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Staphylococcus aureus enterotoxin B-induced endoplasmic reticulum stress response is associated with chronic rhinosinusitis with nasal polyposis

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ABSTRACT

Objective: *Staphylococcus aureus* enterotoxin B (SEB) might participate in the pathogenesis of chronic rhinosinusitis with nasal polyps (CRSwNP). However, the exact mechanism of polyp formation in CRSwNP remains unclear. Since the endoplasmic reticulum (ER) stress response is closely associated with chronic inflammation, we investigated the association between ER stress and SEB in the pathogenesis of CRSwNP.

Design and methods: Twenty-three CRSwNP patients with eosinophilic polyps (EP) or non-eosinophilic polyps (NEP) and 10 healthy subjects who were undergoing septoplasty were enrolled in this study. ER stress response was investigated using immunohistochemical staining and Western blotting.

Results: We show in this study that there are significantly more SEB-positive cells and higher production of reactive oxygen species (ROS) in the epithelial layer of EP than NEP or control tissue. Both SEB and protein A were detected strongly in tissues from patients with CRSwNP. We observed SEB induced the ER stress response in RPMI 2650 cells. GRP78 elevation by SEB was reduced by ROS scavenger pretreatment. In addition, the induction of GRP78 and p47 phox was increased significantly in EP compared with NEP or control mucosa.

Conclusions: SEB may induce ER stress via ROS production in CRSwNP. Therefore, we suggest that SEB-induced ER stress may play important roles in the pathogenesis of nasal polyposis.

Introduction

Chronic rhinosinusitis (CRS) is a very complex inflammatory disease of the nasal and paranasal sinuses, which is often associated with asthma [1,2]. Clinically, CRS can be classified into CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP) [3]. CRSwNP is a multifactorial disease. Patients with CRSwNP are associated with bacterial infections or fungal (molds) infection [4,5]. Although numerous host and environmental factors are considered predisposing factors [6], the exact mechanism of polyp formation in CRSwNP is not fully understood.

Some research has implicated bacterial colonization and bacterial products such as *Staphylococcus aureus* exotoxins (SEs) in the pathogenesis of CRS with or without NP [7–10]. Staphylococcal exotoxins A (SEA), SEC1-C3, SED, and *Staphylococcus aureus* enterotoxin B (SEB), activate T cells with a subsequent massive inflammatory response (they function as superantigens), thereby contributing to the etiology of CRSwNP [1,11]. Although SEs increase the production of IL-8 in human nasal epithelial cells [12], little is known about their effect on nasal epithelial cells in the pathogenesis of nasal polyposis. SEB was known to be involved in the pathogenesis of nasal polyposis [13]. Membrane vesicles (MVs) from *S. aureus* can deliver toxins to host cells and contribute to bacterial pathogenesis [14]. We therefore examine the relationship between SEB and nasal polyposis. However, little is known about the roles of staphylococcal components underlying the pathogenesis of nasal polyposis.

Recent studies have indicated that epithelial cells from patients with CRSwNP play important roles triggering mucosal inflammation and oxidative stress [15,16], and secrete numerous proinflammatory cytokines and mediators [17]. Major inflammatory and stress signaling networks are linked to the endoplasmic reticulum (ER) pathway [18,19]. The ER stress response can be induced by perturbations of ER homeostasis, such as increased protein synthesis, the accumulation of misfolded proteins, or impaired ER redox status [20]. ER stress plays a

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role in inflammation in obesity, insulin resistance, type 2 diabetes, and many other chronic metabolic disorders [21]. Reactive oxygen species (ROS) generated by inflammation can induce ER stress [22] and also are involved in the development of nasal polyposis [23,24]. Therefore, we examined the relationship between ER stress and SEB in the pathogenesis of CRSwNP.

Materials and methods

Ethics

An informed consent was obtained from each patient and control subject before collecting the study material. The study was approved by the Institutional Review Board of the Chungnam National University Hospital (IRB's approval number: 1105-88).

Reagents and cell culture

Staphylococcus aureus enterotoxin B (SEB) was obtained from List Biological Laboratories INC. (Campbell, CA, USA) and N-acetyl-L-cysteine (NAC) was purchased from Calbiochem (San Diego, CA, USA). The human nasal epithelial cell line RPMI 2650 (KCLB 10030, Korea) were maintained in Dulbecco's modified Eagle's medium (Lonza, Walkersville, MD, USA) supplemented with 10% FBS, penicillin (100 IU/mL), streptomycin (100 μg/mL) and 1% L-glutamine. The cells were cultured at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in 75-cm^2 culture flasks. Results were obtained from RPMI 2650 at passage number 27. After reaching confluence, cells were plated in 12-well dishes at a concentration of 1×10^5 cells per well.

Patients

Twenty-three CRSwNP patients and 10 control subjects who were undergoing septoplasty were enrolled in this study. Inferior turbinate mucosa from control subjects and nasal polyps from the CRSwNP patients were collected for immunofluorescence staining and ROS detection. Inferior turbinate samples from patients without sinus disease undergoing septoplasty or rhinoseptoplasty were collected as controls (controls: $n = 10$; median age 27 years; range 18–45 years, four females, six males), none of whom had a history of allergy, asthma, or aspirin sensitivity. Patients who used oral or nasal corticosteroids or other medications (e.g., antibiotics or antileukotrienes) for 4 weeks before sample collection were excluded from the study. We also excluded patients with a recent upper respiratory tract infection, and patients undergoing revision surgery. This enrollment of subjects was possible because all subjects in experimental groups (EP and NEP) were patients with CRSwNP. CRSwNP are relatively stable disease except acute exacerbation periods. Samples from patients with CRSwNP [$n = 23$; median age 45 years; range 34–78 years; 10 non-eosinophilic nasal polyps (NEPs), 13 eosinophilic nasal polyps (EPs)] were obtained during functional endoscopic sinus surgery. The nasal polyps of CRSwNP patients were sub-classified as either EPs or NEPs according to the result of H&E stain. The polyp was defined as an EP if the proportion of eosinophils exceeded 10% of the total infiltrating cells in the tissue. Otherwise, the polyp was defined as a NEP. A total of 13 patients with EPs, and 10 patients with NEPs were recruited. The diagnosis of sinus disease was based on history, clinical examination, nasal endoscopy, and computed tomography of the paranasal sinuses. Patients who used oral or nasal corticosteroids or other medications (e.g., antibiotics or antileukotrienes) for 4 weeks before sample collection, those with a recent upper respiratory tract infection, and patients undergoing revision were excluded from the study.

Histology and imunohistochemical staining

Paraffin-embedded tissue samples were soaked in xylene to remove the paraffin wax and then sequentially in solutions of 100%, 95%, and 70% ethanol for rehydration. For histological staining, samples were stained with hematoxylin–eosin (H&E). Antigen unmasking was performed by heating the slides in a Decloaking Chamber (Biocare Medical, Concord, CA) to 120 °C. A protein block [1% fetal bovine serum (FBS) in phosphate buffered saline (PBS) and 0.3% Triton X-100 for 30 min] was then applied to the tissue to prevent non-specific protein binding. Endogenous peroxidase activity was blocked by incubation of the sections in 1% H_2O_2 in PBS for 20 min. Primary antibodies [rabbit anti-SEB antibody (Abcam, Cambridge, MA, USA), rabbit anti-protein A (Sigma, St Louis, MO, USA) or rabbit anti-GRP78 antibody (Cell Signaling)] were used at a dilution of 1:100 at room temperature and the sections were rinsed in PBS and then incubated with biotinylated anti-rabbit IgG and streptavidin peroxidase complex (Vector). After further washing, the slides were stained with diaminobenzidine (DAB; Vector, Burlingame, CA). Rabbit IgG (Abcam, Cambridge, MA, USA) was used as isotype control in place of the primary antibodies. The slides were then observed under a fluorescence microscope (Olympus, Tokyo, Japan). SEB-, protein A- or GRP78-positive cells were counted per high-powered field (HPF, × 400) at three different sites in the tissue and the mean values with range were calculated and presented. The experiments were performed in triplicate, repeated at least twice, and judged in a double-blind fashion by at least two observers.

Western blotting analysis

Western blot analysis was performed as previously described [25]. Anti-GRP78/BiP and anti-phospho (Ser-51)-eIF2α were purchased from Cell signaling and anti-p47phox and anti-β-actin were purchased from Santa Cruz Biotechnology. The secondary antibodies used in the study are goat anti-rabbit-IgG-HRP (Cell signaling), rabbit anti-mouse-IgG-HRP (Calbiochem). Membranes were developed using a chemiluminescent reagent (ECL; Millipore Corporation, Billerica, MA, USA) and subsequently exposed to chemiluminescence film to visualize proteins. Actin is shown as a control for protein loading. In some experiments, RPMI 2650 cells were pretreated with indicated concentrations of the free radical scavenger N-acetylcysteine (NAC, Sigma) for 30 min before SEB stimulation.

Measurement of ROS

Intracellular superoxide production was measured using dual-fluorescent probe dihydroethidium (DHE; Molecular Probes). Cells were resuspended in 2 μM DHE and incubated for 30 min at 37 °C in the dark. Cells were centrifuged to be washed and resuspended in 4% paraformaldehyde to fix cells. For detection of ROS in tissue sections, sections of paraformaldehyde-fixed EP, NEP and deviated nasal septum (DNS) were deparaffinized, rehydrated, and incubated with DHE (5 μM) for 30 min at 37 °C followed by acquisition of images with a fluorescence microscope.

Statistics

All statistical analyses were performed using GraphPad Prism 5 (GraphPad, Inc., San Diego, CA). A one-way ANOVA followed by Tukey's test was used to obtain significant differences among at least three groups.