Ubiquitin E3 Ligase A20 Facilitates Processing Microbial Product in Nasal Epithelial Cells

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Background: Ubiquitin A20 contributes to the homeostasis. Results: A20 facilitates endosome/lysosome fusion and protein degradation in epithelial cells. Conclusion: A20 is required in degradation of absorbed proteins in epithelial cells. Significance: A20 is a critical molecule in maintaining the homeostasis in the body.

Microbial products play a role in the pathogenesis of allergic diseases; ubiquitin E3 ligase A20 (A20) is an important molecule in regulating inflammation in the body. The present study aims to elucidate the role of A20 in processing the absorbed microbial products in nasal epithelial cells. Human nasal mucosal specimens were collected from patients with or without chronic rhinitis and analyzed by immunohistochemistry. Human nasal epithelial cell line, RPMI2650, was employed to assess the role of A20 in processing the absorbed staphylococcal enterotoxin B (SEB). The RPMI2650 cells absorbed SEB in the culture. The increase in A20 was observed in RPMI2650 cells in parallel to the absorption of SEB. A20 is a critical molecule in the degradation of SEB in the nasal epithelial cells by promoting the tethering of endosomes and lysosomes. A20 plays a critical role in processing of the absorbed SEB in nasal epithelial cells.

Staphylococcus aureus is a common opportunistic pathogen; its colonization frequently occurs in the anterior nares (1) and also occurs in the axilla, perineum, rectum, and throat (2). Its virulence factor, such as the staphylococcal enterotoxin B (SEB),4 acts as a superantigen, which is associated with the pathogenesis of a number of skewed immune responses. It is reported that SEB-specific IgE is detected in patients with allergic diseases, such as allergic rhinitis (3) and allergic dermatitis (4). Others observed that SEB had the immune adjuvant effect to promote the sensitization to specific antigens (5, 6). However, the process of SEB absorption into the body is not fully understood.

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On the surface of the airway mucosa, there is a thin layer of epithelium; the epithelial cell bodies and the tight junctions form the epithelial barrier. The epithelial barrier physically separates the exterior environment and the deep tissue of the airway mucosa. It has been described that up to ~10^14 commensal bacteria live in the lumen of an adult human intestine (7). How exogenous antigens and microbial products pass across the epithelial barrier to be absorbed into the deep tissue in the body is to be further elucidated. Published data indicate that exposure to SEB does not affect the transepithelial resistance (TER) (8), which implies that SEB does not alter the paracellular permeability of the epithelial barrier. Our previous studies indicate that the exogenous antigens can be transported across the epithelial barrier to get into the deep tissue via the intracellular pathway (9, 10). Whether nasal epithelial cells absorb SEB via the intracellular pathway is not clear.

In previous studies, we (9, 10) and others (11) noted that that the endocytosed foreign antigens could be wrapped by the plasma membrane to form endosomes. The endosomes are defined as a membrane-bound compartment inside eukaryotic cells. It is an endocytic membrane transport pathway by which the endocytic cargo can be transported from the plasma membrane to the lysosome. The endocytic molecules from the plasma membrane can follow this pathway to lysosomes to be degraded.

Three types of endosomes are described, the early, late, and recycling endosomes (12). The early endosomes mature into the late endosomes; during the course of time, the endosomes become increasingly acidic mainly through the activity of the V-ATPase (13). The late endosomes finally fuse with lysosomes. The endocytic substance can be degraded by the hydrolyase in lysosomes. The process of the fusion of endosome/lysosome is not fully understood yet.

Recent studies indicate that the ubiquitin E3 ligase A20 (A20) plays a role in the fusion of endosome/lysosome (14). A20 is both a ubiquitin ligase and a deubiquitinating enzyme; whether these activities are required for degrading SEB remains to be investigated. Thus, we designed and carried out this project. The results indicate that nasal epithelial cells endocytosed SEB, which increased the expression of ubiquitin E3 ligase A20 (A20,
in short) in the cells; the A20 facilitated the degradation of SEB in the epithelial cells.

MATERIALS AND METHODS

Reagents—SEB, NH4Cl, and cycloheximide were purchased from Sigma-Aldrich (Shanghai, China). Antibodies of IgE, CD23, SEB, A20, EEA1, LAMP2, A20 shRNA, and fluorescence-labeled secondary antibodies were purchased from Santa Cruz Biotechnology (Shanghai, China). Reagents for quantitative real time RT-PCR (qRT-PCR) were purchased from Invitrogen (Shanghai, China). RPMI2650 cell line was purchased from ATCC (Manassas, VA). The fluorescein isothiocyanate (FITC) labeling kit was purchased from Thermo Scientific (Beijing, China).

Cell Culture—Human nasal epithelial cell line, RPMI2650 cells, was grown in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum, 100 µg ml⁻¹ streptomycin, 100 µg ml⁻¹ penicillin, and 200 mM l-glutamine in a humidified 37 °C incubator with 5% CO₂. When the cells reached 80–90% confluence, they were trypsinized with 0.05% trypsin-EDTA solution. For the SEB degradation assay, cells were cultured in Transwell filter inserts (polycarbonate membrane, 0.4-µm pore size, 1.12-cm² surface area, Corning Costar Co.). To inhibit the lysosome function, 50 mM NH4Cl was included in the culture medium.

Recording Transepithelial Resistance—TER of RPMI2650 monolayers was determined using the Millicell-ERS apparatus (Millipore, Bedford, MA).

Assessment of Permeability of RPMI2650 Monolayers—Following published procedures (15), we cultured RPMI2650 cells for 2 weeks in Transwell inserts to confluence (TER = 100 ohm/cm²). SEB (10 µg/ml) was added to the apical chambers. Samples were taken from the basal chambers 24 h later. The levels of SEB in the samples were determined by ELISA.

Enzyme-linked Immunoassay (ELISA)—ELISA was performed to assess the levels of SEB and A20. Sample proteins (20 µg/ml), standard proteins, or bovine serum albumin (BSA, using as an irrelevant protein and a negative control) were added to 96-well plates (0.1 ml/well for every reagent); the plates were incubated at 4 °C overnight. 5% skim milk was added to each well for 30 min to block nonspecific binding. The primary antibodies (10 ng/ml) were added to the wells and incubated at 4 °C overnight. HRP-labeled secondary antibodies (5 ng/ml) were added and incubated at room temperature for 1 h. 3’,5’-Tetramethylbenzidine was added and incubated for 15 min; the reactions were stopped by adding 2 M H2SO4. Washing with TBS for three times was performed after each step. The optical density of each well was measured with a microplate reader (Bio-Tek, Shanghai, China). The optical density value of the negative control wells was subtracted from the optical density value of each sample well and the standard wells. The results were calculated against the standard curve.

Western Blotting—Protein samples (60 µg/well) were fractioned in SDS-PAGE and transferred onto nitrocellulose membrane. The membrane was incubated with primary antibodies (10 ng/ml) at 4 °C overnight and followed by adding HRP-labeled secondary antibodies (5 ng/ml). The positive immunoreaction was visualized with the ECL Plus Western blotting reagent (Amersham Biosciences; Shanghai, China).

qRT-PCR—The A20 gene expression in RPMI2650 cells was assessed by qRT-PCR. The total RNA was converted to cDNA with reverse transcriptase with the primer of A20 (forward, gaggacacaagtgtggaac; reverse, tccagtgtatctcggctc; National Center for Biotechnology Information (NCBI), NM_006290.2). The quantitative PCR was performed with SYBR Green master mix (Qiagen, Shanghai, China) in a Bio-Rad thermocycler (Bio-Rad Biotech, Shanghai, China). The results were expressed as the percentage of the housekeeping gene β-actin.

RNA Interference of A20—A20 gene was knocked down in RPMI2650 cells with the shRNA of A20 (nonspecific shRNA was used as a control) following the manufacturer’s instructions. The gene knockdown effect was presented in Fig. 3C.

Immunocytochemistry—Following our established procedures with minor modifications (16), we stained A20, the absorbed SEB, endosomes, and lysosomes in RPMI2650 cells in an Eppendorf tube. The cells were fixed with 2% paraformaldehyde for 2 h and incubated with the primary antibodies for 1 h followed by incubating with fluorescence-labeled secondary antibodies for 1 h. The cells were smeared onto a slide and observed under a confocal microscope.

Image Analysis—The positive immune staining was quantitatively analyzed with the software Photoshop CS5. The results were expressed as pixels.

Collection of Nasal Epithelial Samples—10 healthy subjects (5 male, 5 female; age, 23–30 years old), 10 patients with allergic rhinitis (5 male, 5 female, age, 25–32 years old), and 10 patients with non-allergic chronic rhinitis (5 male, 5 female; age, 24–29 years old) were recruited into this study at our outpatient clinic. The nasal epithelial samples were collected by scratching on the surface of the inferior turbinate with a plastic spoon (3 mm in diameter). The samples were processed for the extraction of RNA and protein immediately. The use of human tissue in this study was approved by the Human Study Ethic Committee at Shanxi Medical University. Informed, written consent was obtained from each subject.

Cycloheximide Chase Assay—The cultured epithelial cells exposed to SEB in the culture were incubated with cycloheximide (70 µg/ml) for different periods of time. The cells were collected at the end of culture; the total cell extracts were analyzed by Western blotting.

Labeling SEB with FITC—For the endocytosis of SEB of the epithelial cells, SEB was labeled with FITC employing the FITC-labeling kits following the manufacturer’s instructions.

Statistical Analysis—All data are presented as mean ± S.D. Differences between means were evaluated by Student t test and considered statistically significant at p < 0.05. All experiments were repeated at least three times.

RESULTS

SEB by Nasal Epithelial Cells—To elucidate whether nasal epithelial cells absorbed SEB, the nasal epithelial cell line, RPMI2650, was exposed to SEB in culture for 0–30 min. The total proteins in the RPMI2650 cells were extracted and analyzed by ELISA. The results showed that the cells could absorb SEB in a SEB dose-(Fig. 1A) and exposure time-depen-
A20 of Nasal Epithelial Cell

FIGURE 1. Nasal epithelial cells absorb SEB. RPMI2650 cells were cultured in the presence of SEB. The cellular extracts were assessed for SEB absorption by ELISA. A, the bars indicate the levels of SEB in the cell extracts. B, cells were treated with SEB (10 μg/ml) in culture for 0–30 min; the bars indicate the levels of SEB in the protein extracts of the cells. The x axis indicates the time points at which the cells were collected. C, the cells were cultured in the presence of FITC-labeled SEB (10 μg/ml) and collected at the indicated time points; the cells were smear on slides and observed with a confocal microscope. The representative confocal images indicate the endocytosis of SEB (in green) of the cells at the indicated time points. Saline: the cells were treated with saline instead of SEB. The original magnification of the image: ×630. The data represent three separate experiments.

Absorption of SEB Increases Production of A20 by Nasal Epithelial Cells—Recent studies indicate that A20 plays a role in the endosome/lysosome fusion (14); the latter is a critical procedure in degradation of the absorbed proteins in cells. To see whether human nasal epithelial cells express A20, we collected nasal epithelial samples and analyzed by qRT-PCR and Western blotting. The results showed that the A20 expression was detected in healthy subjects and that this expression was significantly less in patients with chronic non-allergic rhinitis and allergic rhinitis (Fig. 2, A and B). The results were corroborated by the data from a cell line study. The expression of A20 was also observed in the human nasal epithelial cell line, the RPMI2650 cells, at naive status; exposure to SEB in culture markedly increased the expression of A20 in the cells in a SEB dose- and exposure time-dependent manner (Fig. 2, C and D).

A20-facilitated Degradation of SEB by Nasal Epithelial Cells Is A20- and Lysosome-dependent—Published data indicate that A20 plays a critical role in the prevention of immune inflammation in the body (17) and facilitates protein degradation in the cells (14). We postulated that the increase in A20 in nasal epithelial cells upon uptake of SEB may be associated with the degradation of the absorbed SEB in the cells. Because SEB could be absorbed into RPMI2650 cells (Fig. 1), the absorbed SEB could be either degraded within the cells or converted to the basal side of the cells. We next cultured the RPMI2650 cells in Transwell inserts to confluence. SEB was added to the apical chambers. Samples were taken from the basal chambers 24 h later. The contents of SEB in the basal chambers were assessed by ELISA using this test as a reference parameter to reflect the degradation of SEB by RPMI2650 cells. The results showed that the levels of SEB in samples from A20-sufficient RPMI2650 cells were below the detectable levels, implying that the SEB was degraded within the cells. To test the role of A20 in the degradation of SEB in nasal epithelial cells, the A20 gene was knocked down in a batch of RPMI2650 cells and then exposed to SEB in the Transwell system. Abundant SEB was detected in the basal chambers. To clarify whether the absorption of SEB altered the nasal epithelial paracellular permeability, we recorded the TER of RPMI2650 monolayer in Transwell inserts after the addition of SEB. The results showed that TER in the SEB-treated group was not significantly different from the control group (Fig. 3). The results indicate that the exposure to SEB does not alter the paracellular space of the nasal epithelial monolayer.

We next further tested the role of the lysosome in the degradation of endocytic SEB in the epithelial cells. With the same experimental setting above, we added the lysosome inhibitor, NH4Cl, to the culture medium; the SEB flux through the epithelial monolayers was carried out. The results showed that even the A20-sufficient epithelial monolayers showed the high permeability to SEB (Fig. 3). The data indicate that A20 may not be directly involved in the degradation of the endocytic SEB; lysosome activities are required in the degradation of endocytic SEB.

Endosome/Lysosome Fusion in Nasal Epithelial Cells Requires A20—To further understand the role of A20 in the degradation of the endocytic SEB, we stained the SEB-treated RPMI2650 cells with antibodies against the markers of endosome, lysosome, and SEB; the cells were observed under a confocal microscope. As shown in Fig. 4 and Table 1, SEB+ EEA1+ and SEB+ EEA1+ LAMP2+ immune products were observed in the A20-sufficient cells (Fig. 4A). In A20-deficient cells, more SEB+ EEA1+ immune products and fewer SEB+ EEA1+
LAMP2⁺ immune products were observed as compared with A20-sufficient cells (Fig. 4B). The results indicate that A20 plays a role in the fusion of endosome and lysosome. To confirm this inference, another batch of cells was stained with antibodies of A20, EEA1, and LAMP2. The results showed that A20⁺ EEA1⁺ and A20⁺ EEA1⁺ LAMP2⁺ immune products, but no A20⁺ LAMP2⁺ immune products, were observed (Fig. 4C). The results indicate that A20 is involved in the fusion of endosome and lysosome.

**DISCUSSION**

The present data reveal a novel mechanism by which nasal epithelial cells process the absorbed microbial product, SEB. Upon the absorption of SEB, nasal epithelial cells express A20, which facilitates the fusion of endosome and lysosome and promotes the degradation of the absorbed SEB. Deficiency of A20 interferes with the fusion of endosome and lysosome, resulting in the endocytic SEB being un-degraded and being transported across the epithelial barrier.

SEB is a superantigen; it is capable of activating T cells directly to trigger skewed immune response (18). On the other hand, SEB can also act as an immune adjuvant to promote immune responses such as the induction of the hypersensitivity (5, 6). SEB is produced by *S. aureus*. The nasal mucosa is one of the sites at which *S. aureus* commonly colonizes (19), which can be a source of SEB. Applying SEB to the nasal mucosa directly facilitates the development of nasal hypersensitivity (20) and airway mucosal sensitization (21). This information implies that the nasal epithelial cells can absorb SEB from the nasal cavity. The present study has proved the inference that nasal epithelial cells indeed absorb SEB. We expected that SEB would be converted to the basal side of the epithelial monolayers. However, this was not shown by the data. The amounts of SEB in the basal chamber of the Transwell were below detectable levels. The phenomenon indicates that the amounts of SEB converted to the basal chambers of Transwell inserts are very small if there is any.

The amounts of A20 in the epithelial cells were also increased upon exposure to SEB, which was in parallel to the absorption of SEB in the cells. This is in line with previous studies; Hitotsumatsu *et al.* (22) reported that the expression of A20 was...
increased in macrophages upon the exposure to a microbial product, peptidoglycan. The biological significance of the increase in A20 is to limit the activities of the nuclear factor-κB in the cells to avoid potential injury caused by the microbial products (22). The present results indicate that the increase in A20 may facilitate the degradation of the absorbed SEB in the epithelial cells. The subsequent data support the inference; the A20-deficient nasal epithelial monolayers converted significantly more amounts of SEB to the basal chambers of Transwell inserts. This inference is also supported by a previous study that indicates A20 plays a role in tethering endosomes and lysosomes to facilitate the endosome/lysosome fusion (14). Our data are in line with the study by showing that the SEB-carrying endosome/lysosome fusion was inhibited by knockdown of A20 in the nasal epithelial cells. Furthermore, we observed much lower levels of A20 in the nasal epithelium from patients with chronic allergic and non-allergic rhinitis; the data indirectly indicate that the insufficient amount of A20 may contribute to the pathogenesis of the chronic inflammation in the nasal mucosa. This inference is supported by a recent publication indicating that knocking out the gene of A20 from intestinal epithelial cells resulted in severe inflammation in the intestine (17).

The nasal epithelium directly faces the exterior environment. The airborne antigens and microbial products can physically contract the nasal epithelial cells. As shown by the present study, the nasal epithelial cells can endocytose SEB, an example of microbial products. The results indicate that the nasal epithelial cells should have the ability to degrade the endocytic cargo. The results showed that the nasal epithelial cells did have such a capacity to degrade the endocytic SEB. The results also showed that after being endocytosed, the SEB was wrapped by the plasma membrane to form endosomes. Our previous studies also showed a similar phenomenon in which intestinal epithelial cells endocytosed protein antigens to form endosomes in

**TABLE 1**

Quantified data of immune staining in nasal epithelial cells

The immune positive staining particles in Fig. 4 were counted with confocal microscopy. The data were presented as mean (S.D.). Cells were analyzed individually. 30 cells were analyzed for each group. *, p < 0.01, as compared with the A20⁺ group.

| Immune staining   | Number of particles | SEB/EEA1* | SEB/EEA1/LAMP2* |
|-------------------|---------------------|-----------|-----------------|
|                   |                     | %         | %               |
| A20⁺ cell         | 866                 | 44.26 (5.8)| 51.63 (4.8)     |
| A20⁻ cell         | 897                 | 85.23 (8.2)*| 5.44 (6.1)*     |

| Immune staining   | Number of particles | A20/EEA1 | A20/EEA1/LAMP2 |
|-------------------|---------------------|----------|----------------|
|                   |                     | %        | %              |
| A20⁺ cell         | 882                 | 41.55 (5.3)| 55.6 (7.4)    |

* Numbers in parentheses indicate number of cells.
the cells (10, 23); such endosomes were also observed in rat nasal epithelial cells (24).

The present data show that the endocytic SEB can be degraded within the naive nasal epithelial cells, but cannot be degraded in the A20-deficient epithelial cells. The endocytic SEB is thus transported across the epithelial monolayer. Similar phenomena were observed previously; under allergic environment, the epithelial layer actively transports specific antigens to the subepithelial region (9, 24). We also observed that the expression of A20 was significantly less in the nasal mucosa of patients with allergic rhinitis; thus, these epithelial layers may have hyperpermeability to airborne antigens and microbial products. Further investigations are needed to prove this.

After endocytosis, the early antigen-carrying endosomes fuse each other to form late endosomes and finally fuse to lysosomes (12, 13); the endocytic antigens are thus degraded by the acidic hydrolases of lysosomes (12, 13). In the present study, we observed an interesting phenomenon that most SEB-carrying endosomes fused with lysosomes in the A20-sufficient epithelial cells, whereas far fewer SEB-carrying endosomes fused with lysosomes in those A20-deficient epithelial cells. The results imply that A20 is required in the fusion of endosome and lysosome. Similar phenomena were also observed in previous studies; Li et al. (14) found that A20 was capable of targeting an associated signaling molecule such as TRAF2 to the lysosomes for degradation. We also observed that the endocytic SEB could also pass across the A20-sufficient epithelial monolayer in the presence of lysosome inhibitor. The results indicate that SEB is not degraded by A20 directly, but by the A20 deficiency-interfered endosome/lysosome fusion, which was supported by the immunocytochemistry data as shown by the present study.

In summary, the present study reveals that nasal epithelial cells can endocytose SEB, which can be degraded in the cells with the aid of A20. Knockdown of A20 increases the SEB flux to the basal side of the nasal epithelial monolayers by interfering with the fusion of endosome/lysosome in the epithelial cells.

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