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Deficiency of ubiquitin A20 promotes antigen transport across airway epithelial cells via a transcellular pathway

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ABSTRACT

The epithelial barrier dysfunction is associated with the pathogenesis of a number of diseases. Ubiquitin E3 ligase A20 (A20) plays a critical role in maintaining the homeostasis in the body. This study aimed to investigate the role of A20 in the degradation of endocytic antigens in airway epithelial cells. The expression of A20 in the human nasal epithelial cell line, RPMI 2650 cells (Rpcs), was evaluated. The role of A20 in maintaining the intracellular permeability in Rpc monolayers was assessed in Transwells. The endosome/lysosome fusion in epithelial cells was observed by immunocytochemistry. On the absorption of antigen, the expression of A20 was increased in Rpcs. The knockdown of the A20 gene in Rpcs increased the amounts of the endocytic antigens across the Rpc monolayers. A20 was required in the process of the endosome/lysosome fusion. The antigens transported to the basal compartment by A20-deficient Rpc monolayers still kept strong antigenicity. The nasal epithelial cell line, Rpcs, expresses A20 that facilitates the degradation of endocytic antigens in Rpcs by facilitating the endosome/lysosome fusion.

Under the physiological condition, the epithelial barrier restricts the absorption of macromolecular antigens and other noxious substances in order to maintain the homeostasis of the body. The dysfunction of the epithelial barrier results in antigens with strong antigenicity being absorbed into the deep tissue, which possibly initiates the skewed immune responses such as allergic rhinitis [1], allergic asthma [2], and food allergy [3]. The mechanism underlying the epithelial barrier dysfunction is not fully understood yet.

Exposure to cockroach is associated with the development of airway allergy, such as allergic rhinitis and allergic asthma, and more than 80% of children in the industrial countries are sensitized to cockroach allergens [4]. In the cockroach allergens, Bla g 2 (or Bla for short) is of particular importance, eliciting the immunoglobulin E (IgE)[1] responses in 50 to 70% of patients with cockroach allergy [5].

Endocytosis is the major pathway by which epithelial cells absorb foreign antigens [6]. After sensitization, epithelial cells express CD23; the latter facilitates the absorption of specific antigens [7]. At the early stages, the endocytic antigens are small vesicles, which further form the early endosomes with a marker of the early endosome antigen 1 (EEA1) [8] and then form the late endosomes that fuse with lysosomes with a marker of lysosome-associated membrane protein-2 (LAMP2) [4]; the ubiquitination plays a role at this stage [9]. Lysosomes contain acid hydrolase enzymes that break down the absorbed cargo such as foreign antigens. Thus, the absorbed antigens should be degraded within epithelial cells before releasing out to the basal compartment. However, under pathological conditions, such as in sensitization, foreign antigens do avoid being degraded in epithelial cells and arrive at the deep tissue to initiate the skewed immune responses [2]; the mechanism is to be further elucidated.

Recent reports indicate that the ubiquitin E3 ligase A20 contributes to maintaining homeostasis in the body [10]. Deficiency of A20 in epithelial cells results in severe mucosal inflammation via weakening its nuclear factor kappa B (NF-κB) inhibitory and anti-apoptotic effect, leading to the dysfunction of epithelial barrier [11]. The mechanism by which the deficiency of A20 in the epithelial barrier dysfunction occurs is to be further investigated. In this study, we hypothesized that the deficiency of A20 might affect the degradation of the endocytic antigens in epithelial cells. Thus, from employing an airway epithelial cell model, the results

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[1] *Abbreviations used:* IgE, immunoglobulin E; EEA1, early endosome antigen 1; LAMP2, lysosome-associated membrane protein-2; NF-κB, nuclear factor kappa B; IL-4, interleuken 4; IFN-γ, interferon gamma; HRP, horseradish peroxidase; shRNA, short hairpin RNA; RT–PCR, reverse transcription polymerase chain reaction; FITC, fluorescein isothiocyanate; Rpc, RPMI 2650 cell; TER, transepithelial resistance; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; PBMC, peripheral blood mononuclear cell; DC, dendritic cell; CFSE, carboxyfluorescein succinimidyl ester; PBS, phosphate-buffered saline.

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revealed that the deficiency of A20 interfered with the endosome/lysosome fusion, which resulted in the increase in the amounts of antigen to be transported across the epithelial monolayers.

Materials and methods

Reagents

The mite antigen Bla P1 (Bla for short) was purchased from Virogen (Watertown, MA, USA). Antibody of Bla P1 was purchased from Antibody Research (St. Charles, MO, USA). Antibodies of A20, EEA1, LAMP2, interleuken 4 (IL-4), interferon gamma (IFN-γ), fluorescence (or horseradish peroxidase [HRP])-labeled second antibodies, and short hairpin RNA (shRNA) of A20 were purchased from Santa Cruz Biotech (Shanghai, China). Real-time reverse transcription polymerase chain reaction (RT–PCR)-related reagents were purchased from Invitrogen (Shanghai, China). Immune cell purification reagent kits were purchased from Miltenyi Biotec (Shanghai, China). Fluorescein isothiocyanate (FITC)-labeled dextran-4000 was purchased from Sigma–Aldrich (Shanghai, China). The endotoxin levels in all reagents were detected using the Limulus assay (Limulus amebocyte lysate QCL 1000, BioWhittaker, Walkersville, MD, USA). All of the reagents used in this study contained less than 0.2 U endotoxin/10 μg reagents.

Cell culture

Human nasal epithelial cell line, RPMI 2650 cells (Rpcs), were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 200 mM L-glutamine in a humidified incubator at 37 °C with 5% CO_2. The cells were trypsinized with 0.05% trypsin–EDTA (ethylenediaminetetraacetic acid) solution when the cells reached 80 to 90% confluence. The cells were seeded on the inserts of Transwells (polycarbonate membrane, 0.4 μm pore size, 1.12 cm^2 surface area, Corning Costar, Corning, NY, USA). On confluence, the Rpc monolayers were used for further experiments.

TER recording

Transepithelial resistance (TER) of Rpc monolayer was recorded using the Millicell ERS apparatus (Millipore, Bedford, MA, USA).

Assessment of permeability of Rpc monolayers

Based on the published procedures [12], we cultured Rpcs for 2 weeks in Transwell inserts to confluence (TER $\geqslant$ 100 ohm/cm^2). Bla (10 μg/ml) was added to the apical chambers. Samples were taken from the basal chambers 24 h later. The levels of Bla in the samples were determined by enzyme-linked immunosorbent assay (ELISA).

FITC–dextran-4000 (5 μg/ml) was added to the apical chamber of Transwells, and 30-μl samples were taken from the basolateral side when it was sampled at 2 h. The contents of dextran were determined by a spectrophotometer (Beckman Coulter DU650, Shanghai, China) at an excitation wavelength of 492 nm and an emission wavelength of 515 nm.

ELISA

ELISA was employed to determine the levels of Bla, A20, IL-4, and IFN-γ. Sample proteins (20 μg/ml), diluted standard solutions, or a control antigen (bovine serum albumin, BSA) were added to 96-well plates (0.1 ml/well for each reagent); the plates were incubated at 4 °C overnight. Skim milk (5%) was added to each well 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 were added and incubated at room temperature for 1 h. 3,3′,5,5′-Tetramethylbenzidine (TMB) was added and incubated for 15 min; the reactions were stopped by adding 2 M H_2SO_4. Washing with Tris-buffered saline (TBS) three times was performed after each step. The plates were read by a microplate reader (Bio-Tek, Shanghai, China). The optical density (OD) value of the negative control wells was subtracted from each sample well. The results were calculated against the standard curve.

Western blotting

Samples (60 μg protein/well) were fractioned in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose membrane. The membrane was incubated with primary antibodies (10 ng/ml) at 4 °C overnight, followed by adding HRP-labeled secondary antibodies (5 ng/ml). The positive immunoreaction was visualized with the ECL Plus Western blotting reagent (Amersham, Shanghai, China). The results were recorded with X-ray films.

Quantitative real-time RT–PCR

The expression of the A20 gene in Rpcs was assessed by quantitative real-time RT–PCR (qRT–PCR). The total RNA was converted to complementary DNA (cDNA) with reverse transcriptase with the primer of A20 (forward: gagagcacaatggctgaaca; reverse: tccagtgtgtatcggtgcat; NCBI: NM_006290.2). The qRT–PCR was performed with SYBR Green Master Mix (Qiagen, Shanghai, China) in a Bio-Rad thermocycler (Bio-Rad Biotech, Shanghai, China). The results are expressed as percentages of the housekeeping gene β-actin.

RNA interference of A20

The A20 gene was knocked down in Rpcs with the shRNA of the A20 (nonspecific shRNA was used as a control) following the manufacturer's instructions. Briefly, when the Rpcs in Transwells reach 3/4 confluent, the cells were stimulated by the antigen Bla first (in order to increase the expression of A20 in the cells), and the lentiviral vectors carrying the shRNA of A20 or control vectors were added to the inserts. The Rpc monolayers were kept culturing. After reaching confluence, the monolayers were used in the designed experiments. The gene knockdown effect, presented in Fig. 2C (see Results), occurred 48 h after the transduction and lasted for at least 2 weeks (we observed the effects in separate experiments).

Immunocytochemistry

Following our established procedures with modifications [13], A20, the endocytic Bla, endosomes, and lysosomes were stained in Rpcs in an Eppendorf tube. The cells were fixed with 2% paraformaldehyde for 2 h, incubated with the primary antibodies (0.5 μg/ml) for 1 h, and followed by incubating with fluorescence-labeled secondary antibodies (0.3 μg/ml) for 1 h. The cells were smeared onto a slide and observed under a confocal microscope with a 630× objective. When the positive staining of interest was observed in the cells, the images were further enlarged using the built-in enlarging software tag. A total of 30 images (each image for one cell) were photographed from each group.