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Eosinophil Cationic Protein (ECP) Is Processed during Secretion

Charlotte Woschnagg, Jenny Rubin, and Per Venge

The eosinophil granulocyte is an inflammatory cell involved in allergic diseases such as asthma and rhinitis. Eosinophil cationic protein (ECP) is a basic and potentially cytotoxic granule protein that is released from the eosinophil upon activation. The aim was to study secretion of molecular variants of ECP from blood eosinophils with the hypothesis that the stored noncytotoxic ECP is altered into cytotoxic species upon release from the cell. Eosinophil granulocytes were purified to >95% from venous blood from birch pollen allergic subjects, with symptoms of rhinitis, and from healthy control subjects during the birch pollen season. The cells were stimulated with IL-5, GM-CSF, or serum-opsonized Sephadex particles. Concentration of ECP in cells or supernatants was measured by means of a fluoroenzyme immunoassay, and ECP heterogeneity was studied using an affinity capture assay with the surface-enhanced laser desorption/ionization-time of flight mass spectrometry technique. Extracts of unstimulated eosinophils contained 10 major ECP variants, with molecular masses ranging from 16.1 to 17.7 kDa. Stimulation with particles mainly induced the secretion of two molecular variants at 16.1 and 16.3 kDa, while cytokine stimulation gave rise to a different secretion profile. ECP variants in the pellet extracts remained unaffected by cell activation. The modifications of secreted ECP were partly explained by differences in N-linked glycosylations. Secretion of ECP from eosinophils involves protein modification. The molecular masses of released ECP have acquired the masses of the cytotoxic species.


The eosinophil granulocyte takes part in the body’s natural defense against invading parasites (1), as well as in other inflammatory diseases like allergic asthma and gastrointestinal disorders (2). Once attracted to the site of inflammation the eosinophil becomes activated and, as a result of this, secretes several tissue-toxic mediators. These are either basic and granule-stored proteins (eosinophil cationic protein (ECP)), eosinophil peroxidase (EPO), eosinophil protein/eosinophil-derived neurotoxin, and major basic protein (MBP) or reactive oxygen-free radicals (3). The eosinophil also produces a wide array of different cytokines, chemokines, and lipid mediators and is therefore, in addition to being an effector cell, thought to play an immunoregulatory role in inflammatory processes as well as taking part in tissue remodelling (4, 5).

ECP is one of several highly basic proteins present in the secretory granules of the eosinophil. ECP belongs to the superfamily of RNases and has both cytotoxic and noncytotoxic effects. The mechanism for killing target cells is by the formation of transmembrane pores and channels. The molecular mass of the protein. High levels of ECP are found in body fluids of patients with allergic and other inflammatory diseases, as a sign of the involvement of the eosinophil in the process. ECP secretion is induced in vitro by secretagogues such as immunoglobulins, complement factors, and serum-opsonized particles (6, 7). Blood eosinophils from allergic subjects are primed in vivo during allergen challenge, giving rise to increased migratory responses (8), adhesiveness (9), degranulation (10), and an altered oxidative metabolism (11, 12).

In a previous study we showed that eosinophil extracts from healthy blood donors contain several variants of ECP with molecular masses ranging from 15.9 to 17.2 kDa when analyzed by means of a sensitive and specific surface-enhanced laser desorption/ionization-time of flight mass spectrometry (SELDI-TOF MS) technique (13). Preliminary in vitro studies of fractionated ECP, purified from buffy coats, demonstrated that only some of these molecular variants displayed cytotoxic activity. Highly glycosylated ECP was generally less toxic than the less glycosylated variants. Enzymatic deglycosylation of the noncytotoxic variants resulted in active protein species with molecular masses ranging from ~15.7 to 16.3 kDa (14). Our studies, however, showed that the cytotoxic activity of ECP was mainly associated with the molecular masses of 16.1 and 16.3 kDa. In this study we aimed to investigate the secretion of variants of ECP from blood eosinophils and to study whether these were modified by the secretory process and affected by in vivo priming of the eosinophils by allergen exposure of allergic subjects. Our hypothesis was that stored noncytotoxic ECP would be converted into active, cytotoxic variants upon priming and release from the eosinophils. We used the analysis of specific molecular masses as indirect markers of cytotoxic ECP.

Materials and Methods

Reagents

IL-5 and GM-CSF (R&D Systems) were diluted in Gey’s buffer (pH 7.4) before use to give a final concentration of 100 ng/ml. The MACS system and anti-CD16 beads were from Miltenyi Biotec.
Percoll (Pharmacia) was diluted with PBS to a 67% (v/v) solution before use.

**Serum opsonization of Sephadex G-15 particles**

Serum opsonization of Sephadex G-15 particles (GE Healthcare Bio-Sciences) was performed as described (10), with some modifications. Forty-two-milligram Sephadex G-15 particles were dissolved in 250 µl of PBS. Normal human serum was added (50%, v/v) and the suspension was incubated at 37°C for 10 min. After centrifugation at 1000 rpm for 10 min, the particles were washed twice in PBS and resuspended in 500 µl of Gey’s buffer.

**Patient and control groups**

Eight birch pollen allergic patients with seasonal symptoms of rhinitis and a control group consisting of seven healthy, nonallergic blood donors were studied during the Swedish birch pollen season. The allergic subjects presented elevated blood eosinophil counts (362 × 10⁶/L; range, 100–600) compared with the nonallergic controls (228 × 10⁶/L; range, 100–400) during the period of pollen exposure (p = 0.025), indicating an ongoing inflammatory process.

The study was approved by the Medical Ethical Committee at Uppsala Academic Hospital, and all blood donors gave a written informed consent.

**Preparation of eosinophil suspensions**

Eosinophils were isolated from 80 ml of peripheral venous blood from each donor using the MACS system, as described by Hansel et al. (15).

Venous heparinized blood was diluted 1/1 in PBS before loaded on 67% Percoll (density of 1.085 g/ml). After centrifugation at 1000 × g for 30 min, mononuclear cells were removed and the erythrocytes in the cell pellet were hypotonically lysed for 60 s. The obtained polymorphonuclear neutrophils were incubated for 1 h at 4°C with anti-CD16 beads and the suspension was placed in a MACS column for the final separation. Eosinophil purity was >95% in all cases.

**Cell counting and quantification**

Cells were stained with Türk’s dye and counted under the light microscope. Cell viability was measured using trypan blue exclusion. Total blood cell counts were performed with a Technicon H1 cell counter. Differential counts were obtained using a cytocentrifuge preparation (Cytospin; Shandon Southern Instruments) stained with May-Grunewald-Giemsa, then examined under the light microscope.

**Release and extraction of ECP from eosinophils**

The release assay was performed with some modifications according to the method described previously (10).

The eosinophils were pelleted by centrifugation for 10 min at 600 × g at 4°C and resuspended in Gey’s buffer to a final concentration of 1 × 10⁶/ml. The cell suspension was divided in three: total cell extract, unstimulated cells, and stimulated cells. The cells for the total cell extract were kept on ice, while 200 µl of Gey’s buffer or stimuli (IL-5, GM-CSF, or opsonized particles) was added to the other cell suspensions. The stimulated cells were incubated for 30 min at 37°C, with careful mixing every 10 min. The cells were thereafter put on ice to stop the reaction and then centrifuged for 10 min at 600 × g in 4°C. The supernatant was collected and the granule proteins were extracted from the remaining cell pellet of both the unstimulated and stimulated cells by adding 100 µl of 0.5% cetyltrimethylammonium bromide (CTAB; Merck) in 0.9% NaCl containing Complete (Boehringer Mannheim) and pervanadate (0.6 mM) for 1 h in room temperature (RT). The suspension was centrifuged for 10 min at 600 × g in 4°C and the supernatants were collected and stored at −70°C until assayed for ECP.

The concentration of ECP in the samples was quantified by fluorometric microculture cytotoxicity assay described in Larsson et al. (17) was used. The small-cell lung cancer cell line NCI-H69 was cultured in RPMI 1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin (all from Sigma-Aldrich). The cells were pelleted and washed once with RPMI 1640 medium containing 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin but without FCS. The cells were separated by AccuMax treatment for 15 min at 37°C and then washed twice more with RPMI 1640 medium containing 100 U/ml penicillin and 100 µg/ml streptomycin. Cells were counted and seeded into V-shaped 96-well microtiter plates, 10,000 cells/well. Purified ECP in a total volume of 20 µl of NaAc buffer (pH 5.5) was added in duplicates to the wells before the cells were seeded. ECP concentrations in the wells ranged from 0.6 to 10 µg/ml. Eight wells containing cells and NaAc buffer (pH 5.5) served as buffer control. Two wells of cells and 0.01% Triton X-100 served as positive control for cytotoxicity. Plates were incubated for 72 h at 37°C, 5% CO₂ and were then centrifuged for 5 min at 200 × g. Medium was removed and the cells washed once with 200 µl/well PBS before the addition of fluorescein diacetate (Sigma-Aldrich) (10 µg/ml). Plates were incubated at 37°C, 5% CO₂ for 30 min before reading fluorescence with filters set at 485 and 538 nm for excitation and emission, respectively (Fluoroscan 2; Lab-systems). The fluorescence generated is proportional to the number of cells with intact plasma membrane in the well, and data are presented as survival index (SI %). Survival index is calculated as: SI = [(fluorescence in test well − mean fluorescence in 12 blank wells)/(mean fluorescence in 8 control wells − mean fluorescence in 12 blank wells)] × 100 (%).

Criteria for a successful assay included a fluorescent signal in control wells of more than five times mean blank values. Dose-response experiments were repeated once. The means ± SEM of four results at each concentration are presented in Fig. 4B.

**Statistics**

The Wilcoxon signed rank test, the Mann–Whitney U test, or the χ² test was used, and p values <0.05 were considered significant. All calculations were performed on a personal computer by means of the statistical packages Statistica (Statsoft) or MedCalc (MedCalc Software).

**Results**

**Stimulus-induced secretion of ECP from blood eosinophils**

IL-5, GM-CSF, and serum-opsonized particles all induced ECP secretion from the eosinophils obtained from all subjects studied.
The particle-induced secretion was increased in eosinophils obtained from the allergic subjects during allergen season (10.5, 5.8–18.0) as compared with the healthy controls (6.2, 0.39–16.0) (p = 0.037), whereas the secretions induced by IL-5 and GM-CSF were similar (Table I).

The effects of in vivo priming on the secretion of ECP

As shown above, the allergic subjects presented elevated blood eosinophil counts and increased release of ECP from the purified eosinophils as signs of an ongoing allergic inflammatory process during the birch pollen season. The profiles of ECP in cell extracts and supernatants, however, were similar to the profiles of the nonallergic controls, suggesting that in vivo priming had no impact on the molecular modifications of secreted ECP. The results are therefore from now on presented as one group only, including both allergic and nonallergic subjects (supplemental Fig. 1).

Affinity capture of ECP from eosinophil extracts and supernatants with SELDI-TOF MS

The spectra of ECP in pellet extracts of unstimulated and particle-stimulated cells showed up to 10 separate peaks, with masses ranging from 16.1 to 17.7 kDa, with no differences between the extracts. The 10 peaks differed in molecular masses of 164–196 Da. In supernatants of unstimulated and particle-stimulated cells, two major peaks with masses of 16.1 and 16.3 kDa and two minor peaks of 17.2 and 17.4 kDa were predominant, but with no discernible differences between the supernatants (Fig. 1A).

Similar to the spectra of unstimulated or particle-stimulated eosinophils, the spectra of ECP in extracts of cytokine-stimulated cells showed up to 10 separate peaks, with masses ranging from 16.1 to 17.7 kDa. In supernatants of cytokine-stimulated cells, two major peaks with masses of 16.3 and 17.4 kDa were seen, but with no discernible differences between the supernatants (Fig. 1A).

The cytotoxicity of purified ECP was determined by a dose-response assay. The dose-response curves of both ECP pools are shown in Fig. 4B. The LMW-ECP pool displayed a potent cytotoxic effect against the NCI-H69 cells, in contrast to the effect of the HMW-ECP. The cytotoxicity of the two pools differed significantly at 2.5 and 5 μg/ml (p < 0.05, t test).

Discussion

In this study, it was for the first time shown that the secretion of ECP from eosinophils involves modifications of the protein and that the modifications probably result in the generation of cytotoxically active molecules. It is also suggested that the modifications of ECP take place during the departure of the molecules from the cell, since the protein that remains inside the activated cell is unchanged. These conclusions were based on our findings of a shift in molecular masses of the secreted ECP resulting in the accumulation of molecular masses of the cytotoxically active moiety. It was also shown that the secretion profile of ECP varies between different secretagogues and that the mechanism by which the protein is secreted from the cell is not affected by in vivo priming.

The secretion of ECP was induced by both soluble and insoluble stimuli in this study. Serum-opsonized Sephadex particles are potent secretagogues and are known to activate granulocytes through the C3b receptor (18). The C3b-induced degranulation was increased in the cells from the allergic patients with seasonal symptoms as compared with the cells from healthy subjects, which was shown previously (10). IL-5 and GM-CSF are members of the same hemopoietic cytokine family by sharing a common receptor chain, the β-chain, which is thought to be the signal transducer part of the receptor complex (19). IL-5 and GM-CSF have been reported to directly induce degranulation of eosinophil-derived neurotoxin, another eosinophil-specific basic protein, in vitro in human eosinophils (20), while these cytokines only have been shown to act as enhancers on the stimulation-induced ECP degranulation (21). Here it was shown that the hemopoietic cytokines indeed can function as secretagogues for ECP on their own, but contrary to secretion induced by serum-opsonized particles, the cytokine-induced secretion was unaffected by in vivo priming of the eosinophils.

Eosinophils secrete and release their granule contents by means of three known mechanisms: cytolysis, regulated exocytosis, and piecemeal degranulation (22). Piecemeal degranulation is suggested to be a selective mobilization and secretion of granule proteins by means of transport of vesicles (23) and is induced by
agents such as cytokines (24), but also by allergen exposure of human eosinophils (25). Regulated exocytosis is characterized by fusion of granule membranes with the plasma membrane, which allows the release of the granule contents to the outside of the eosinophils (26). Regulated exocytosis was shown to be induced by the interaction of eosinophils with serum-opsonized particles (27). Our observations of differences in profiles of the secreted ECP may reflect that different secretion mechanisms are involved in this process dependent on the stimulus used. With cytokine stimulation, ECP might be modified as part of the process of piece-meal degranulation, whereas particle stimulation induces regulated exocytosis and a different processing of ECP.

Analyses of total cell extracts showed that human blood eosinophils contain at least 10 major molecular variants of ECP, with masses ranging from 16.1 to 17.7 kDa. However, in previous results only five variants were detected (13). The differences in these results could partly be due to method development, including the use of a new and more sensitive SELDI-TOF MS system, making it possible to analyze the samples with higher resolution and better accuracy.

Stimulation with different agents caused the secretion of different isoforms of ECP, while the protein that remained inside the cell was unaffected. These results are intriguing, since a selective secretion of different variants of an eosinophil protein never has been described. Even in our own previous study no differences between secreted and granule-stored ECP was seen (13). In the present study, in contrast to the previous, the samples contained both protease and phosphatase inhibitors, indicating that the selective secretion process involves several kinds of protein modifications. The results also suggest that ECP is modified during the process of secretion. Thus, it seems that most ECP is stored in an inactive form in the granules, to protect the cell from getting destroyed, and as it leaves the cell ECP becomes modified and thereby cytotoxic. Indeed, previous work showed that the cytotoxic activity of ECP was associated with variants of molecular masses ranging from 15.75 to 16.3 kDa (14) and that only a minority of ECP purified from eosinophil granules had cytotoxic activity (28). Preliminary studies indicated that the main cytotoxic activity was associated with the molecular species of molecular masses of 16.1 and 16.3 kDa (14), which are identical to the ECP species released from the eosinophils. In this study we confirm that the cytotoxic properties of ECP is predominantly associated with low molecular weight ECP of similar masses of those of secreted ECP, lending support to our hypothesis that ECP is activated at secretion.
The heterogeneity of ECP is mainly due to N-linked oligosaccharides, and the use of glycosidases caused deglycosylation, both of recombinant (28) and of native (14) ECP. This decreased the molecular masses of the protein and changed the functionality by increasing its cytotoxicity. In this study, the use of N-glycosidase F caused further reductions in the molecular masses of ECP, which indicated that the conversion into the putative cytotoxic molecules did not include complete, but rather partial, deglycosylation of the protein. In a previous study, we showed that the N-linked carbohydrate chains of ECP are in part made up of sialic acid, galactose, and acetylglucosamine (13), and we speculate that these molecules in various combinations are cleaved off ECP at the process of secretion. The challenge will be to identify the actual enzymatic principles responsible for this conversion to inhibit the cytotoxicity of ECP in diseases characterized by increased numbers of activated eosinophils.

Blood eosinophils from patients with allergy are primed in vivo during allergen challenge, causing changes in several cellular functions as mentioned before. The mechanisms leading to the state of priming of the cells are still unclear, although the hemopoietic cytokines IL-3, IL-5, and GM-CSF have been shown to prime the eosinophil in vitro (29). Our hypothesis was that priming would change the functionality of the stored ECP. As mentioned earlier, the allergic subjects in this study presented elevated blood eosinophil counts and released higher amounts of ECP from the cells as compared with the healthy controls, both signs of an ongoing

**FIGURE 3.** A, Enzymatic deglycosylation of secreted ECP from unstimulated and particle-stimulated cells. Purified eosinophils were treated with buffer or serum-opsonized Sephadex particles for 30 min at 37°C. Supernatants and total cell extracts were incubated with or without N-glycosidase F at 37°C overnight and the samples were thereafter analyzed by means of SELDI-TOF MS. See Materials and Methods for further details. The samples are shown pairwise, with untreated samples on top of samples deglycosylated by the N-glycosidase F enzyme, in a gel view. The figure shows one representative experiment of four subjects. B, Enzymatic deglycosylation of secreted ECP from cytokine-stimulated cells. Purified eosinophils were stimulated with IL-5 (100 ng/ml) or GM-CSF (100 ng/ml) for 30 min at 37°C. Supernatants and total cell extracts were incubated with or without N-glycosidase F at 37°C overnight and the samples were thereafter analyzed by means of SELDI-TOF MS. See Materials and Methods for further details. The samples are shown pairwise, with untreated samples on top of samples deglycosylated by the N-glycosidase F enzyme, in a gel view. The figure shows one representative experiment of four subjects.

**FIGURE 4.** A, SELDI-TOF MS analysis of purified ECP with the 614 mAb. One sample of high m.w. (HMW-ECP) and one of low m.w. (LMW-ECP) were selected for the experiment. The m.w. of the peak of highest peak intensity is indicated in each spectrum. B, The cytotoxic activity of the HMW-ECP and LMW-ECP pools. The figure shows a mean of two dose-response experiments at 0.6–10 μg/ml ECP. The cytotoxicity of the two ECP pools differed significantly at 2.5 and 5 μg/ml (p < 0.05, t test), respectively.
inflammatory process. However, no differences in ECP secretion profile between the groups were seen. Thus, we conclude that although eosinophils from allergic subjects with symptoms during allergen exposure release higher amounts of ECP as compared with healthy cells, the way that ECP is secreted and modified is independent of priming and the state of inflammation.

We conclude that secretion of ECP from eosinophils involves protein modification and that this modification mainly takes part at the departure from the cell, while the protein that remains inside the activated cell is unchanged. It was also shown that the secretion profile of ECP changes into molecular masses identical to the masses of cytotoxicity active ECP. Thus, our results indicate that stored noncytotoxic ECP is converted into cytotoxically active molecules upon release from the cell.

Disclosures

The authors have no financial conflicts of interest.

References


