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Expression of eosinophil peroxidase in the immature basophil cell line KU812-F

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Abstract Although peroxidase activity in basophils can be detected by optical and ultrastructural cytochemistry, its characteristics remain to be determined. We have demonstrated the characteristics of peroxidase activity induced in the immature basophil cell line, KU812-F. Ultrastructually, peroxidase activity was detected in granules as well as in the perinuclear space and endoplasmic reticulum. Immunocytochemisty revealed that KU812-F cells were stained by anti-eosinophil peroxidase antibodies, and eosinophil peroxidase mRNA, not myeloperoxidase, was detected in the cells using Northern hybridization and reverse transcription–polymerase chain reaction. Eosinophil peroxidase can be one of the molecules shared with eosinophils and basophils. The biological function of eosinophil peroxidase detected in basophils remains uncertain.
Keywords: Basophils; Eosinophil peroxidase; Immunocytochemistry; KU812-F; mRNA.

1. Introduction

Peroxidase activity is found in the cytoplasmic granules of neutrophils, monocytes and eosinophils [1,2]. Myeloperoxidase (MPO) is present as a heme-containing glycoprotein in the primary granules of neutrophils and granules of monocytes [3], and it plays an important role in the oxygen-dependent microbicide system [4]. Eosinophil peroxidase (EPO) is also a heme-containing glycoprotein which is present in granules of eosinophils, and it acts as a potent toxin for parasites [5] as well as a mediator of several hypersensitivity reactions [6]. Although MPO and EPO have similar enzymatic functions and high homology of amino acid sequences, their genes are mapped in different locations [7,8]. EPO is detected in eosinophils of patients with congenital MPO deficiency [9], therefore EPO is under separate genetic control from MPO. Enzymatic reaction of EPO, but not MPO, is resistant to inhibition by potassium cyanide [10].

Some works have detected peroxidase activity in basophils by cytochemical reaction at the optical and electron microscopical level [11–13]. The biochemical function of peroxidase activity detected in basophils remains unknown, and the molecule of peroxidase in basophils has not been identified.

It has been demonstrated that the ability of the immature basophil cell line KU812-F [14]—one of the subclones of KU812 established from a patient during blast crisis of chronic myelogeneous leukemia (CML) [15]—to differentiate to basophils is enhanced by the addition of interleukin 3 (IL-3) [16]. In this report, we demonstrate peroxidase activity and expression of EPO mRNA in KU812-F.

2. Materials and methods

2.1. Cell culture

KU812-F cells were maintained in RPMI 1640 medium (Nipro, Tokyo, Japan) supplemented with 10% fetal bovine serum (FCS, Sanko Junyaku, Tokyo, Japan) in a CO2 incubator (5% CO_2) at 37°C. The cells were harvested after 3 days of cultivation

with 20 ng/ml of recombinant human IL-3 (rhIL-3; provided kindly by Kirin Brewery Co. Ltd., Tokyo, Japan). The HT93 cell line was used as a positive control for eosinophil peroxidase and myeloperoxidase expression. The cell line established recently in our laboratory, from a patient with acute promyelocytic leukemia, was myeloperoxidase-positive and differentiated to eosinophils by the addition of all-*trans* retinoic acid (ATRA) and granulocyte-colony stimulating factor (G-CSF) to the culture [17]. The differentiation-induced HT93 to eosinophils in the culture developed eosinophil peroxidase in the cytoplasm. HT93 cells were maintained in RPMI 1640 medium supplemented with 10% FCS. The cells were harvested after 5 days of cultivation with 10 μM ATRA (Sigma, St. Louis, MO) and 50 ng/ml of G-CSF (kindly provided by Kirin Brewery Co. Ltd., Tokyo, Japan).

2.2. Cytochemistry

Cells were stained with May–Grunwald–Giemsa as well as 1% toluidine blue in absolute methanol [18] to observe the metachromatic change. The cytochemical optical peroxidase reaction was tested using diaminobenzidine (DAB).

2.3. Ultrastructual peroxidase reaction

The harvested cells were fixed in 1.25% glutaraldehyde in cold Gey's buffer (pH 7.2) (Gibco BRL, USA) for 10 min at room temperature. After three washes using Gey's buffer, cells were incubated in DAB solution containing 20 mg of DAB and 0.1 ml of 1% H_2O_2 in 10 ml of 50 mM Tris–HCl buffer. The pH was readjusted to 7.6 with 1 M sodium hydroxide. Incubation was carried out in a dark room at room temperature for 1 h. Then the cells were rinsed in several changes of 0.1 M phosphate buffer (pH 7.2) and postfixed in 1% osmium tetroxide in phosphate buffer for 30 min.

2.4. Northern hybridization and reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA was extracted using ISOGEN™ (Nippon Gene, Toyama, Japan) from HT93 and KU812-F cells. Total RNA (20 μg) was separated on 1% agarose gel in a formaldehyde–MOPS buffer (pH 7.0), and transferred onto a nitrocellulose membrane filter. The filter was hybridized with ^{32}P-labeled cDNA fragments of eosinophil peroxidase (nucleotide position 2006–2390 in the cDNA; Gene Bank X14346) and myeloperoxidase (nucleotide position 1430–1870 in the cDNA; Gene Bank M19507) in a solution containing 50% formamide, 5× SSC, 50 mM sodium phosphate (pH 6.5), 5× Denhaldt's solution, 250 μg/ml heat-denatured salmon sperm DNA at 42°C, according to the method described by Thomas [19]. We performed RTPCR to confirm the expression of EPO mRNA in KU812-F cells. First strand cDNA was synthesized in a total volume of 20 μl using 1 μg of total RNA and 100 pmol of random hexadeoxynucleotide with 10 U of RAV-2 reverse transcriptase (Takara, Tokyo, Japan). cDNA (0.5 μl) was amplified in a reaction volume of 20 μl containong1Uof rTaq polymerase (Takara, Tokyo, Japan) and 0.2 mM dNTP with primers for EPO (sense: 5'-CACGGTTT CAAGGGACATC, antisense: 5'-CTTT TCTTGCCTGGGGTG) and MPO (sense: 5'-TCTTACTTCGGGAGCACAAC, antisense: 5'CTGCAATTTGGTTCTGACGA). As an internal control, β-actin gene was amplified with sense primer (5'-ATCATG TTTGAGACCTTCAA) and antisense primer (5'-CATCTCTTGCTCGAAGTCCA). The PCR condition was 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, sample-dependent annealing temperature (EPO 60°C, MPO 55°C, β -actin 54°C) for 1 min and 72°C for 1 min. PCR products were electrophoresed on 3% agarose gel and stained with ethidium bromide.

2.5. Immunocytochemistry

The expression of MPO and EPO in KU812-F cells was examined using monoclonal antibodies. Cells were fixed in pure methanol for 5 min on a glass slide, and then stained by the direct or indirect immunofluorescence method for MPO or EPO, respectively. In brief, cells were incubated with anti-eosinophil peroxidase (anti-EP, IgG2a; Nichirei, Tokyo, Japan) at room temperature for 2 h, washed, then stained using fluorescein isothiocyanate

(FITC)-conjugated goat anti-mouse IgG (Becton Dickinson, USA) at room temperature for 30 min in the dark, or cells were directly stained using FITC-conjugated anti-myeloperoxidase (Caltag, San Francisco, USA) at room temperature for 2 h.

3. Results

3.1. Basophil differentiation of KU812-F

About 30% of KU812-F cells showed metachromasia by toluidine blue staining before culturing. After 3 days of cultivation with IL-3, metachromasia-positive cells increased to 46%, and the cells showed basophilic granules (Fig. 1a,b). Therefore, the ability of the cell line to differentiate to basophils was enhanced by the addition of IL-3 in the culture.

3.2. Peroxidase reaction of KU812-F

About 5% of KU812-F cells cultured with IL-3 showed a peroxidase reaction in cytochemical examination (Fig. 2a). Ultrastructual cytochemistry showed a positive reaction in the granules, with a stippling appearance, as well as in the rough endoplasmic reticulum and perinuclear space (Fig. 2b).

3.3. Northern hybridization and RT-PCR

We performed Northern hybridization using ^{32}P-labeled cDNA fragments of EPO and MPO. A strong signal of EPO mRNA was detected in the lane of HT93 cells cultured with ATRA and G-CSF at about 2500 base. Faint signals were detected in the lanes of the control KU812-F cells and KU812-F cultivated with IL-3 (Fig. 3a). On the other hand, the signal of MPO mRNA was detected in HT93 cells at about 3200 base, but not in KU812-F cells (Fig. 3b). EPO mRNA expression was also observed in KU812-F cells cultured with IL-3 using RT-PCR as well as in HT93 cells cultured with ATRA and G-CSF. On the other hand, MPO mRNA expression was observed only in HT93 cells cultured with ATRA and G-CSF (Fig. 4).

3.4. Immunocytochemistry

A portion of the KU812-F cells cultured with IL-3 for 3 days was stained with anti-EP antibody and exhibited fluorescence of the granules and perinuclear space (Fig. 5). The proportion of EPO-positive KU812F was identical with that of cytochemical peroxidase reaction -positive cells. On the other hand, MPO was absent in these cells (data not shown).

4. Discussion and conclusion

Basophils play an important role in allergic reaction in cooperation with eosinophils, mast cells, memory T-cells and B-cells. Because of the low frequency of the cells in blood, the kinetics and function of basophils have been poorly understood. Peroxidase activity of basophils has been shown by cytochemical reaction [11–13]. However, the biochemical function of the activity remains uncertain, and the molecule has not been identified.

In the present study, we demonstrated that the immature basophil cell line, KU812-F, had peroxidase activity in the cytoplasmic granules. Furthermore, we demonstrated using immunocytochemistry that they expressed eosinophil peroxidase. We also detected the expression of EPO mRNA using Northern hybridization and RT-PCR.

Dvorak suggested that basophils differentiated from cultured cord blood did not synthesize peroxidase, and their peroxidase activity detected in the cytoplasmic granules originated from the vesicular uptake of peroxidase molecules released from eosinophils [20]. However, we demonstrated that KU812-F cells had peroxidase activity not only at the cytoplasmic granules, but also in the rough endoplasmic reticulum and perinuclear space (Fig. 2b), without co-cultivation with eosinophils. Hence, the peroxidase activity detected in the granules was produced by the KU812-F cells themselves. Moreover, we demonstrated the expression of EPO mRNA in the KU812-F cells using Northern hybridization and RT-PCR (Fig. 3a and Fig. 4). These results reveal that KU812-F cells themselves synthesized EPO.

Several reports indicate that both eosinophils and basophils differentiate from their common precursor. Schmidt et al. demonstrated hybrid granulocytes containing eosinophilic and basophilic

granules in peripheral blood of patients with CML [21]. Joshua et al. also reported that eosinophil/basophil hybrid granulo -cytes differentiated from cultured normal cord blood [22]. KU812-F may have common characteristics of eosinophils and basophils, or part of the cells may differentiate to eosinophils. However, there have been no reports which demonstrated the differentiation of KU812-F to eosinophils. Moreover, we did not in fact observe any eosinophils in the cultured KU812-F cells. KU812-F cells are cultured leukemic cells and their characteristics should not be identical with normal human basophils, so we also examined purified basophils from human peripheral blood by immunocytochemistry and found that half of them had peroxidase activity and EPO antigen (unpublished).

The positive percent of EPO was less than that of toluidine blue metachromatic cells in cultured KU812F (Fig. 1b, Fig. 2a, Fig. 5); moreover, in the experiments done using Northern hybridization, signals of EPO mRNA in KU812-F were faint, and there was no difference between the signal intensities of the control and IL-3 induced KU812-F cells (Fig. 3a). These results may be due to the partial expression of EPO in human basophils, while almost all human eosinophils do have EPO in the granules.

Eosinophils and basophils have common cytoplasmic proteins, such as eosinophil major basic protein and Charcot–Leyden crystal protein [23]. Recently, it was shown that both eosinophils and basophils have high affinity IgE receptors [24] and macrophage inflammatory protein-1 [25]. The biological function of EPO produced by basophils remains uncertain. It may inactivate the slow reacting substance, as EPO produced by eosinophils [26]. Further investigation is necessary to elucidate the functional relationship and differentiation of eosinophils and basophils.

Acknowledgements

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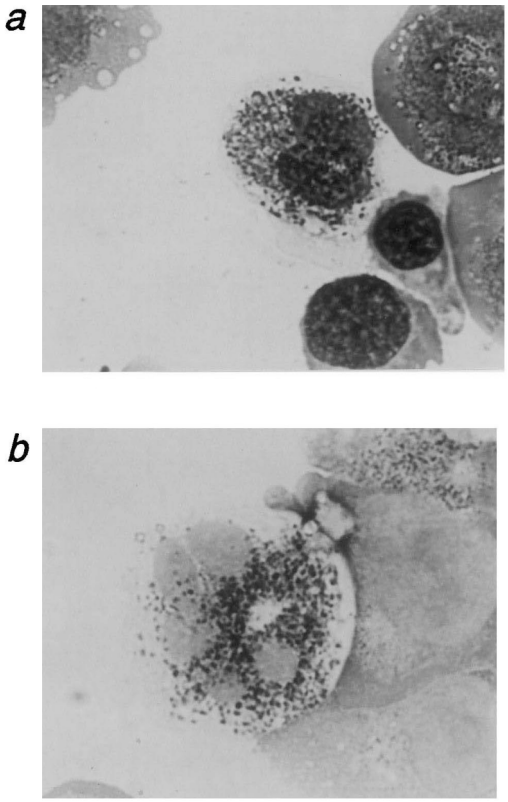

Fig. 1. Morphology of KU812-F cell line cultured with IL-3 for 3 days. May-Grunwald-Giemsa staining (a) and toluidine blue staining (b).

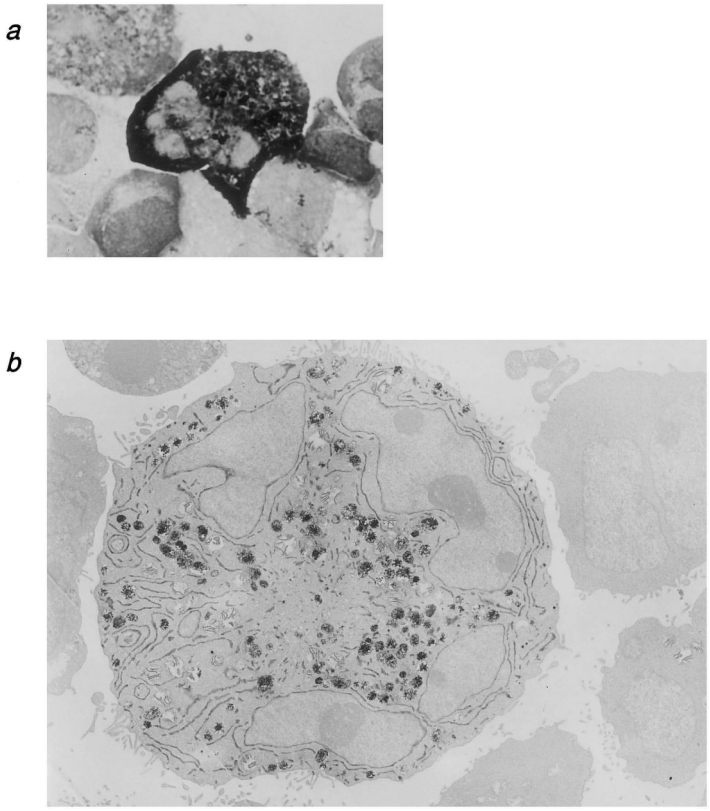

Fig. 2. Peroxidase reaction of KU812-F cell line. (a) Optimal peroxidase reaction was positive in some KU812-F cells. (b) Ultrastractual peroxidase reaction was positive at the granules with a stippling appearance, as well as rough endoplasmic reticulum and perinuclear space (×5300).

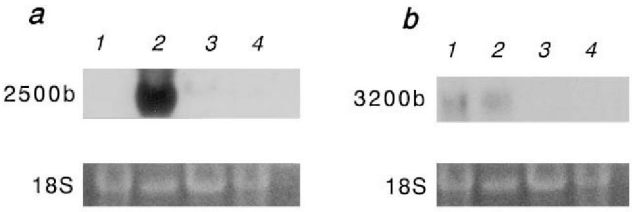

Fig. 3. Northern blot hybridization of MPO mRNA and EPO mRNA. Lane 1, HT93; lane 2, HT93 with ATRA and G-CSF; lane 3, KU812-F; lane 4, KU812-F with IL-3. Total RNA (20 μg) was separated on 1% agarose–formaldehyde gel, and transferred onto a nitrocellulose membrane filter. The filter was hybridized with 32Äup0Änosupersub P-labeled cDNA fragments: (a) hybridization with eosinophil peroxidase cDNA fragment; (b) hybridization with myeloperoxidase cDNA fragment. Ethidium bromide staining of 18S rRNA is shown for integrity and load of total RNA from all cells.

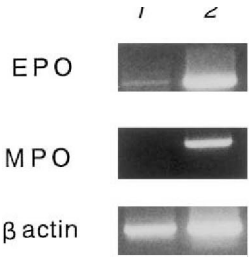

Fig. 4. RT-PCR of MPO and EPO. Lane 1, KU812-F with IL-3; lane 2, HT93 with ATRA and G-CSF. PCR products were electrophoresed on 3% agarose gel and stained with ethidium bromide. Lengths of products were 383 bp for EPO, 441 bp for MPO. As an internal control, β-actin gene was amplified.

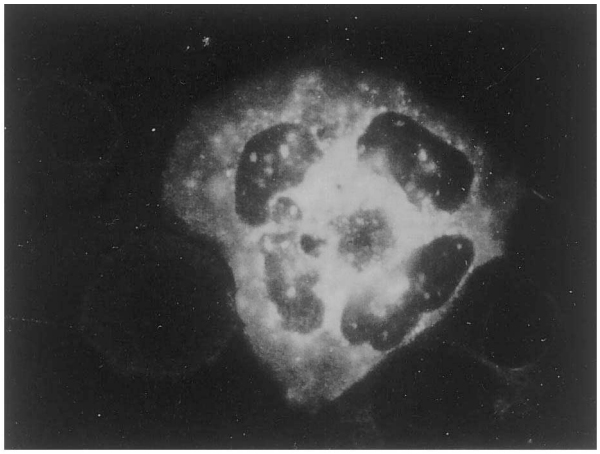

Fig. 5. Immunocytochemistry using the anti-eosinophil peroxidase antibody. A part of KU812-F cells cultured with IL-3 for 3 days was stained using the anti-eosinophil peroxidase antibody and FITC-conjugated second antibodies. Antibodies were demonstrated at the granules and perinuclear space.