Eosinophil decranulation as an allergy activation marker
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Chapter 11

Discussion and Summary
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Introduction

The eosinophilic granulocyte is a type of white blood cell, that, like all other blood cells, originates in the bone marrow. Immature eosinophils in bone marrow differentiate to mature cells, which subsequently circulate in the blood, migrate into a tissue or target organ and finally die by apoptosis. Under normal circumstances, the eosinophil count in blood is below $0.5 \times 10^9/l$.

The eosinophilic granulocyte is involved in several processes; e.g. as an effector cell in the defense against parasite larvae and as an inflammatory cell in allergic reactions in the skin, in the lungs, in the nose and in the intestinal tissues. When the eosinophilic granulocyte binds activating factors, the cell is activated. Activation results in a certain degree of degranulation. During degranulation strongly positively charged proteins are released, e.g. eosinophil protein X (EPX) and eosinophil cationic protein (ECP). The release of proteins is increased if the eosinophilic granulocyte has contacted certain mediators at an earlier stage. These mediators are produced locally in inflamed tissue and induce pre-activation or priming of eosinophils. Earlier studies have shown that blood eosinophils of allergic patients are pre-activated for chemotaxis and oxygen radical formation. It is not yet clear whether the circulating eosinophils of patients with disorders in which the eosinophil plays a role, are already pre-activated to degranulation when they are in the blood circulation.

Objective of the study

Studies to evaluate the priming state of eosinophils with regard to degranulation have failed, because these cells release only a small fraction of their total granule protein content during *in vitro* activation. However, it has been claimed that the release of these
proteins during blood clotting in vitro can be used as a test to establish the priming state of eosinophils (Venge, 1993).

Thus, the amount of products of eosinophil degranulation in serum, like ECP or EPX, might yield insight in the pre-activation state of these cells in healthy and diseased individuals. The aim of this study was to determine the usefulness and additional value of measurement of degranulation proteins (e.g. ECP and EPX) in serum and morphologic characteristics of the eosinophils in relation to the absolute number of eosinophilic granulocytes in blood. For example, we studied whether the concentration of granule proteins in serum is either a better differentiation marker between patients and healthy persons or shows earlier than the eosinophil count an effect of therapy. In clinical studies we have evaluated the value of these parameters for diagnosis or for establishing the effects of therapy in several groups of patients.

When starting the study it became clear that for the clinical evaluation not only the protein assays were important, but even more the pre-analytical phase of serum sample preparation. A number of variables important for a correct interpretation were evaluated, e.g. the clotting time and temperature of the blood sample.

Pre-analytical conditions and test methodology

ECP and EPX are present in very low concentrations in EDTA plasma samples. Higher concentrations of ECP and EPX are detected in serum. ECP and EPX in serum originate both from in vivo and from in vitro degranulation of eosinophils. The in vitro contribution is the dominant factor. For interpretation of serum concentrations of granule proteins originating from eosinophils it is necessary that procedures for blood collection and pre-analytical processing of the samples are standardized. The influence of temperature during clotting and the clotting time, the dependency on eosinophil count, differences and similarities between the granule proteins (ECP and EPX) and differences between detection methods for ECP are described in chapters 2, 3, 4 and 5.
ECP and EPX are present in the same granules. Therefore, we expected no differences in release during degranulation. Indeed, ECP and EPX release showed a similar dependency on clotting temperature and eosinophil count (chapters 2 and 3). For this reason, only one granule protein, ECP, was measured in the following studies.

Measuring the ECP concentration in serum affords an indirect parameter for eosinophil degranulation \textit{in vivo}. For a correct interpretation of results it is necessary to standardize the clotting process. Temperature of clotting influences the results; \textit{e.g.} clotting at 37°C results in ECP concentrations that are 5 - 10 times higher compared to samples clotted at 4°C. Additionally, the clotting time has been standardized. After 2 hours of clotting at 37°C, ECP concentrations in serum are stable. After standardizing clotting time and temperature, ECP concentrations in various sera show less variability.

The reference range for serum ECP concentrations, independent of eosinophil counts, covers a large area (12 - 99 μg ECP/l) (chapter 4). The variation in results should even be higher when using different analytical techniques to measure the ECP concentration. To establish reference ranges, it is important to know whether various methods yield similar results (chapter 5). Comparing results from serum samples with normal ECP concentrations, we found a rather good correlation between two commercially available methods (DPC and Kabi Pharmacia). At higher concentrations of ECP, the method of DPC yields lower values than the method of Kabi Pharmacia. Control samples, consisting of purified ECP or a part of ECP in an aqueous solution, showed higher results when using the method of DPC compared to results obtained with the method of Kabi Pharmacia.

We found a strong correlation between the values of ECP in serum and the number of eosinophils in the blood used for clotting. Therefore, we have also calculated the serum ECP values per eosinophil, in an attempt to eliminate the variation induced by the eosinophil numbers. In this way, we established a reference range for the serum ECP per eosinophil ratio of 61 to 367 μg ECP per 10⁹ eosinophilic granulocytes. This range is narrower than that of the reference range of serum ECP.
For measuring the total content of ECP per eosinophil, eosinophils were lysed in a solution of Cetyl-trimethyl-ammonium-bromide (CTAB). We found total amounts of 0.8 to 6 pg ECP/eosinophilic granulocyte (chapter 6). More ECP was present in eosinophils from individuals with a low eosinophil count in blood. In other studies, the total ECP content has not been quantified in relation to the eosinophil count. The variation in the ECP amounts in literature is high: from 4.0 to 13.5 pg/eosinophilic granulocyte. This may be due to the fact the total amount has been established by cell lysis in various solutions.

**Characteristic features of eosinophil populations with respect to degranulation**

Previous studies have described the hypothesis that circulating eosinophils of allergic patients are pre-activated and therefore release more ECP during *in vitro* clotting than do eosinophils of apparently healthy individuals. However, it is not known whether circulating eosinophils are pre-activated for degranulation *in vitro* and show a similar degranulation as eosinophils in affected tissues. We established that both in apparently healthy individuals and in patients a strong relationship was found between the eosinophil counts in blood and the ECP concentrations in serum. The higher the concentration of eosinophils in blood, the higher was the serum ECP concentration. We concluded that it is important to interpret serum ECP results in relation to the eosinophil count.

A parameter has been defined that yields a useful marker for the state of eosinophil activation, *i.e.* the serum ECP per eosinophil ratio (chapter 6). This ratio concerns the amount of ECP released per eosinophilic granulocyte. The ECP release per eosinophil during *in vitro* clotting showed an inverse trend with respect to the eosinophil count. When the number of eosinophils in blood was low, the ECP release per eosinophil was
rather high. A study with patients with an allergic predisposition (chapter 4) showed no
differences in the serum ECP/eosinophil ratio compared to apparently healthy
individuals. The serum ECP per eosinophil ratio in asthmatic patients, in patients with
colitis ulcerosa and in patients with hay fever also did not show obvious differences
compared with a healthy population. During therapy, the eosinophil count decreased and
the serum ECP per eosinophil ratio increased. Therefore, the additional value of the
serum ECP concentration over the blood eosinophil count has not been demonstrated.
Serum ECP only reflects the eosinophil count in the blood.

To elucidate the observation that eosinophils that are present in low numbers in the
blood release more ECP per eosinophil during \textit{in vitro} clotting, we also determined the
content of ECP per eosinophil. This total amount of ECP per eosinophil was obtained by
lysis in a solution of CTAB (chapter 6). The total amount of ECP per eosinophil in
relation to the blood eosinophil number showed an analogous inverse trend compared to
the serum ECP per eosinophil ratio. When the number of eosinophils in the blood was
high, the ECP content of the eosinophils was rather low. Independently of the eosinophil
count in blood, the amount of ECP released during \textit{in vitro} clotting at $37^\circ$C was about
3\% of the total amount of ECP in the eosinophils (chapter 6). Therefore, no signs of pre-
activation of ECP release during \textit{in vitro} clotting was established by our studies.

Proliferation and terminal maturation of eosinophilic granulocytes occur in bone
marrow. After the myelocytic stage, the cells enter a large storage pool in the bone
marrow. A few days later they are released into the blood. When eosinophils are needed
(e.g. in allergic diseases or in host defense) the eosinophilic granulocytes migrate at an
increased rate from the blood into the tissues (Smith and Goetzl 1980). At higher
eosinophil blood concentrations, more eosinophils, which might include younger cells,
have been released from bone marrow. The increased release of ECP by eosinophilic
granulocytes at low eosinophil concentrations is probably caused in part by the fact that
eosinophils in individuals with a low eosinophil count will contain a higher amount of
ECP per eosinophil. This might be due to the longer time of eosinophil maturation in the
bone marrow storage pool in case of patients with a low blood count of circulating eosinophils.

Clinical studies

To study the clinical usefulness of the serum ECP concentration and the ECP released per eosinophil as potential markers of eosinophil activation, compared to the eosinophil count, several patient groups were investigated. The groups concerned adult asthmatic patients (chapter 7), patients with colitis ulcerosa (chapter 8) and children with hay fever (chapter 9). The asthmatic patients, who did not use corticosteroids at the start of the study and later on were treated with inhalation steroids, were monitored for one year. Patients with colitis ulcerosa were monitored before and during 12 weeks of treatment with oral corticosteroids. In hay fever patients the effects of a new method of therapy, which involves triggering the immune system (immunotherapy), were studied and compared with a control group treated only with corticosteroids. During immunotherapy, small amounts of allergen are applied. As a result of frequent injections of low amounts of allergen, a decrease in the allergic reactions in these patients is observed.

We tried to determine differences between patients and healthy adults by using the serum ECP measurement. In most cases, it was possible to differentiate between these groups of individuals on the basis of the eosinophil counts in their blood. An additional value of serum ECP as a potential marker of activation to diagnose an allergic disease, such as asthma or hay fever, has not been found. For both parameters, a large overlap between the patient group and the control group exists. Therefore, these parameters cannot be used for confirmation of a disease in an individual. Also, ECP serum concentration as a marker of activation was shown to be unfit for predicting the disease activity state in patients treated with corticosteroids or
immunotherapy. Alterations in serum ECP concentration as well as the eosinophil counts in blood demonstrated the effect of treatment. The serum ECP per eosinophil ratio was rather constant. An unexpected increase was observed when the eosinophil count was decreased to below 0.05 x 10^9/l.

Under corticosteroid therapy, a striking decrease in the eosinophilic granulocyte count in the blood occurred. The number of eosinophilic granulocytes in blood and the serum ECP concentration are both suitable laboratory parameters to show the effects of corticosteroid therapy. After 3 months of corticosteroid inhalation therapy, a decrease in these parameters was detectable. Eosinophil blood counts decreased to half the original value, while the ECP serum concentrations decreased to 65%. Oral corticosteroid therapy (chapter 9) showed for both parameters after two weeks treatment a decrease amounting to 35% of the initial values. The difference in effects might be explained by different sensitivities for corticosteroids in the two patient groups. However, the calculated ratio of ECP release per eosinophil did not show differences before and after the start of corticosteroid therapy. Therefore, measuring the ECP concentration in serum offers no additional value over counting the number of circulating eosinophils as a parameter for judging the clinical situation of the patient.

It is remarkable that the number of neutrophilic granulocytes in the circulation increases after the start of the corticosteroid therapy. A possible explanation for an inverse effect on blood neutrophil and eosinophil counts can be found in the acceleration or slowing down of programmed cell death (apoptosis). Other in vitro studies have shown that apoptosis in eosinophils is increased and in neutrophils is decreased in case of treatment with corticosteroids (Matsukura et al. 1996, Meagher et al. 1996, Nittoh et al. 1998).

**Morphological features of eosinophils**

Treatment with corticosteroids does not only influence the number of eosinophilic granulocytes but also the maturation process and apoptosis of these cells. Therefore, it
was worthwhile to investigate with a light microscope the morphological appearance of eosinophilic granulocytes before and during therapy. The eosinophilic granulocyte is characterized by big, round granules with an affinity for acid dyes like eosine. Eosinophil granules appear in the promyelocytes in the bone marrow. The nucleus of eosinophilic granulocytes consists of two lobes. In chapter 10 we describe which differences were observed when studying the eosinophilic granulocytes in a blood smear with a light microscope. The number of vacuoles, the percentage of granulation, the diameter of the cell, the lobe/cell surface ratio and the number of lobes per nucleus were used as markers of activation and differentiation. Eosinophils of patients with a high eosinophil blood count turned out to have an identical appearance to eosinophils from individuals with lower number of eosinophils in their blood, except that the former cells had an increased diameter. In general, undifferentiated granulocytes are larger than mature granulocytes (Baggiolini 1980).

Corticosteroid therapy induces a significantly lower concentration of the eosinophilic granulocytes in the blood of most patients. These eosinophils contained less lobes per nucleus, a marker of maturation, than the eosinophils present before starting therapy. This observation led to the conclusion that during corticosteroid treatment more immature or younger eosinophils are present in the blood. Corticosteroid treatment did not show effects on morphologic parameters reflecting activation. We hypothesize that during treatment with corticosteroids older (more mature) eosinophils are attracted to the tissues and are not found in the circulation. Another possibility is a suppression of eosinophil production; the cells therefore stay longer in the bone marrow, and more fully mature cells are present in the bone marrow.

Conclusion

In this study, we observed that in several conditions in which the eosinophilic granulocyte plays a role in pathology, the cells are present in high concentrations in
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blood. It is not possible to detect a higher activation state of eosinophils by means of the degranulation parameter ECP in serum. Both parameters (eosinophil blood counts and ECP release during blood clotting) followed the same trend. Therefore, useful changes in the serum ECP per eosinophil ratio were not observed. After standardizing pre-analytical conditions, an analogous trend for serum ECP concentrations and blood eosinophil counts was observed. An additional value of serum ECP to the eosinophil count was not established in this study. The additional parameter, serum ECP per eosinophil ratio, showed hardly any variation in the various patient groups. The hypothesis, described in the literature, that eosinophils of allergic or asthmatic patients would release more ECP during in vitro clotting because of their pre-activated state, has not been substantiated by our study. Small morphological differences were observed between eosinophils in case of increased and decreased eosinophil blood counts. Thus, our observations have revealed a difference in the differentiation and maturation of the eosinophils, but not an essential difference in the activation state of the eosinophils. Just like the eosinophil blood counts and serum ECP concentrations, morphological markers show a remarkable overlap between patients and healthy individuals. Therefore, the individual predicting value of these markers to diagnose or follow treatment in a patient is low.

It appears to be difficult to determine the activity of the eosinophil population (quality) besides the number of circulating eosinophils (quantity) with simple diagnostic measurements.

Suggestions for further investigations

To understand the process of eosinophil degranulation during blood clotting and to determine the clinical value of serum ECP measurements, it should be studied which particular factor, released during in vitro blood clotting, induces eosinophil degranulation.
Because measuring ECP in serum yields only an indirect measure for eosinophil activation, it may be better to study the eosinophil degranulation markers in body fluids, for example broncho-alveolar lavage fluid, sputum or faeces, in which pre-analytical activation (e.g. *in vitro* clotting) is not necessary. However, it is questionable whether these fluids will represent the activity of the eosinophils in the affected tissues. Another option is to examine the concentration of ECP or EPX in urine samples. But measurement of ECP in urine is also an indirect parameter; again it is questionable what the concentration tells us about the degranulation activity in the affected tissue. It might represent ECP released in the inflamed tissues but also ECP released by eosinophils present in the kidney.

A more direct determination would be measuring the amount of degranulation markers in a small piece of affected tissue (biopt). However, measuring in a tissue biopt does not belong to the category of easy-to-use laboratory methodologies for diagnosing and monitoring disorders.
References


