparable airway NO data in normal subjects and those with disease states [3,4]. Because the human paranasal sinuses are a major source of intrinsic NO production, the monitoring of fractional concentrations of exhaled NO (FeNO) in nasal airways can be a reliable marker of sinus inflammation [5–7]. Nasal NO levels are reported to be decreased in most patients with chronic rhinosinusitis (CRS); however, some contradictions still remain in the findings pertinent to classification of CRS types. In addition, it is still unclear to what extent nasal NO levels contribute to CRS pathology [8–10].

* Corresponding author at: Department of Otolaryngology, Hiroshima University School of Medicine, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan. Tel.: +81 82 257 5252; fax: +81 82 257 5254.
E-mail address: takeno@hiroshima-u.ac.jp (S. Takeno).

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In the present study, we assessed the oral and nasal FeNO levels in a population of normal subjects, patients with eosinophilic chronic rhinosinusitis (ECRS), and non-eosinophilic CRS (non-ECRS) patients. In Japan, ECRS has been proposed as a subtype of CRS with an intractable clinical course accompanied by the infiltration of numerous activated eosinophils into the paranasal sinus mucosa [11–13]. Eosinophil infiltration is commonly accompanied with histological abnormalities such as fibrosis, thickening of the basement membrane, and epithelial detachment. While the clinical characteristics of ECRS are apparently different from those of non-ECRS, a clear definition that can be used to differentiate each subtype has yet to be established.

We also compared NO production and metabolism pathways of paranasal sinus mucosa between ECRS and non-ECRS patients. The mRNA expressions of three nitric oxide synthase (NOS) isoforms, interleukin-5 (IL-5), and transforming growth factor-beta (TGF-β) were quantitatively analyzed by real-time PCR. The localization of inducible NOS (iNOS) and nitrotyrosine (NT), a marker for oxidized NO metabolites, was immunohistologically examined. There is so far limited information available for FeNO levels in CRS patients in Japan. We found that measurement of oral and nasal FeNO levels was useful in differentiating ECRS from non-ECRS based on the distinctly augmented NO metabolism that underlies ECRS.

2. Patients and methods

2.1. Nitric oxide measurements

Thirty-three ECRS and 16 non-ECRS patients were included in the cross-sectional study of FeNO measurements. Thirty-eight age-matched normal volunteers served as controls. The diagnosis of ECRS was based on clinical symptoms, endoscopic findings, and CT scanning, in accordance with preliminary criteria proposed at the clinical symposium of the 35th Annual Meeting of the Japan Rhinologic Society in 2006 [12]. All patients showed multiple nasal polyps bilaterally, characteristic mucus secretion with high viscosity, and dominant opacification of the ethmoid sinus by CT scanning. Three patients in the non-ECRS group showed a solitary nasal polyp unilaterally without clinical features compatible with ECRS. None of the patients had received topical or systemic steroids for at least 4 weeks before the visit. Patients who had undergone previous sinus surgery were excluded. The CT images were subjected to radiological grading using the Lund-Mackay system [14]. Total sinus scores were calculated bilaterally (range, 0–24). In addition, the E/M ratio (ratio of the averaged ethmoid cells to the maxillary sinus scores) and the PE/AE ratio (ratio of the posterior ethmoid to the anterior ethmoid scores) were calculated as described elsewhere [15].

Oral and nasal FeNO levels were measured before treatment using a handheld electrochemical analyzer (NObreath®, Bedfont Scientific Ltd., Rochester, UK) according to ATS/ERS guidelines [3,16]. For oral FeNO measurements, subjects were advised to exhale for 16 s at a flow rate of 50 mL/s through a mouthpiece. For nasal FeNO measurements, subjects were instructed to exhale transnasally with their mouth closed into a nose adaptor as described elsewhere [17]. Each measurement was performed in triplicate, and the mean value was used for analysis.

2.2. RT-PCR analysis

Ethmoid sinus and nasal polyp specimens were obtained from 18 ECRS and 14 non-ECRS patients who chose surgical therapy and underwent endoscopic sinus surgery. At the time of the surgery, the specimens were divided and either immersed in RNA later™ solution (Ambion, Austin, TX) for real-time RT-PCR, or, alternatively, fixed in 4% paraformaldehyde for immunohistochemistry. Quantitative PCR analysis was performed on the ABI Prisms 7300 system (Applied Biosystems, Foster City, CA, USA). Cellular RNA was isolated using RNeasy mini kits (Qiagen, Valencia, CA). Total RNA was then reverse-transcribed to cDNA using a High Capacity RNA-to-cDNA kit (Applied Biosystems) according to the instructions supplied by the manufacturer. Gene expression was measured on a real-time PCR system using TaqMan Gene Expression Assays.

PCR primers specific for neuronal NOS (nNOS or NOS1; Hs00167223_m1), iNOS (NOS2; Hs01075529_m1), endothelial NOS (eNOS or NOS3; Hs01574659_m1), IL-5 (Hs00174200_m1), and TGF-β, (Hs99999918_m1) were used. Primers for GAPDH (Hs99999905_m1) were used as a reference. PCR cycles were run in triplicate for each sample. Amplifications of the PCR products were quantified by the number of cycles and the results were analyzed using the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta}$Ct). The Ct values for target genes were normalized to the value of GAPDH by calculating the change in Ct ($^{\Delta}$Ct). Ct values of 34 or higher were considered as the lowest limit of detection. The quantities of target gene expression were presented as relative rates compared with the expression of the reference gene GAPDH (ratio: target gene/GAPDH expression).

2.3. Immunohistochemistry

Primary antibodies used were anti-human iNOS mouse monoclonal antibody (clone 2D2-B2; R&D Systems, Minneapolis, MN) and anti-nitrotyrosine mouse monoclonal antibody (clone 39B6; Santa Cruz, CA). Immunostaining was carried out on 5-μm-thick cryostat sections using mucosal specimens from the same patients as described in RT-RCR. For antigen retrieval, sections were immersed in Histo VT One (Nacalai Tesque, Kyoto, Japan) at 70 °C for 40 min. The slides were then incubated overnight at 4 °C with the primary antibodies. Color development was performed using the streptavidin-biotin amplification technique (ChemMate EnVision kit; Dako, Glostrup, Denmark). Peroxidase activity was visualized by the diaminobenzidine solution. Sections were counterstained with Mayer's hematoxylin. Control specimens developed without the primary antibody were used to verify that nonspecific binding was not detectable. Consecutive sections were stained with hematoxylin-eosin (HE) in order to view the mucosal pathology and to assess the degree of eosinophil infiltration.

We carried out semi-quantitative analysis to compare the immunohistological distribution of NT-positive cells in the submucosal layer between the groups. Cell counts were made in five fields at a magnification of 400 from randomly selected sections blind to the clinical diagnosis. The study protocol was approved by the Institutional Review Board at the Hiroshima University School of Medicine and written informed consent was obtained from all patients..

2.4. Data analysis

For multiple comparisons, screening of data for differences was first carried out using ANOVA. If the analysis gave a significant result, further comparison was done by the Mann–Whitney *U* test for between-group analysis. Correlation coefficients were calculated by the Spearman method. P values < 0.05 were considered to indicate statistical significance.

3. Results

3.1. Comparison of FeNO levels between non-ECRS and ECRS patients

The clinical characteristics of the study population are summarized in Table 1. A significant difference between the non-ECRS and the ECRS groups was found in the baseline data of