



Tryptase disturbs endocytic allergen degradation in intestinal epithelial cells

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ABSTRACT

It is noticed that mast cell activation can compromise epithelial barrier function, enabling antigens to be transported to the deep tissue of the intestine; the underlying mechanism is to be further elucidated. The current study aimed to investigate the mechanism by which the mast cell-derived mediator, tryptase, interferes with the degradation of the endocytic antigen in the intestinal epithelial cells. Intestinal epithelial cell lines were cultured into monolayers. The transepithelial resistance (TER) and permeability were assessed. The fusion of endosome and lysosome in epithelial cells was observed by immunocytochemistry. The antigenicity of protein antigens was tested by flow cytometry. The results showed that the expression of ubiquitin E3 ligase A20 (A20) was weakly expressed by naive gut epithelial cells and was markedly suppressed on exposure to tryptase in the culture. The presence of tryptase greatly disturbed the fusion of antigen-carrying endosomes and lysosomes in the epithelial cells, resulting in epithelial barrier dysfunction and a large quantity of antigen with functional antigenicity to be transported across the epithelial barrier. We conclude that tryptase can suppress the production of A20 in the intestinal epithelial cell lines, playing a critical role in intestinal epithelial monolayer barrier dysfunction.

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The epithelial barrier consists of epithelial bodies and the tight junctions surrounding the top of the cells. The functions of the epithelial barrier are critical for maintaining the homeostasis of the body [1]. Dysfunction of the epithelial barrier is involved in a number of disorders, including allergic diseases [2], chronic inflammation [3], and stress-related injuries [4]. Although studies on epithelial barrier dysfunction have advanced rapidly during recent years, the underlying mechanism is still not fully understood.

The major function of the epithelial barrier is to prevent the macromolecular substances from being absorbed into the deep tissue of the body; the physical parts (cell bodies and tight junctions) play a critical role in the barrier function. However, epithelial cells are featured with the ability to endocytose substances on physical contact [5]. The endocytic cargo can be degraded within the cells; such a process includes the formation of endosomes, the fusion of endosome and lysosome, and the degradation of the endocytic cargo by the acidic hydrolases in the lysosomes [6]. Thus, any disturbance in the process may result in the incomplete degradation of the endocytic cargo, and consequently antigens with the functional antigenicity may be released out of the epithelial cells to reach the subepithelial region to induce the skewed immune responses.

The ubiquitin E3 ligase A20 (A20)² is also termed as TNFIP3. A20 is involved in the pathogenesis of chronic inflammation, allergic diseases, and malignant tumors [7]. The deficiency of A20 causes intestinal epithelial barrier dysfunction [6,8,9] and initiates intestinal bowel disease-like inflammation [10]. A20 is involved in immune regulation by suppressing the activities of nuclear factor (NF)-κB [11]. Recent studies indicate that the knockdown of A20 interferes with the fusion of the endosome and lysosome so as to compromise the epithelial barrier function [6,8,9]. Yet, the factors causing the suppression of A20 in the epithelial cells are unknown.

Mast cells are one of the inflammatory effector cells. On activation, mast cells release a number of chemical mediators such as histamine, serotonin, and tryptase. The mediators play a critical role in the initiation of inflammation and also compromise epithelial barrier function [12]. Because the deficiency of A20, epithelial barrier dysfunction, and mast cell activation induce tissue inflammation, and A20 deficiency induces epithelial barrier dysfunction, we hypothesize that there may be an association between the activation of mast cells and A20 deficiency. Thus, with a cell culture

² Abbreviations used: A20, ubiquitin E3 ligase A20; NF, nuclear factor; BABIM, bis(5-amidino-2-benzimidazolyl)methane; qPCR, quantitative polymerase chain reaction; shRNA, short hairpin RNA; mRNA, messenger RNA; HRP, horseradish peroxidase; TBS, Tris-buffered saline; TER, transepithelial resistance; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; OD, optical density; DC, dendritic cell; CFSE, carboxyfluorescein succinimidyl ester; SEB, staphylococcal enterotoxin B.

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model, we observed that mast cell-derived tryptase inhibited the expression of A20 in intestinal epithelial cells, further compromising epithelial barrier function.

Materials and methods

Reagents

The tryptase inhibitor, bis(5-amidino-2-benzimidazolyl)methane (BABIM), was purchased from Chemical Book (Shanghai, China). Specific PAR2 agonist peptides (SLIGKV-NH2) and control peptides (VKGILS-NH2) were supplied by Alibata Peptides (Hangzhou, China). An RNeasy kit, an OmniScript RT kit, and SYBR Green qPCR (quantitative polymerase chain reaction) Mastermix were purchased from Qiagen (Shanghai, China). Cell isolation reagent kits were purchased from Miltenyi Biotec (Shanghai, China). PAR2 short hairpin RNA (shRNA) and control shRNA were purchased from Santa Cruz Biotech (Shanghai, China).

Quantitative real-time reverse transcription PCR

Total RNA was isolated from the collected gut epithelial cells using the RNeasy kit following the manufacturer's instructions. RNA was reverse transcribed and amplified using SYBR Green qPCR Mastermix with a MiniOpticon Real-Time PCR Detection System (Bio-Rad, Shanghai, China). The messenger RNA (mRNA) levels among the samples were determined by the relative quantification $2^{-\Delta\Delta Ct}$ method using β -actin as the reference gene, as described previously [13].

Western blotting

The proteins were extracted from the experimental cells; the extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk for 1 h and incubated with the first antibodies (50–100 ng/ml) overnight at 4 °C. After washing with TBST (Tris-buffered saline with Tween 20), the membranes were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies (50 ng/ml) for 1 h at room temperature. The immune complexes were developed with enhanced chemiluminescence (ECL).

Gene silencing

The PAR2 genes were silenced in the epithelial cells with commercial reagent kits. Briefly, the epithelial cells were cultured in the inserts of Transwells. When the cells reached 70% confluence, the gene silencing reagents were added to the inserts following the manufacturer's instructions. Approximately 3 to 5 days later, the cells reached confluence. The expression of the target genes was suppressed, as checked by Western blotting (see Fig. 1C in Results). The effect of the gene silencing lasted at least 10 days, which was sufficient for the experiments.

Immunocytochemistry

Cells were fixed with 2% freshly prepared paraformaldehyde for 1 h at room temperature and were blocked with 5% skim milk for 30 min. The cells were then incubated with the first antibodies (0.5–1 μ g/ml) containing 0.1% Triton X-100 for 1 h at room temperature, followed by the fluorescence-labeled secondary antibodies (0.5 μ g/ml) for 1 h at room temperature. Washing with Tris-buffered saline (TBS) was performed after each step. The cells were smeared onto a glass slide, mounted with cover slips, and

observed with a confocal microscope with the objective of $\times 630$. The images were further enlarged with the built-in software when necessary. In total, 30 images were photographed per group. The positive staining particles were categorized to OVA⁺, OVA⁺ EEA1⁺, and OVA⁺ EEA1⁺ LAMP2⁺ types and counted. To avoid observer bias, the slides were coded; the observers were not aware of the codes.

Cell culture

Human gut epithelial cell lines, T84 cells, HT-29 cells, and Caco-2 cells (American Type Culture Collection, Manassas, VA, USA) were seeded on the inserts of Transwells and cultured in Dulbecco's modified Eagle's medium (DMEM)/F-12 with 10% fetal calf serum (FCS), 15 mM Hepes, 100 U/ml penicillin, and 100 μ g/ml streptomycin in a 5% CO₂ environment at 37 °C. The media were changed every 3 days.

Measurement of HT-29 monolayer barrier function

The transepithelial resistance (TER) was recorded, and the OVA flux was performed in Transwells. After reaching the confluence, the TER of HT-29 monolayer was recorded with an ohmmeter. To test the permeability of HT-29 monolayer to protein antigens, OVA (10 μ g/ml) was added to the apical chambers of Transwells; the supernatant samples were taken from the basal chambers 24 h later. The amount of OVA in the samples was determined by enzyme-linked immunosorbent assay (ELISA).

ELISA

Samples were added to a 96-well microplate at 20 μ g/ml protein (or standard proteins or bovine serum albumin [BSA] using as an irrelevant protein) and incubated at 4 °C overnight. The plate was blocked by adding 5% skim milk for 30 min. The first antibodies were added to the plate at 50 ng/ml and incubated for 1 h at room temperature. After washing with TBS, the HRP-labeled secondary antibodies (10 ng/ml) were added to the plate and incubated for 1 h at room temperature. After washing, 0.1 ml of 3,3',5,5'-tetramethylbenzidine (TMB) was added to each well and incubated for 15 min; the reactions were stopped by adding 25 μ l of 2 M H₂SO₄. The optical density (OD) of each well was determined by a microplate reader (Bio-Tek, Shanghai, China). The OD value of the BSA wells was subtracted from each sample well. The contents of target proteins were calculated against the standard curve.

Stimulation with PAR2 peptides

Some of the epithelial cells were stimulated with PAR2 peptides (active or control peptides) in the inserts of Transwells.

Overexpression of A20

The whole sequence of human A20 was synthesized by Alibata Biotech (Hangzhou, China). The sequence (or control sequence) was subcloned into the pET-32 α (+) vector (Dingguo Biotech, Beijing, China) and then transferred into HT-29 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The cells were treated with 50 ng/ml ampicillin for 1 week. The drug-resistant cells were expanded for further experiments.

Test of antigenicity

The CD3⁺ CD4⁺ CD25[−] T cells and dendritic cells (DCs) were isolated from the DO11.10 mouse spleen (Beijing Experimental

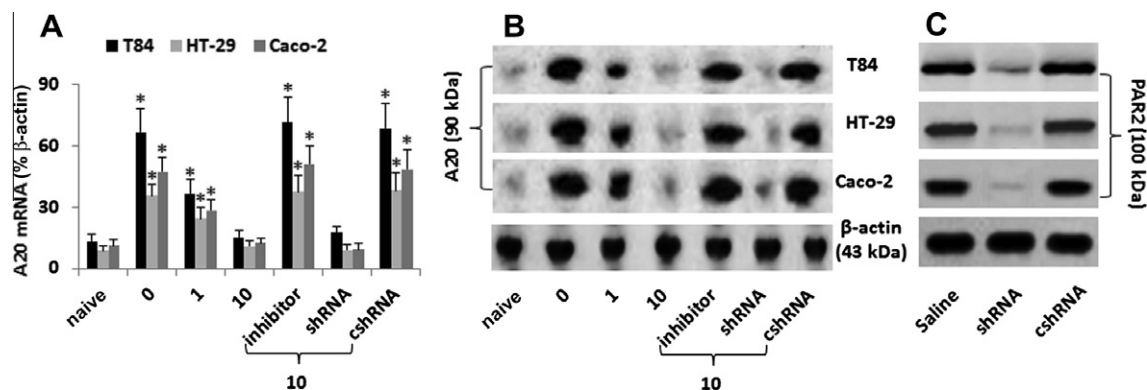


Fig. 1. Tryptase inhibits A20 expression in gut epithelial cells. Gut epithelial cell lines were cultured in the presence of SEB (10 μ g/ml for all groups except the naive group) and tryptase (0–10 μ g/ml) for 48 h. The cell extracts were analyzed by quantitative real-time reverse transcription PCR and Western blotting for the levels of A20. (A) The bars indicate the mRNA levels of A20 in the cell extracts of gut epithelial cells. (B, C) The immune blots indicate the contents of A20 (B) and PAR2 (C, gene silencing results) in the cell extracts of gut epithelial cells. Naive: naive gut cell lines; 0, 1, and 10: tryptase doses (μ g/ml); inhibitor: in the presence of tryptase inhibitor (BABIM, 10 μ M); shRNA (cshRNA): cells transduced with lentiviral vectors carrying shRNA of PAR2 (or control shRNA); saline: cells treated with saline and used as controls. The data in panel A are presented as means $\pm$ standard deviations. * P < 0.01 compared with naive group. The data represent three separate experiments.

Animal Institute) using commercial reagent kits following the manufacturers' instructions. The T cells were labeled with carboxy-fluorescein succinimidyl ester (CFSE), and DCs were cultured at a ratio of 10:1 in the presence of protein samples (collected from the basal chambers of Transwells) or BSA (an irrelevant antigen) for 4 days. The CFSE dilution assay was carried out with a flow cytometer.

Statistics

The data are presented as means $\pm$ standard deviations. The difference between two groups was assessed by a Student's *t* test or with analysis of variance (ANOVA) if there were more than two groups. P < 0.05 was set as the significance criterion.

Results

Tryptase inhibits expression of A20 in gut epithelial cells

Recent reports indicate that epithelial cells express A20 on the endocytosis of protein antigen or microbial toxins, resulting in the disintegration of the epithelial barrier [8,9]. Because epithelial barrier dysfunction also occurs in allergic disorders [14], and tryptase plays a critical role in the pathogenesis of allergy [15], we wondered whether tryptase interfered with the expression of A20 in epithelial cells to compromise the barrier function. To this end, we exposed the staphylococcal enterotoxin B (SEB)-pulsed [6] epithelial cell monolayers to tryptase in the culture. In line with published data [6], the SEB-pulsed gut epithelial cells expressed high levels of A20. The exposure to tryptase resulted in suppression of A20 expression in T84 cells in a dose- and time-dependent manner. Because tryptase can cleave the PAR2 receptors to activate the target cells [16], we next knocked down the PAR2 gene from the gut epithelial cells and then exposed the cells to SEB and tryptase. The expression of A20 was still increasing in the PAR2-deficient gut epithelial cells (data not shown); however, the exposure to tryptase did not suppress the expression of A20 in the cells (Fig. 1). The results indicate that tryptase can activate PAR2 to suppress the expression of A20 in gut epithelial cells.

Tryptase disturbs endosome/lysosome fusion

Published data indicate that A20 plays a role in the fusion of endosome and lysosome in the cells [6,8,17,18]; we wondered

whether the exposure to tryptase could also affect the endosome/lysosome fusion. To this end, we exposed HT-29 cells to OVA (a model allergen) in the culture with or without the presence of tryptase. The cells were then observed by immunocytochemistry. The results showed that without the presence of tryptase, the OVA was endocytosed by HT-29 cells and was surrounded by the EAA1⁺ immune positive staining. The phenomenon indicates that HT-29 can endocytose OVA; the OVA can be surrounded by the plasma membrane to form endosomes. We also observed that more than 80% of endosomes were also LAMP2⁺, indicating that the endosomes have fused with lysosomes. However, in the presence of tryptase, significantly fewer OVA-carrying endosomes fused with lysosomes; this phenomenon indicates that tryptase interferes with the fusion of endosome/lysosome. The results imply that tryptase disturbs the fusion of endosome/lysosome by inhibiting the expression of A20 because A20 plays a critical role in the fusion of endosome/lysosome [6,8,17,18]. To further confirm the inference, we made HT-29 cells overexpress A20 and then exposed to OVA and tryptase in the culture. Indeed, the tryptase-disturbed fusion of endosome/lysosome was prevented (Fig. 2).

Tryptase increases gut epithelial permeability

We next observed whether tryptase compromised epithelial barrier function. With permeability and TER as the indicators of the barrier function, we observed that tryptase significantly increased the permeability to OVA in HT-29 monolayers; the TER was also markedly decreased. The effect of tryptase on gut epithelial barrier function was abolished in PAR2-deficient HT-29 monolayers or the cells overexpressed A20. Such an effect of tryptase was mimicked by PAR2 peptides (Fig. 3). The results indicate that exposure to tryptase can compromise the gut epithelial barrier function by activating PAR2 pathway and suppressing A20 in the cells.

Antigens conserve antigenicity after passing tryptase-treated gut epithelial monolayers

We further observed whether the antigens still conserved the antigenicity after passing the HT-29 monolayers in Transwells. To this end, we concentrated the proteins from the basal chambers of Transwells. The proteins were added to the culture of OVA-specific T cells (labeled by CFSE) in the presence of DCs. After a 4-day culture, the cells were analyzed by flow cytometry. The results

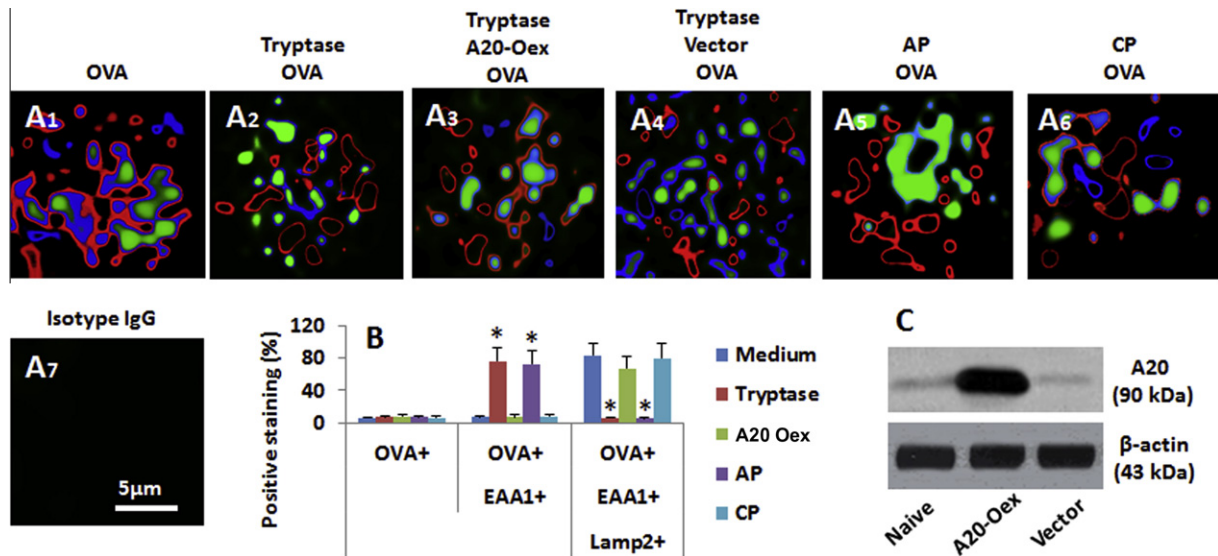


Fig. 2. Tryptase disturbs fusion of endosome/lysosome. (A) HT-29 cells were cultured to monolayers and treated with reagents in the culture, as indicated above each panel. (A1) OVA (10 μ g/ml); (A2) tryptase (10 μ g/ml); (A3) A20-Oex: overexpressing A20; (A4) vector: transfected with empty vector; (A5, A6) AP or CP: active peptides or control peptides of PAR2 (100 μ M). The cells were collected and observed by immune staining and confocal microscopy. The representative confocal images show the positive staining of OVA (in green), EAA1 (a marker of endosome, in blue), and LAMP2 (a marker of lysosome, in red). Panel A7 is an isotype immunoglobulin G (IgG) control. (B) The bars indicate the counts of positively stained OVA* particles with or without costaining with EAA1 or EAA1/LAMP2. The data are presented as means $\pm$ standard deviations from 30 images per group. * P < 0.01 compared with medium group. (C) The immune blots show the A20 overexpression. The data represent three separate experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

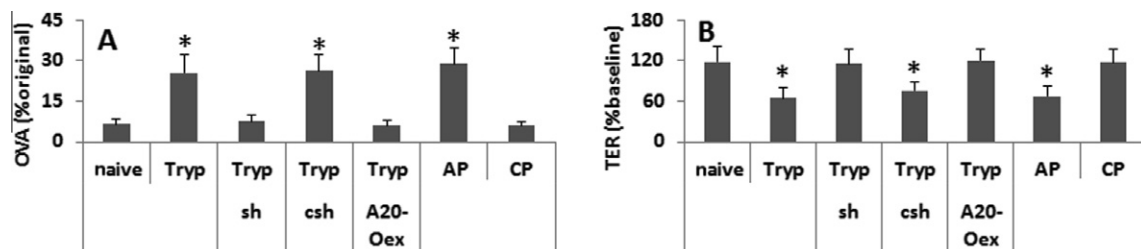


Fig. 3. HT-29 monolayer barrier function. The OVA flux (A) and TER (B) were used as the indicators of epithelial barrier function of HT-29 monolayers. The y axis of panel A shows the amount of OVA in basal chambers of Transwells sampled at the 24-h time point, which was normalized to the percentage of the amount of OVA originally added to the apical chambers (10 μ g/ml). The y axis of panel B indicates the TER, which was recorded at the 24-h time point and is normalized to the baseline of TER (recorded at the time point 0 h). The x axis presents the treatments of HT-29 monolayers in the culture. Naive: cells treated with medium alone; Tryp: tryptase (10 μ g/ml); sh: shRNA of PAR2; csh: control shRNA; A20-Oex: cells overexpressing A20; AP (CP): active peptides (or control peptides) of PAR2 (100 μ M). The data are presented as means $\pm$ standard deviations from three separate experiments. * P < 0.01 compared with naive controls.

showed that proteins from the tryptase-treated group markedly increased the T cell proliferation, which could be blocked by knocking down the PAR2 gene from the monolayers or mimicked by treating the monolayers with PAR2 peptides (Fig. 4). The results indicate that after passing the tryptase-treated gut epithelial monolayer, the antigens still conserve the antigenicity.

Discussion

Tryptase can be detected in the body under physiological conditions and is highly elevated under pathological conditions of certain diseases such as allergic diseases [19]. Mast cells are the major source of tryptase in the body. In line with recent reports [6,8,9], we observed that A20 is a critical molecule in the fusion of endosome and lysosome in epithelial cells and plays an important role in the degradation of the endocytic antigens. The current data also add novel information to the data set of the function of tryptase by showing that tryptase could disturb the fusion of

endosome/lysosome in gut epithelial cells by suppressing the expression of A20, resulting in an increase in epithelial barrier permeability; thus, a large quantity of antigen with functional antigenicity was transported to the basal chambers of Transwells, similar to the subepithelial region in the body.

It is well documented that mast cell activation can compromise epithelial barrier function [20]. Tryptase is one of the mast cell-derived mediators in the induction of epithelial barrier dysfunction [19]. One of the major physiological functions of tryptase is to cleave certain proteins such as PAR2 [21]. On activation of mast cells, the levels of tryptase in the tissue may be elevated [19], which may result in inflammation in the tissue. The current study adds novel information to the function of tryptase by showing that tryptase can suppress the expression of A20 in the gut epithelial cells; consequently, the suppression of A20 interferes with the fusion of endosome and lysosome and indirectly disturbs the endocytic antigen degradation.

The etiology of allergy is unknown, although it is clear that the skewed Th2 polarization plays a crucial role in the pathogenesis of

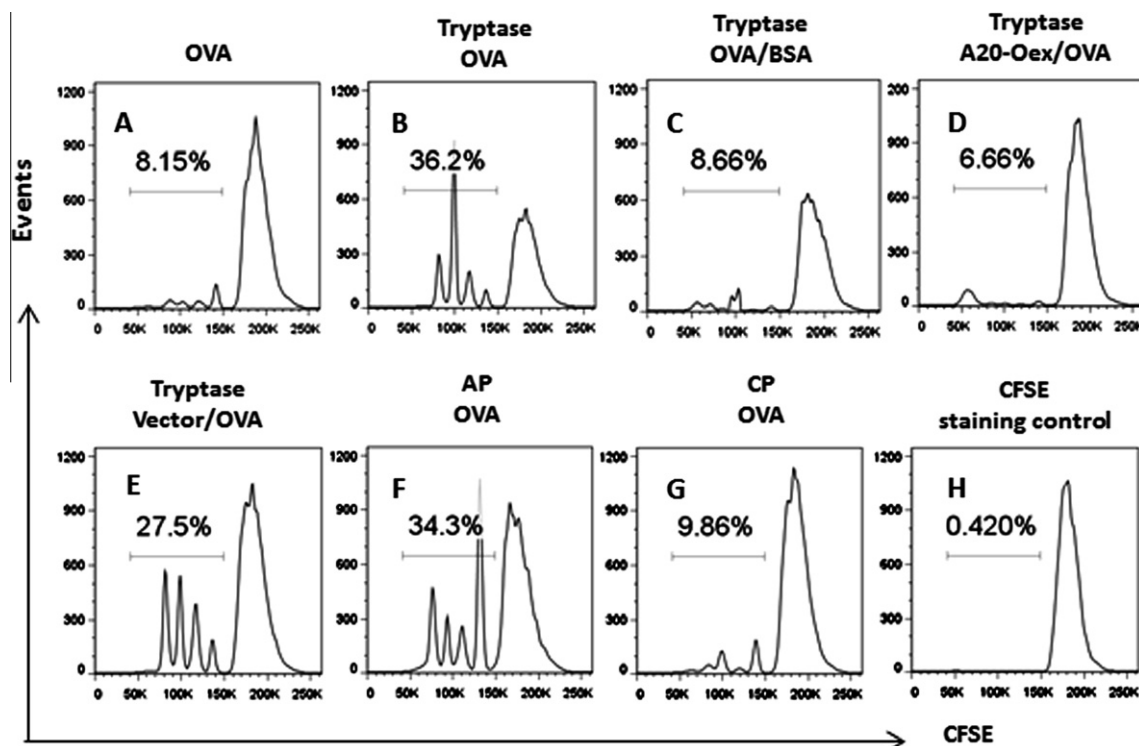


Fig. 4. Antigenicity test. The T cell proliferation was tested by CFSE dilution assay using as an indicator of the antigenicity of the antigen passing through HT-29 monolayers. CD3⁺ CD4⁺ CD25⁻ T cells were isolated from the spleen of DO11.10 mice; the cells were cultured in the presence of DCs (T cells/DCs = 10:1) and the protein extracts for 4 days. The protein extracts were concentrated from the supernatants of the basal chambers of Transwells, in which the treatments of HT-29 monolayers are annotated above each panel. (A) OVA (10 µg/ml). (B, C) Tryptase (10 µg/ml). (D) A20-Oex: overexpressing A20. (E) vector: transfected with empty vector. (F, G) AP or CP: active peptides or control peptides of PAR2 (100 µM). (H) CFSE staining control.

allergic diseases. To initiate the Th2 responses, the presence of antigens with functional antigenicity is required. The current data indicate that exposure to tryptase can induce epithelial barrier dysfunction to increase the permeability to antigens. Mast cells are one of the major sources of tryptase in the body. It is noteworthy that mast cells are residential cells in the body. Not only allergic reaction but also a number of other events can activate mast cells; for example, psychological stress can activate mast cells to release tryptase [22], compromising epithelial barrier function in nonallergic rats [23].

In summary, the current data indicate that tryptase can suppress the expression of A20 in gut epithelial cells; this interferes with the fusion of endosome and lysosome and results in epithelial barrier dysfunction. The data also imply that A20 may have a therapeutic potential in the restoration of epithelial barrier function; this is to be further investigated.

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