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File ID	59062
Filename	Chapter 10 Evaluation of alterations in morphology of eosinophils

SOURCE (OR PART OF THE FOLLOWING SOURCE):

Type	Dissertation
Title	Eosinophil decranulation as an allergy activation marker
Author	C.J. Admiraal
Faculty	Faculty of Medicine
Year	2001
Pages	208
ISBN	909014997X

FULL BIBLIOGRAPHIC DETAILS:
http://dare.uva.nl/record/91491

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Chapter 10

Evaluation of alterations in morphology of eosinophils

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Summary

To diagnose and monitor patients with allergic diseases, we have studied not only the number of circulating eosinophils but also morphologic features that may reveal additional information concerning the activation state of the eosinophils. To establish the morphologic characteristics of eosinophils and the number of circulating eosinophils eleven apparently healthy individuals and nine individuals with eosinophilia and allergic complaints were included in this study. Effects of corticosteroids on the parameters mentioned above were studied in five newly diagnosed patients with COPD. The number of nuclear lobes, the cell size, the number of vacuoles and the density of granulation were considered as characteristic features of eosinophils. Our results indicate that patients with an eosinophil concentration above 0.5 x 10^9/l and allergic complaints have an eosinophil population with an increased diameter. Treatment with corticosteroids did not affect morphological characteristics such as the number of vacuoles, the density of granulation, the cell diameter and the nucleus/cell surface ratio. However, the number of lobes per nucleus, a marker of eosinophil maturation, was significantly decreased under the influence of corticosteroids. This observation led to the conclusion that during corticosteroid treatment more immature or younger eosinophils are present in the blood.

Introduction

The eosinophilic granulocyte was first observed in human blood in 1879 by Paul Ehrlich (1). Since then, deviations of blood eosinophil counts have been documented in a number of diseases (2, 3, 4, 5, 6). Eosinophilic granulocytes are terminally differentiated effector cells of the immune system. Bone marrow eosinophilic promyelocytes and myelocytes are still capable of mitosis (7). Maturation concerns generation of a continuous flow of cells via myelocyte and metamyelocyte stages to an eosinophilic granulocyte. A substantial bone marrow storage pool of mature eosinophilic cells can be mobilized when needed (8). After finishing maturation, eosinophilic granulocytes leave the bone marrow and move into the blood circulation (chapter 1, figure 1).

Eosinophilic granulocytes are activated to release granule proteins and toxic enzymes. The eosinophil may reveal morphologic features with regard to the activation state. Immature eosinophils undergo a series of changes during maturation *in vivo* or *in vitro* (9). Eosinophilic myelocytes are rather large cells (10-18 μm diameter), which demonstrate a size reduction during maturation (10). Diameters of a mature eosinophilic granulocyte amount to 10-16 μm, which is similar to that of neutrophilic granulocytes (5). The nucleus is characteristically bilobed. The most characteristic morphologic feature of the eosinophilic granulocyte is its content of distinctive cytoplasmic granules (11), which stain avidly with acid dyes, such as eosin. Several studies concerning morphologic features of eosinophils have been performed by application of electron microscopy techniques (9, 12). This microscopic evaluation reveals small numbers of primary granules, lipid bodies and small granules in addition to the large specific granules (chapter 1, figure 2).

Activated eosinophils remain polymorphonuclear. Increased numbers of cytoplasmic lipid bodies, vesicles, tubules and glycogen appear. In eosinophils found in tissues, the number of empty specific granules (*e.g.* vacuoles) is increased (9). The presence of a

higher number of cytoplasmic vacuoles and loss of the dense core of specific granules is associated with previous secretion of granule contents. As a result, so-called tissue eosinophils can be recognized in the circulation as degranulated eosinophils (12). It has been shown that activated (hypodense) eosinophils contain less granules (13).

To diagnose and monitor patients with allergic diseases it would be useful to have additional parameters available that will not only reflect the presence of eosinophils but also their state of activation. To investigate this state of activation, we have studied the amount of released granule proteins, *i.e.* Eosinophil Cationic Protein, during blood clotting *in vitro*, and light microscopy of the cells. Light microscopy instead of electron microscopy may provide an easy method to establish deviations in morphology. Apparently healthy individuals and patients with allergic complaints were studied to evaluate the possibility of patient recognition by these eosinophilic activation markers. To monitor the effect of corticosteroids, patients with COPD were selected. These individuals were newly diagnosed COPD patients, who started with inhalation of corticosteroids after the first visit. Corticosteroid treatment has an opposite effect on the number of circulating neutrophils compared with eosinophils. Therefore, the effects on morphologic parameters in neutrophils were also investigated in these COPD patients.

Patients and methods

To establish morphologic characteristics of eosinophils and the serum ECP concentration, blood samples were drawn from 11 apparently healthy individuals (aged 18 - 65 years) and 9 individuals with eosinophilia (eosinophil count > 0.5 x 10^9/l) who did have allergic complaints but did not use corticosteroids. To monitor the effect of corticosteroids, newly diagnosed patients with COPD (n = 5) were selected. After the first visit, these patients received corticosteroids by inhalation. From each subject a serum sample and an anticoagulated blood sample was drawn (Vacutainer®, ref. 367703

SST with clot activator and ref. 367652 with K_3EDTA as an anticoagulant, Becton Dickinson, Plymouth, UK). After venepuncture, the blood samples were clotted immediately for serum preparation during 120 ± 10 minutes in a water bath of 37°C. After incubation, the samples were centrifuged for 10 minutes at 1350 x g at room temperature. Serum samples were stored at -20°C until the serum ECP concentration was assayed. Serum ECP concentrations were established by application of a fluorescence enzyme immuno-assay kit (Kabi Pharmacia, Uppsala, Sweden).

In the anticoagulated blood samples, eosinophilic granulocytes were counted on a Sysmex NE-8000 Haematology Analyser (Charles Goffin Medical Systems BV, Tiel, The Netherlands). Five blood smears were prepared for microscopic morphology evaluation, after staining with May-Grünwald Giemsa dye solution.

Morphologic features were established by a total of 14 experienced microscopists. A microscopist evaluated ten eosinophilic granulocytes per individual. Evaluation whether differences in morphology exist between apparently healthy individuals and patients with eosinophilia and allergic complaints was established by 9 microscopists. Effects of corticosteroid treatment on morphologic characteristics were investigated by 5 microscopists. Eight microscopist of the first group (n = 9) established morphologic features of one apparently healthy individual and one patient with eosinophilia. One microscopist of the first group (n = 9) evaluated 3 apparently healthy individuals and one patient with eosinophilia. This evaluation was performed without knowledge of the patient or healthy control nature of the samples by the microscopist. To monitor the effects of corticosteroids, results before and during treatment of one patient were investigated by one microscopist (n = 5).

For classification of morphologic features of eosinophils, the number of nuclear lobes, the number of vacuoles and the density of granulation were established. A nuclear lobe is defined to be present if the connection between two lobes is less than one third of the maximal width of the lobe. The density of granulation was established by comparison of the amount of granules in relation to the cytoplasm. Granules are orange-stained while the cytoplasm is coloured pink after staining with May-Grünwald Giemsa dye solution.

The density of granulation was divided in to four groups; 100% (whole cytoplasm orange-stained), 75 - 99%, 50 - 74% and less than 50% (half of the cytoplasm orange-stained).
Because variability between microscopists exists, we also established eosinophil morphology features by application of an automated blood-cell recognising system. By means of this blood cell evaluation system, the diameter and the nucleus/cell surface ratio of each scanned eosinophil was determined.
The same morphologic features as those described above were also determined by light miroscopy in the neutrophilic granulocytes of the COPD patients.

Statistics

For statistical analysis the computer program SPSS (Windows, release 6.1) was used. The statistical significance of differences between results from each group of subjects was assessed by application of ANOVA analysis of variance and T-test when appropriate. P-values below 0.05 were considered statistically significant.

Results

The number of vacuoles, the granulation density and the number of nuclear lobes in eosinophilic granulocytes did not reveal significant deviations when comparing results of patients with eosinophilia and apparently healthy control individuals (table I). In the patients with eosinophilia, larger eosinophilic granulocytes (mean diameter 15.3 μm) were observed than in apparently healthy individuals (mean diameter 12.4 μm). The nucleus/cell surface ratio was similar for both groups.

Table I: Characteristic measures of eosinophil morphology (expressed as mean ± standard deviation). Results refer to patients with eosinophilia (n = 9, [eosinophil] > 0.5 x 10^9/l) compared with apparently healthy controls (n = 11, [eosinophil] < 0.5 x 10^9/l). The density of granulation was divided into four groups: 100% (group 1), 75 - 99% (group 2), 50 - 74% (group 3), < 50% (group 4).

morphologic characteristics	apparently healthy subjects	patients with eosinophilia	p-value
vacuoles	1.2 ± 1.0	1.2 ± 0.8	n.s.
granulation density (group)	2	2	n.s.
nuclear lobes	2.1 ± 0.3	2.4 ± 0.2	n.s.
diameter (μm)	12.4 ± 3.0	15.3 ± 0.6	<0.02
nucleus/cell surface ratio (%)	39.4 ± 8.1	42.2 ± 3.4	n.s.

As a result of corticosteroid inhalation used by COPD patients (n = 5), the eosinophil blood counts significantly decreased, but the serum ECP concentrations remained unaffected during 4 weeks of steroid therapy (table II). The number of vacuoles, the granulation density, the nucleus/cell surface ratio and the cell diameter did not show alterations within 4 weeks of treatment with corticosteroids, whereas the number of lobes per nucleus decreased.

Neutrophil counts demonstrated an increasing trend during 4 weeks of steroid therapy. Morphologic characteristics, such as number of nuclear lobes, number of vacuoles per neutrophil and percentage of granulation did not show significant differences (table II).

Table II: Characteristics of eosinophil and neutrophil morphology (expressed as mean ± standard deviation) observed by light microscopy. Results concern 5 patients with COPD before and during inhaled corticosteroid therapy. The density of granulation was divided into four groups: 100% (group 1), 75 - 99% (group 2), 50 - 74% (group 3), < 50% (group 4).

morphologic characteristics	before corticosteroid therapy	after corticosteroid therapy for 4 weeks	p-value
eosinophil count (10^9/l)	0.2 ± 0.1	0.1 ± 0.1	<0.04
ECP (μg/l)	56 ± 23	52 ± 40	n.s.
ECP/eos (μg/10^9)	168 ± 44	709 ± 669	n.s.
eosinophil vacuoles	0.96 ± 1.0	0.6 ± 0.5	n.s.
eosinophil granulation density (group)	2	2	n.s.
nucleus/cell surface ratio	48.3 ± 5.0	49.2 ± 3.5	n.s.
diameter (μm)	13.6 ± 2.3	14.0 ± 2.7	n.s.
eosinophil nuclear lobes	2.4 ± 0.2	2.1 ± 0.2	<0.05
neutrophil count (10^9/l)	4.4 ± 0.8	7.2 ± 4.1	n.s.
neutrophil nuclear lobes	4.8 ± 0.3	4.9 ± 0.2	n.s.
neutrophil vacuoles	0.06 ± 0.13	0.28 ± 0.19	n.s.
neutrophil granulation density (group)	4	4	n.s.

Discussion

Arneth (14) has stated that more cells with a high level of maturation are present in patients with an eosinophil count in blood exceeding 0.5 x 10^9/l. We were unable to confirm this statement with the observation that patients with eosinophilia indeed have eosinophils with a significantly increased number of nuclear lobes and nucleus/cell surface ratio compared with apparently healthy individuals. However, in our study, the diameter of these cells in patients with eosinophilia was increased compared to the diameter of eosinophils of apparently healthy individuals. In patients with allergic complaints and eosinophilia, the occurence of a high number of eosinophils in blood may be a consequence of the requirement of marrow reserves to be transported to the tissues. In general, undifferentiated cells are larger compared to more mature eosinophils (15). It is not known whether younger eosinophils in blood are also larger compared to eosinophils in their last stage of life in blood. Probably, the group of patients with eosinophilia possesses more eosinophils in blood with a less differentiated appearance, recently released from bone marrow, than apparently healthy individuals do. Differences according to the activation state of eosinophils were not supported by morphologic deviations as observed by microscopic evaluation. The granulation density and the number of vacuoles in eosinophils from patients with eosinophilia were in agreement with the results obtained with eosinophils from apparently healthy subjects.

Glucocorticoids are anti-inflammatory drugs, which decrease the number of circulating eosinophilic granulocytes (16, 17, 18, 19, 20). Despite the extensive prescription of inhaled or orally supplied glucocorticoids in case of COPD, asthma or ulcerative colitis, the mechanism of corticosteroids on the eosinophilic maturation in bone marrow, the release into the blood and the requirement of eosinophils in tissues is poorly understood. The mediators that are affected by corticosteroids may suppress eosinophil maturation or eosinophil migration to inflammatory tissues (21). Nittoh *et al.* (22) have elucidated that glucocorticoids inhibit the survival of rat peritoneal eosinophils by enhancing eosinophil

apoptosis. In the present study it was shown that treatment of COPD patients with glucocorticoids results in the presence of a lower number of peripheral eosinophils, with a significantly decreased number of nuclear lobes. ECP serum concentrations did not decrease significantly. We conclude that corticosteroid treatment does not have a morphologically detectable effect on the eosinophil activity grade.

The decrease in the number of circulating eosinophils could be due to enhancement of apoptosis of mature eosinophils in tissues and/or an inhibitory effect on maturation in the bone marrow. However, this hypothesis was not confirmed by measuring the diameter of the cell, a morphologic marker of maturation. After 4 weeks of corticosteroid treatment, the circulating cells were of approximately the same size. The nucleus/cell surface ratio was also almost the same before and after 4 weeks of therapy with corticosteroids. However, it is remarkable that patients with COPD have higher nucleus/cell surface ratios than apparently healthy controls and patients with eosinophilia without allergic or inflammatory symptoms.

The possible effect of apoptosis on neutrophilic granulocytes due to corticosteroids (22) is not apparent from morphologic characteristics; significant deviations with respect to the percentage of granulation, the number of vacuoles and the number of nuclear lobes have not been established.

We conclude that the size of the eosinophilic granulocyte, as a parameter of maturation, can be used to discriminate between a group of patients with allergic complaints and eosinophilia and apparently healthy controls. However, a remarkable overlap of this marker was shown between patients and apparently healthy controls. Therefore, the individual predicting value of this marker is low. Effects of corticosteroid treatment in patients with COPD can be evaluated by measuring the number of nuclear lobes, which is considered a marker of maturation.

Other morphologic markers, such as the number of vacuoles and the density of granulation, can not be used, neither to discriminate between a group of apparently healthy controls and patients with eosinophilia nor to evaluate the effects of corticosteroids.

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