Decreased Expression of Eosinophil Peroxidase and Major Basic Protein Messenger RNAs During Eosinophil Maturation

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We evaluated the levels of mRNAs encoding cationic proteins in peripheral blood eosinophils (PBE) purified from patients with eosinophilia and in eosinophils differentiated from cord blood cells (CBC) by culture with recombinant human interleukin-3 (rhIL-3), rhGM-CSF, and rhIL-5. Messenger RNAs encoding eosinophil peroxidase (EPO), major basic protein (MBP), eosinophil-derived neurotoxin (EDN), and eosinophil cationic protein (ECP) were detected by Northern blot hybridization with the respective specific oligonucleotide probes. In mature PBE, MBP mRNA appeared to be absent, whereas EPO mRNA was barely detectable in only 5 of the 19 patients. In contrast, EDN and ECP mRNAs were observed in the PBE of all patients. In CE, EPO, and MBP, mRNAs were abundant in immature eosinophils and their amounts decreased after differentiation toward eosinophils. ECP and EDN mRNAs followed the same patterns, but mRNAs were less abundant at all timepoints studied. Study of mRNA t1/2 during the time course of differentiation indicated that changes in the stability of the different mRNAs were not responsible for the variations observed in the steady-state levels. Together, these results suggest that regulation of expression differs among EPO, MBP, EDN, and ECP mRNAs during the time course of eosinophil differentiation.

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PURIFICATION of eosinophils from patients with eosinophilia has led to identification of eosinophil subpopulations differing by their cell density. Hypodense eosinophils can be distinguished from normodense eosinophils by impaired oxidative metabolism, increased cytotoxic function against parasite targets, and increased expression of various membrane receptors. All these biologic properties involve newly or preformed eosinophil mediators such as lipid mediators (LTC4, PAF-acether) or cationic proteins (eosinophil peroxidase, major basic protein, eosinophil-cationic protein, eosinophil-derived neurotoxin; EPO, MBP, ECP, and EDN, respectively). The biochemical and functional properties of these proteins have been well studied. MBP is a 14-Kd protein that is toxic to helminth parasites, tumor cells, and host cells. MBP has no recognized enzymatic activity. EPO, so called for its peroxidase activity, consists of two polypeptides of 15 and 55 Kd and is toxic to parasites, bacteria, and tumor or host cells. ECP and EDN are proteins of 18-21 Kd that share sequence similarity and ribonuclease activity. They have been implicated in causing cell damage and in neurotoxicity. These proteins can be detected in many tissues and biologic fluids. Their release has been observed after activation with various stimuli such as interleukins or immunoglobulins. After immunoglobulin-dependent activation, preferential release of EPO and MBP under IgE stimulation, in contrast to release of ECP under IgG activation, has allowed us to suggest a selectivity in mediator release. Whereas many studies have examined the biologic and biochemical properties of these cationic proteins, considered as preformed proteins, very few reports have studied their synthesis by eosinophils. In this regard, cloning of the various cDNAs encoding cationic proteins and discovery of the potentiality of cord blood cells (CBC) to differentiate into eosinophils in the presence of interleukins have recently provided useful tools to approach this question.

We evaluated the levels of mRNAs encoding EPO, MBP, EDN, and ECP in peripheral blood eosinophils (PBE) from patients with eosinophilia. The surprising results obtained with the PBE led us to measure the levels of the mRNAs encoding the four cationic proteins at various stages of eosinophil maturation present in a CBC culture system.

MATERIALS AND METHODS

Reagents. Recombinant human interleukin-5 (rhIL-5) produced in yeast was a gift from Dr J. Tavernier (Roche Research, Gent, Belgium). rhIL-3 produced in Escherichia coli and rhGM-CSF were a gift from K. Rüedi (Sandoz, Basel, Switzerland).

Source of human eosinophils. Eosinophils were obtained from heparinized venous blood of patients with eosinophilia of various etiologies: four patients with parasitic infections, two patients with allergic diseases, and 13 patients with hypereosinophilic syndrome. Informed consent was obtained from all participants.

Purification of eosinophils from patients. As described previously leukocytes were obtained after dextran sedimentation. The leukocyte-rich supernatant was collected, washed in Tyrode’s buffer, and layered onto a discontinuous metrizamide gradients (Nyegaard, Oslo, Norway). After centrifugation at 1,200g for 45 minutes at 15°C, cell fractions were collected from each layer. The degree of purity of eosinophils was estimated by cytofluorimetric preparations stained with Giemsa (RAL 555, Société Chimique Pointet Girard, Clichy, France). Cell preparations containing more than 85% eosinophils were used in these studies.

CBC cultures and eosinophil differentiation in liquid medium. Mononuclear cells were isolated from umbilical CBC samples by centrifugation over Ficoll-Hypaque solution as described previously. Cells were cultured at 37°C in RPMI 1640 medium ( Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) 50 μmol/L, 2 mercaptoethanol, 2 mmol/L-glutamine, and antibiotics. Eosinophil differentiation was obtained by addition of a mixture of rhIL-3 (2 × 10-10 mol/L), rhGM-CSF...
was replaced every week. Cells were enumerated once weekly.

was demonstrated by incubation of the slides in phosphate buffer containing (2 x 10^{-10} mol/L), and rhIL-5 (1 U/mL). Medium containing IL was replaced every week. Cells were enumerated once weekly. Differential counts of cells were based on 400 cells in cytocentrifuged preparations (Shandon, Pittsburgh, PA) stained with Giemsa.

**EPO staining.** EPO was specifically detected by a technique based on the resistance of EPO enzyme to cyanide. Cells were cytocentrifuged and fixed for 30 seconds in formalin-acetone. EPO was demonstrated by incubation of the slides in phosphate buffer containing 3.3' diaminobenzidine tetrahydrochloride, NaCN, and H2O2. Cells were counterstained with hematoxylin. To evaluate the percentage of EPO-positive cells, 300 cells from randomly selected fields were counted every week.

*Isolation and Northern blot analysis of total cellular RNA.* Total RNA from PBE purified from patients with eosinophilia or from cultures of eosinophils was isolated with the guanidine isothiocyanate-cesium chloride method. Ten micrograms total RNA was fractionated on a 1.2% agarose/formaldehyde gel and transferred to nylon membrane (Hybond-N, Amersham, Les Vles, France) in 10× SSC (SSC is 0.15 mol/L NaCl, 0.015 mol/L Na3 citrate). After ultraviolet cross-linking, the filters were hybridized with 32P-labeled probes in hybridization mix [40% formamide, 10% dextran sulfate, 4× SSC, 20 mmol/L Tris-HCl pH 7.4, 1× Denhardt's solution, 20 μg/mL denatured salmon sperm DNA, 0.1% sodium dodecyl sulfate (SDS)]. After hybridization, the filters were washed twice in 2× SSC, 0.05% SDS at 42°C, and twice in 1× SSC, and 0.05% SDS at 55°C for 30 minutes each time.

The same blots were used to test MBP, EPO, EDN, ECP, and actin probes in identical conditions and to limit the amounts of RNA used in these experiments. Quantitation of hybridization was determined by scanning densitometry. To study the stability of mRNAs, cells were exposed to actinomycin D for 0 to 10 hours at day 16 and at day 27 before isolation of total RNA.

**Probes.** Oligonucleotides were synthesized on a DNA Synthesizer (Cyclone plus, Milligen/Biosearch, Burlington, MA), on the basis of the published sequences: EPO, 5'-ACACCTCGTTCTGC-CCACGAAACCTGTCCTCGGCTTCGAA; MBP, 5'-TGCCGGCATTTGGAATTCGATCGAGGGGGGTCA-GACT; EDN, 5'-GTCGACATTTTTGAGAGTTTATT-TAGTACGAGATGCTATATTTGAGT; ECP, 5'-CCACGCAAGAGCTGTCACGCCGGACGCTGTGATCCTGCC-TCCTAAC; and actin probe was radiolabeled with a 32P-dCTP (3,000 Ci/mmol; Amersham) by the random priming method.

**RESULTS**

*Northern blot analysis of mRNAs encoding cationic proteins in PBE.* The amounts of total RNA isolated from highly purified PBE were very low, ranging between 20 and 40 μg/10⁸ eosinophils. These RNA samples were isolated and tested individually for each probe soon after purification. The limited amounts of RNA obtained for each patient did not allow us to repeat the procedure to compare RNA from all patients on the same Northern blot. Therefore, a composite figure representing the results of seven patients in 19 preparations tested is shown in Fig 1. Northern blot analysis of PBE RNAs was performed with EPO, MBP, EDN, and ECP oligonucleotide probes. Highly purified populations of eosinophils were used to prevent potential hybridization of EDN and ECP probes with neutrophil RNA. Very weak hybridization signals were detected with the EPO probe. Studies performed on preparations of eosinophils obtained from different patients (n = 19) showed that EPO mRNA was present in only five cases (three are shown in Fig 1, lanes c through e). The transcripts of MBP were undetectable (or present at levels below detectable limits) in all preparations of eosinophil tested (n = 15). In contrast, mRNAs encoding EDN and ECP were highly expressed in all eosinophil populations (n = 15). No correlation with the cell density of eosinophils was noted.

*Eosinophil differentiation from CBC.* The kinetics of appearance of eosinophils in culture was followed using EPO-specific staining in 27 cultures of CE; mean values ± SD of EPO⁺ cells are shown in Fig 2A. A homogeneity was observed in the kinetics of differentiation at the beginning and end of the cultures, but variations in the timing of differentiation were underscored by SD at days 15 and 21. The percentage of EPO-positive cells in parallel with the nature of the contaminating cells and the evolution of eosinophil differentiation (Table 1). During the first week of culture with rhIL-3, rhGM-CSF, and rhIL-5, the total cell number did not vary, whereas EPO-positive cells began to appear. At day 7, EPO-positive cells consisted of 25% promyelocytes and 10% myelocytes. From day 8 to day 21, the total cell numbers greatly increased as the percentage of eosinophils increased and reached a maximum of 90% at
were evaluated at various times of the culture. The percent-age of eosinophils (mean ± SD). Cells were cultured with rhlL-3 (2 × 10⁻¹⁰ mol/L), rhGM-CSF (2 × 10⁻¹⁰ mol/L), and rhlL-5 (1 U/mL) for 34 days, and differentiation was followed by EPO-specific staining. (B) Eosinophil differentiation in one representative culture of CBC. Cells were cultured with rhlL-3, rhGM-CSF, and rhlL-5 for 34 days. The percentage of EPO⁺ cells and the total number of viable nonadherent cells were evaluated at various times of the culture.

Fig 2. (A) Kinetics of eosinophil differentiation in 27 CBC cultures (mean ± SD). Cells were cultured with rhlL-3 (2 × 10⁻¹⁰ mol/L), rhGM-CSF (2 × 10⁻¹⁰ mol/L), and rhlL-5 (1 U/mL) for 34 days, and differentiation was followed by EPO-specific staining. (B) Eosinophil differentiation in one representative culture of CBC. Cells were cultured with rhlL-3, rhGM-CSF, and rhlL-5 for 34 days. The percentage of EPO⁺ cells and the total number of viable nonadherent cells were evaluated at various times of the culture.

day 21. During this period, every step of eosinophil lineage (promyelocyte, myelocyte, metamyelocyte, and segmented eosinophils) could be identified in the culture. The number of cells then began to decrease, and a low number of monocytes appeared in the cultures.

Northern blot analysis of cationic protein mRNAs in cultured eosinophils. The results of one representative culture in seven experiments are shown in Fig 3. Cells were harvested at different times of culture for extraction of total cellular RNA. Northern blots hybridized with EPO, MBP, EDN, ECP, and actin probes are shown and the band intensities normalized to actin signal hybridization are shown in arbitrary units on the graph (Fig 3). The results shown in Fig 3 were obtained on the same Northern blot. Although one point of the kinetics study was deleted (between day 21 and 34) to eliminate degraded RNA, the integrity of hybridization signals obtained with the various probes (particularly at day 34) indicates the absence of RNA degradation in the other points of the kinetic study (Fig 3). At day 0, no cationic protein mRNA was detected in the cells with the four oligonucleotide probes. The intensity of the hybridization signals of the four mRNA species increased until day 21 and paralleled cell growth and eosinophil differentiation. The maximum amounts of mRNAs were detected at approximately day 21 for each probe. The levels of mRNAs encoding the four proteins decreased dramatically at day 34. The absence of contaminating neutrophils observed in the differential cell counts (Table 1) allowed us to discard participation of these cells in the Northern blot results.

Stability of cationic protein mRNAs. The stability of EPO, MBP, EDN, and ECP mRNAs was measured at day 16 and 27 of differentiation by exposing the cells for 0 to 12 hours to actinomycin-D, which blocked greater than 95% of total RNA synthesis. Cell viability was more than 90% during the experiments, as determined by Trypan blue exclusion (data not shown). Using the oligonucleotide probes, we measured the t½ of the four cationic protein mRNAs in two different experiments. They were more than 12, 10 to 11, 5 to 6, and 7 to 8 hours for EPO, MBP, EDN, and ECP mRNAs, respectively, at day 16 of culture. To determine whether the differences in mRNA levels could be attributed to variations in the stability of mRNAs during the course of differentiation, the t½ of mRNAs was calculated at day 27, after the decrease in the different messengers and before their complete disappearance. As shown in Fig 4, the t½ of EPO, MBP, EDN, and ECP mRNAs was quite similar (>12, 8 to 9, 4 to 5, and 4 to 5 hours, respectively) at day 27 in comparison to day 16.

DISCUSSION

We report the existence of variations in the steady-state levels of mRNAs encoding the different cationic proteins of eosinophil granules. First, our study performed on a significant number of patients with eosinophilia (n = 19) clearly indicates the very low amounts of total RNA that could be extracted from highly purified blood eosinophils (percentage of purity ranging between 85% and 98%), perhaps owing to their degree of differentiation, because increased amounts of RNA could be isolated from eosinophils differentiated in vitro from CBC. Besides these quantitative aspects, our results suggest that, surprisingly, mRNAs encoding EPO and MBP were absent or barely detectable in mature blood eosinophils, in contrast to EDN and ECP mRNAs, which were easily detectable. The absence of EPO and MBP mRNAs in eosinophils from patients with eosinophilia led us to ensure that the result was not due to a
Fig 3. Northern blot analysis of CBC transcripts. Total RNAs were isolated from CBC at days 0, 8, 12, 21, and 34. Blots were hybridized with probes encoding EPO, MBP, EDN, ECP, and actin. Messenger RNA species were detected at the corresponding molecular size (EPO, 3.3 to 3.5 kb; MBP, EDN, and ECP, ~1 kb). Exposure time of the autoradiograph was 48 hours at -70°C. Band intensities were determined by densitometry scanning, reported to the value obtained at day 0 with the actin probe, and expressed in arbitrary units in the corresponding graph.

Fig 4. Analysis of the $t_{1/2}$ of cationic protein mRNAs during eosinophil differentiation. The $t_{1/2}$ was measured at days 16 (□) and 27 (○) of culture by incubation of cells with actinomycin-D for the indicated times; 10 μg equalized RNA was subjected to Northern blot analysis and hybridized with EPO, MBP, EDN, ECP, and actin probes. Band intensities were determined by densitometry scanning and reported in percentage of the value obtained at time 0 of actinomycin-D treatment. All values were normalized to actin hybridization. The $t_{1/2}$ was calculated as the time when 50% of the mRNA was degraded.

technical problem. The purification procedure of eosinophils on metrizamide gradients was not responsible for the absence of MBP and EPO mRNAs because detectable levels of these mRNAs were obtained before or after centrifugation of CBC on the same gradients (data not shown). Furthermore, the ability of the oligonucleotide probes used in this study to hybridize to target mRNA was controlled by obtaining the appropriate signals with EPO and MBP probes on cultured eosinophils (CE) mRNAs.

The absence or low expression of EPO and MBP mRNAs in PBE prompted us to follow the steady-state levels of cationic protein mRNAs at various stages of eosinophil maturation. The CBC culture with addition of IL-3, GM-CSF, and IL-5 was used as a model system to investigate eosinophilic differentiation. Northern analysis showed that the amounts of all four mRNAs (EPO, MBP, EDN, and ECP) progressively increased until day 21 and then decreased. A comparison between the results obtained with PBE and CE clearly indicates that using the same probes on both types of RNA caused marked differences in the amounts of mRNA encoding the four cationic proteins, which could not be due to technical problems. The reduction in mRNA amounts in CE closely paralleled the increased number of differentiated cells, as observed in the precise follow-up study of differential cell counts, suggesting that eosinophil maturation was accompanied by a profound decrease in the steady-state levels of cationic protein mRNAs. Several mechanisms, such as variations in mRNA stability during the time course of eosinophil differentiation or modifications in the levels of transcription could explain the regulation of the cationic protein mRNAs. No evident differences were noted in the $t_{1/2}$ of mRNAs between immature or differentiated cells, indicating that the stability of mRNAs did not play an important role in the variations of cationic protein mRNA levels. These results suggest that the cationic protein mRNA levels are determined primarily through regulation of transcription. A similar downregulation of mRNA during differentiation has already been described for myeloperoxidase (MPO) in neutrophil lineage.2,3 Differentiation of HL60 cells by dimethylsulfoxide (DMSO) is a model system that has allowed rapid studies of the mechanism of MPO regulation. In HL60 cells, neither a selective decrease in
MPO transcription nor a decrease in mRNA stability could explain the decreased levels of MPO mRNA. Another mechanism involving alteration in posttranscriptional processing of MPO mRNA has been proposed. In addition to many common biologic and biochemical features of EPO and MPO, the two proteins appear to be regulated in the same manner. The absence of RNAs encoding EPO and MBP in PBE in the same cells in which EPO and MBP proteins are detected favors the view that these proteins have long t1/2. Such stability could be related to their translation as precursor proteins. They could be synthesized at an early stage of differentiation and stored in the granules of mature eosinophils until their release.

In our experiments, low levels of EPO mRNAs were detected in 5 of 19 preparations of eosinophils tested from patients with eosinophilia. The detection of EPO mRNA in these patients might suggest the presence of some immature eosinophils in PB. Examination of a larger number of patients with eosinophilia of various etiologies would be useful to examine a possible relation with the pathology.

In contrast to EPO and MBP mRNAs, the detection of EDN and ECP mRNAs in all preparations of PBE suggested that mature eosinophils, in addition to previously described proteins, still possessed the capacity to synthesize EDN and ECP. Variable levels of ECP and EDN mRNAs were detected in individual patients, probably related to the different states of eosinophil activation. The absence of EPO and MBP mRNAs linked to a downregulation of mRNA levels during differentiation suggests the existence of distinct regulatory mechanisms for EDN and ECP mRNA expression.

The recent cloning of the genes encoding EPO, MBP, EDN, ECP, and the comparison of their 5' flanking region sequences have shown intriguing similarities and differences around several nucleotide blocks. Functional analysis of these different regions will certainly provide useful information concerning regulation of the mechanisms controlling transcription of these proteins. In addition, measurement of mRNA levels of lineage-specific proteins represents an important first step in the understanding of the regulation of lineage-specific gene expression. The present study underlines the interesting possibilities arising from CBC cultures. Regulation and control of hematopoiesis by interleukins and regulation of synthesis of various mediators, as well as pharmacologic studies can be envisaged in this experimental system.

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