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Original Article

Analysis of apoptotic cells in allergic and non-allergic nasal mucosa

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Abstract

Background: Although the mechanisms controlling the resolution of inflammatory processes are still not clear, it is thought that a number of inflammatory cells, including neutrophils and eosinophils, and ingestion of these cells by macrophages may be involved in the apoptotic cell process in allergic or non-allergic nasal tissues. We postulated that apoptosis of inflammatory cells may occur *in vivo* in nasal mucosa, thus regulating allergic and non-allergic inflammation, and to test this hypothesis we examined apoptotic cells in the nasal tissue of surgical specimens.

Methods: Human turbinates were obtained after conchotomy performed on patients with nasal obstruction refractory to medication. Nasal tissues were fixed in formalin and embedded in paraffin. The paraffin-embedded tissues were stained for apoptotic cells by terminal deoxynucleotidyl transferase-mediated deoxyuridine 5′-triphosphate (dUTP) nick end-labeling (TUNEL). To identify cell types undergoing apoptosis, double staining was performed by combining TUNEL and immunohistochemistry.

Results: The majority of TUNEL-positive cells were identified as leukocytes. Most TUNEL-positive cells found in these tissues represented granulocytes. A higher proportion of TUNEL-positive cells was found to be macrophages and most TUNEL-positive macrophages had intact nuclei and contained phagocytosed TUNEL-positive material (assumed to be apoptotic cells or bodies) in the cytoplasm.

Conclusions: These experiments represent the demonstration of cell type-specific apoptosis in human nasal mucosa. The results may have an important clinical implication and also promote further investigation to control the apoptosis of these cells in health and disease.

Key words: apoptosis, immunohistochemistry, nose, rhinitis, terminal deoxyribonucleotidyl transferase-mediated dUTP nick end-labeling, TUNEL.

Introduction

Apoptosis is widely accepted as a fundamental biological process implicated in the resolution of inflammation and control of this process may allow the promotion of a new therapy for the regulation of allergic or non-allergic inflammation in nasal diseases in the future. Cytokines, such as interleukin (IL)-3, IL-5 and granulocyte– macrophage colony stimulating factor (GM-CSF), dramatically increase the life span of purified eosinophils by inhibiting their apoptotic cell death *in vitro*.[1] It has been reported that tissue eosinophilia in nasal polyps is associated with inhibition of eosinophil apoptosis.[2] Increased expression of some of these cytokines has been reported in allergic diseases,[3] suggesting that a delay of eosinophil apoptosis may occur *in vivo* in nasal allergy. In addition, Fan *et al*. demonstrated that the induction of eosinophilic apoptosis was critical for reversing tissue eosinophilia in patients with allergic sinusitis.[4] The technique of terminal deoxyribonucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) has enabled *in situ* visualization of individual apoptotic cells while

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preserving tissue architecture.[5] In the present study, we used this technique to analyze apoptotic cells in human nasal mucosa. In addition, we evaluated whether a reduction of inflammatory cell apoptosis was associated with allergic rhinitis.

METHODS

Subjects

Human inferior turbinates were obtained at the time of conchotomy from 10 subjects with perennial nasal allergy (six men/four women; mean (±SD) age 36.3 ± 3.9 years) and from 10 subjects with non-atopic chronic rhinitis (five men/five women; mean age 38.8 ± 5.3 years). Informed consent was obtained from all patients. The diagnosis was confirmed by history, radioallergosorbent test, skin test and nasal provocation test to dust mites (*Dermatophagoides pteronyssinus* and/or *Dermatophagoides farinae*), grass pollen and birch pollen. No patient had a recent airway infection and all medication against rhinitis had been withheld for 2 weeks before surgery. None of the patients had received topical or oral steroids in the 2 months before the study or immunotherapy in the previous 3 years. A group of three healthy volunteers (two men/one woman; mean age 33.0 ± 5.0 years) with no known disease and taking no medication was also studied. Alligator forceps were used to obtain a biopsy from inferior turbinate after use of 1% plain lidocaine for local anesthesia. The fresh nasal tissue was fixed immediately in 10% formalin for paraffin section. Sections (4 μm) were cut and mounted onto poly-L-lysine-coated microscope slides.

Immunohistochemistry

Commercial streptavidin-biotin immunostain kits (alkaline phosphatase LSAB$^+$ kit; DAKO, Glostrup, Denmark) were used to enumerate cells binding to monoclonal antibodies against eosinophil cationic protein (EG2; Pharmacia, Uppsala, Sweden), neutrophils elastase (NP57; DAKO), mast cells tryptase (AA1; DAKO), pan T cell marker (MT1; Bio-Science Products AG, Emmenbrucke, Switzerland) and macrophages (CD68; DAKO). Briefly, sections were dewaxed, treated with 20 μg/mL proteinase K (Takara Biomedicals, Osaka, Japan) for 15 min at room temperature and incubated with levamisole (DAKO) to quench endogenous alkaline phosphatase activity. Sections were then incubated in blocking reagent (Tris-buffered saline (TBS), 1% bovine serum albumin (BSA)). The primary antibody at optimal dilution was applied for 15 min and sections were then washed in TBS. The appropriate bridge antibody, biotinylated goat antimouse immunoglobulins (DAKO), was then applied for 10 min. Sections were rewashed in TBS and streptavidin alkaline phosphatase was applied for 10 min. A further wash in TBS was followed by development in New Fuchsin (DAKO) as a chromogen for signal visualization.

In situ detection of apoptotic cells in nasal tissues

Apoptotic cell death was detected by the TUNEL method using commercial kits (*In situ* Apoptosis Detection Kit; Takara Biomedicals, Japan). Briefly, deparaffinized sections were digested with 20 μg/mL proteinase K for 15 min at room temperature and incubated with 3% H_2O_2 for 15 min to quench endogenous peroxidase activity. Sections were incubated for 60 min at 37°C in a mixture of terminal deoxynucleotidyl transferase (TdT) and dUTP–fluorescein isothiocyanate (FITC) in accordance with the manufacturer's instructions (Takara Biomedicals). The dUTP-FITC-labeled sections were incubated with peroxidase-conjugated rabbit anti-FITC immunoglobulin (DAKO). Bound peroxidase was detected by True blue (Kirkegaard Perry Laboratories, Gaithersburg, MD, USA).

Combination of immunohistochemistry and TUNEL

Deparaffinized sections were digested with 20 μg/mL proteinase K for 15 min at room temperature and incubated with 3% H_2O_2 for 15 min and levamisole for 10 min to quench endogenous peroxidase and alkaline phosphatase activity, respectively. Apoptotic cell death was then detected by the TUNEL method using commercial kits (*In situ* Apoptosis Detection Kit, Takara Biomedicals) with some modifications. Briefly, sections were incubated for 60 min at 37°C in a mixture of TdT enzyme and dUTP-FITC. Then, to detect the phenotype of apoptotic cells undergoing DNA fragmentation, immunostainings were performed by using an alkaline phosphatase LSAB$^+$ kit (DAKO) and sections were developed with New Fuchsin (DAKO). To identify the FITC-labeled TUNEL-positive cells, sections were then incubated with peroxidase-conjugated rabbit anti-FITC immunoglobulin (DAKO) and reacted with True blue (Kirkegaard Perry Laboratories). In some sections, after immunohistochemistry (IHC) and TUNEL, sections were

counterstained with methyl green. Using this method, non-apoptotic cell nuclei were stained green, cellular phenotypical makers were stained red and apoptotic (TUNEL-positive) nuclei appeared blue.

Quantitation

For both IHC and TUNEL, slides were counted under a microscope equipped with an eyepiece graticule. A total of six fields (1 mm^2 each) from each slide were counted by placing the upper edge of the grid at the epithelium. Results are expressed as the number of positively stained cells per mm^2.

Statistical analysis

Values are expressed as the mean±SD. Differences between and among groups were compared using Mann–Whitney *U*-test. $P < 0.05$ was considered significant.

RESULTS

Immunohistochemistry

Using IHC techniques, we observed a great number of T cells in the nasal tissues (Fig. 1). The difference between the numbers of T cells in allergy and non-allergy (109.1 ± 31.0 *vs* 77.1 ± 19.3 cells/mm^2, respectively) was statistically significant. In addition, the number of EG2-positive cells was significantly higher in patients with allergy than in non-allergy or normal volunteers (52.0 ± 16.1, 11.4 ± 6.4 and 10.3 ± 4.8 cells/mm^2, respectively). However, no statistically significant difference was detected in number of other cell types between allergic, non-allergic and normal nasal mucosa.

TUNEL and the combination of IHC and TUNEL

The TUNEL technique demonstrated the number of TUNEL-positive cells in nasal tissues. As shown in Fig. 2, there were no significant differences between the number of total TUNEL-positive cells in allergic, non-allergic and normal nasal mucosa (12.4 ± 4.8, 11.6 ± 2.9 and 9.6 ± 4.9 cells/mm^2, respectively). To identify the cell type that undergoes apoptosis, we stained sections by combining TUNEL and IHC. Figure 3 demonstrates the examples of TUNEL-positive–eosinophil cationic protein (ECP)-positive eosinophils, elastase-positive neutrophils, tryptase-positive mast cells, pan T cell marker-positive

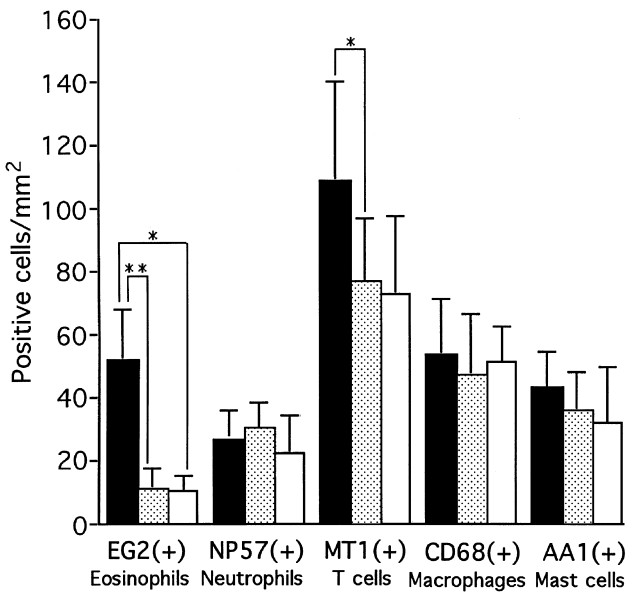

Fig. 1 Numbers of eosinophils, neutrophils, mast cells, T cells, and macrophages in allergic (■; $n = 10$), non-allergic (▨; $n = 10$) and normal (□; $n = 3$) nasal mucosa. Data are the mean±SD. *$P < 0.05$, **$P < 0.01$.

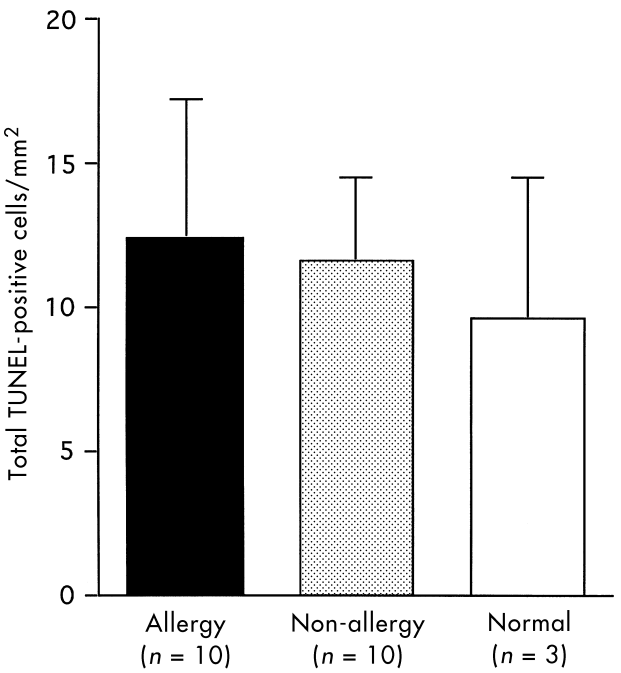

Fig. 2 Numbers of terminal deoxyribonucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)-positive cells in allergic, non-allergic and normal nasal mucosa. Data are the mean ± SD.

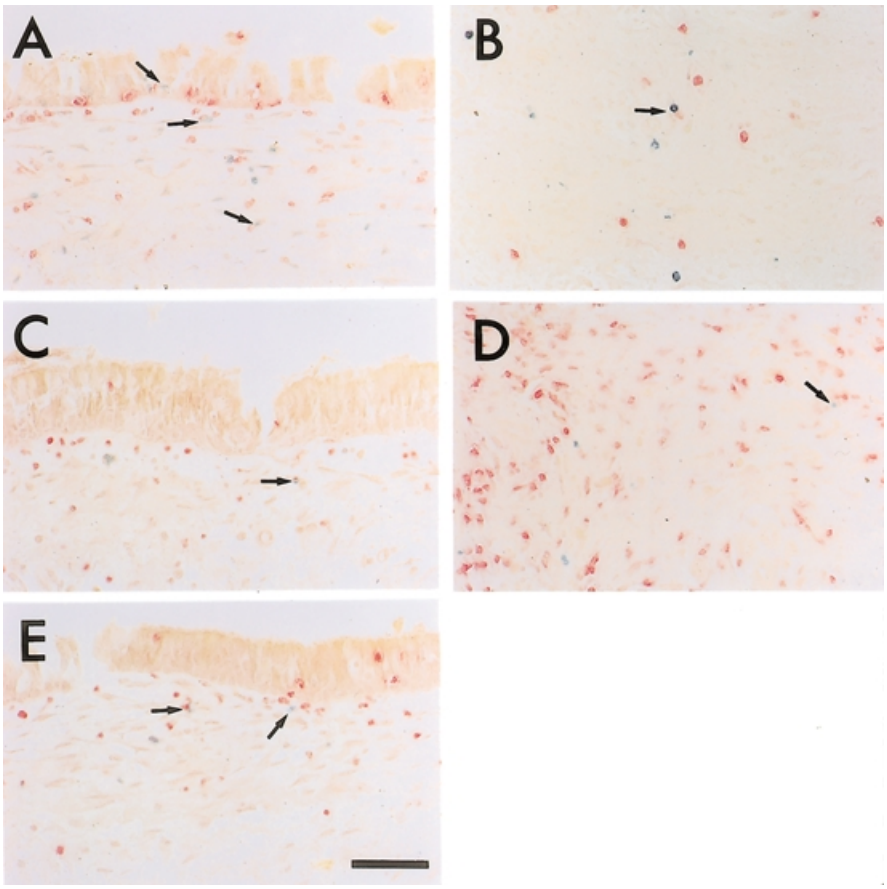

Fig. 3 Examples of combination of immunohistochemistry (IHC) and terminal deoxyribonucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL). Sections were incubated with the following antibodies: (a) eosinophil cationic protein (EG2) antibody, anti-eosinophil cationic protein; (b) NP57 antibody, anti-neutrophil elastase; (c) AA1 antibody, anti-mast cell tryptase; (d) MT1 antibody, anti-pan T cell marker; and (e) anti-CD68 antibody. The IHC-positive cells stain red, while TUNEL-positive cells stain blue. Double-positive cells are indicated by the arrows. Bar, 20 μm.

T cells and $CD68^+$ macrophages. As shown in Fig. 4, the ratio of TUNEL-positive cells was highest for macrophage (11.9 ± 7.4, 10.6 ± 7.0 and 12.9 ± 4.7% for allergy, non-allergy and normal controls, respectively) and was moderately high for eosinophils (7.0 ± 2.9, 7.9 ± 7.4 and 7.1 ± 3.8% for allergy, non-allergy and normal controls, respectively) and neutrophils (5.5 ± 2.6, 8.5 ± 5.4 and 8.8 ± 4.8% for allergy, non-allergy and normal controls, respectively). In contrast, very little apoptosis was associated with mast cells (1.3 ± 2.3, 1.2 ± 1.5 and 1.6 ± 1.3% for allergy, non-allergy and normal controls, respectively) and T cells (1.7 ± 1.2, 1.6 ± 1.2 and 2.2 ± 1.6% for allergy, non-allergy and normal controls, respectively). In each phenotype, there were no significant differences between ratio of TUNEL-positive cells in allergic, non-allergic and normal nasal mucosa. In order to confirm the phagocytosis of apoptotic cells by macrophages, sections were counterstained with methyl green after IHC and TUNEL. We found that the majority of TUNEL-positive macrophages had TUNEL-positive signals located in the cytoplasm, the nuclei of these cells being TUNEL negative (Fig. 5). These findings indicate that apoptosis was not occuring in macrophages themselves and that macrophages had engulfed apoptotic cells or bodies.

Discussion

Apoptosis, a form of programmed cell death, is thought to be critically important in promoting the clearance of inflammatory cells and the resolution of inflammation.[6] Using the technique of combined IHC and TUNEL, we revealed the apoptosis of inflammatory cells and phagocytosis of these cells by macrophages in human nasal mucosa. *In vitro*, macrophages express Fas and readily undergo apoptosis when cultured with anti-Fas.[7] It is possible that macrophage Fas is stimulated by T cells, triggering apoptotic death, because activated T cells have been shown to express the Fas ligand.[8] However, in the present study, most TUNEL-positive signals on

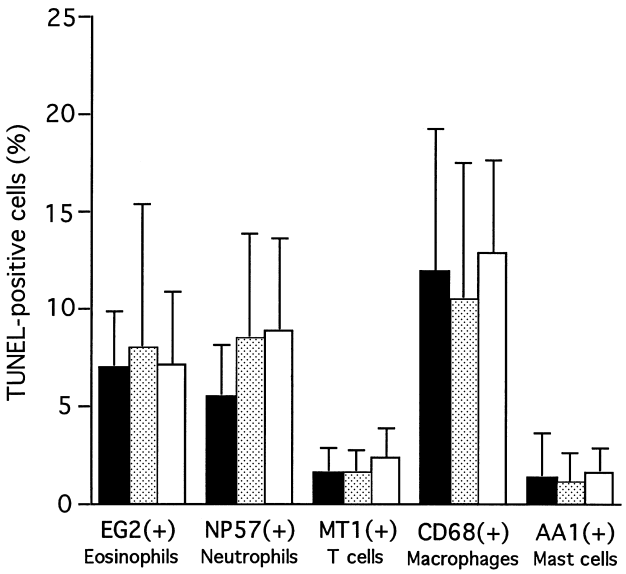

Fig. 4 The percentage of terminal deoxyribonucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL)-positive cells in each phenotype in allergic (■; $n = 10$), non-allergic (▨; $n = 10$) and normal (□; $n = 3$) nasal mucosa. Sections were stained by a combination of immunohistochemistry (IHC) and TUNEL. The percentage of double-positive cells to IHC-positive cells was calculated. Data are the mean ± SD.

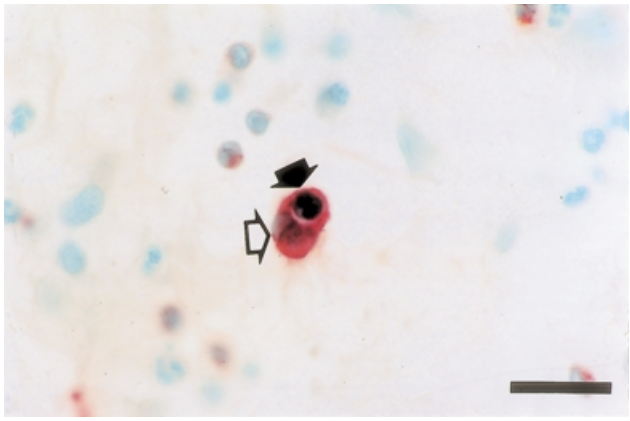

Fig. 5 Micrograph showing phagocytosis of apoptotic cells by $CD68^+$ macrophages. The section was counterstained with methyl green after immunohistochemistry (IHC) with anti-CD68 antibody (red) and terminal deoxyribonucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL; blue). The TUNEL-positive signals were located in the cytoplasm (closed arrow), the nuclei being TUNEL negative (open arrow). This indicates that apoptosis was not occuring in the macrophages themselves and that macrophages had engulfed apoptotic cells or bodies. Bar, 10 μm.

macrophages were located in the cytoplasm and not in the nuclei. This suggests that apoptosis was not occuring in the macrophages themselves. It has been shown that phagocytosis of eosinophils by macrophages was found in eosinophil-infiltrated nasal polyps.[9] In addition, it has been reported that there was a progressive increase in the number of macrophages that engulfed apoptotic cells and bodies in allergen-induced cutaneous late-phase responses.[10] These findings suggest that apoptosis of inflammatory cells and the removal of these cells by phagocytes may occur in allergen-induced inflammation.

One of the characteristic features of allergic inflammation is the tissue eosinophilia and, in the present study, the number of cells expressing EG2 was significantly higher in subjects with allergic rhinitis than in those with non-allergic rhinitis. Although the recruitment of eosinophils has been widely demonstrated, it is still unclear whether tissue eosinophilia is due to a delayed eosinophil apoptosis. It has been reported that the ratio between apoptotic eosinophils and total eosinophils is significantly lower in bronchial biopsy specimens from asthmatic subjects than in those with chronic bronchitis.[11] This suggests a delayed eosinophil apoptosis may be present in allergic diseases. However, in the present study, the ratio between TUNEL-positive eosinophils and total eosinophils was not statistically different in nasal mucosa obtained from control subjects and subjects with allergic rhinitis. On the basis of these findings, we could not confirm the delay of eosinophil apoptosis in nasal allergy.

Mature peripheral T cells generally undergo activation and proliferation when stimulated through the CD3/TCR complex, and stimulation with T cells through the TCR with CD3 antibody in the absence of antigen-presenting cells caused T cell death.[12] Ligation of Fas on activated T cells by either Fas antibodies or recombinant human Fas-ligand results in apoptosis.[13] In the present study, we found a very low number of apoptotic T cells, suggesting that these cells are less susceptible to apoptosis than eosinophils and neutrophils. This evidence confirms previous results obtained by examination of mucosal biopsy specimens of patients with asthma and bronchitis.[11]

Mast cells are virtually unique among hematopoietically derived cells in that they pass the majority of their life cycle within tissues. The number of mast cells in tissue may be regulated by a balance between the number of mast cells arising from hematopoietic precursors and the rate of mast cell death. The regulation of tissue mast cell numbers may be largely related to the local control of

mast cell apoptosis. It has been reported that mast cells undergo apoptosis upon withdrawal of IL-3.[14] In addition, the c-kit ligand, also known as stem cell factor,[15] and interferon-γ[16] promote mast cell survival by suppressing apoptosis. However, the duration of mast cell survival in tissues has not yet been clarified. In the present study, the ratio of TUNEL-positive cells among mast cells was much lower than for other leukocytes, suggesting that mast cells may survive longer than other leukocytes. It may be possible that survival of mast cell is prolonged by IL-3 or stem cell factor because previous studies have shown that stem cell factor[17] and IL-3[3] mRNA is expressed in nasal tissues. The demonstration of mast cell apoptosis may reveal a possible therapeutic approach to the treatment of mast cell-dependent inflammation, including nasal allergy.

The reduced apoptosis of inflammatory cells and, in particular, of eosinophils may play a crucial role in the allergic inflammation and controlling the apoptosis of these cells may provide a new and effective therapy for allergic diseases. It has been shown that eosinophil apoptosis in sputum samples increases after corticosteroid treatment for an exacerbation of asthma and this increase occurred in association with clinical improvement and the resolution of eosinophilic inflammation.[18] Administration of anti-Fas monoclonal antibody to mouse lungs after the induction of lung eosinophilia increased the number of peroxidase-positive macrophages in bronchoalveolar lavage fluid 4–12 h later, which was followed by a marked reduction in the number of eosinophils in the airways.[19] These results suggest that induction of apoptosis in eosinophils is beneficial in the suppression of allergic inflammation.

In conclusion, the present study demonstrates cell type-specific apoptosis in human nasal mucosa. The ability to control the apoptosis of these cells will offer the potential for a new and attractive strategies for the treatment of allergic and non-allergic nasal inflammation in the future.

Acknowledgments

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