Effect of Recombinant Interleukin-5, Interleukin-3, and Eotaxin on Apoptosis in Eosinophilic Granulocytes

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We studied *in vitro* effects of recombinant interleukin-5, interleukin-3, and eotaxin on programmed death of eosinophils from healthy donors and patients with non-Hodgkin's lymphomas associated with severe blood eosinophilia. Interleukin-5 and eotaxin produced the most potent antiapoptotic effect on eosinophils from healthy donors. In patients with non-Hodgkin's lymphomas, spontaneous apoptosis in eosinophilic leukocytes was low and remained unchanged during incubation with recombinant proteins.

Key Words: *apoptosis; eosinophils; recombinant proteins; cytokines*

An increasing interest to apoptosis, or genetically determined cell death, is related to the necessity of revising the mechanisms maintaining immune homeostasis and oncogenesis. Some molecular mechanisms of apoptosis closely related to the system of intracellular and intercellular signaling were estimated in recent years [4]. Impaired apoptosis leads to the development of diseases associated with apoptosis activation or inhibition [5,6].

The balance between pro- and antiapoptotic factors is responsible for the maintenance of the equilibrium between cell proliferation, differentiation, and elimination in the organism [5]. The mechanisms of cytokine-mediated apoptosis are of particular interest in this respect. Cytokines belong to a large group of bioactive substances. Much attention is paid to the effect of cytokines on apoptosis. Many cytokines, including interferon-γ (IFN-γ), tumor necrosis factor-α, interleukin-1 (IL-1), and IL-10, induce apoptosis in normal and malignantly transformed cells [1,5,12]. Various mediators (*e.g.*, IL-2, IL-3, IL-4, IL-10, IFN-γ, and growth factors) trigger the endogenous program of cell protection from apoptosis via Bcl-2, Bcl-x_L, *etc.* [4,5]. Some cytokines (IFN-γ, IL-10) produce opposite effects depending on cell type and function [5].

Many diseases and syndromes are accompanied by a significant increase in eosinophil count in the peripheral blood [1]. The cytokine-induced inhibition of apoptosis in eosinophilic (EP) granulocytes is considered as a major mechanism of the development of hemic and tissue eosinophilia during various diseases [9,10]. It was reported that immunoregulatory molecules secreted mainly by T helper cells Th-2, *e.g.* IL-5, IL-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF) activate EP granulocytes and increase their survival *in vitro* via inhibition of apoptosis [1,11,13,15].

Here we studied the effects of recombinant IL (rIL-3 and rIL-5) and eotaxin on apoptosis in EP granulocytes.

MATERIALS AND METHODS

In vitro experiments were performed on cultured EP granulocytes from 22 healthy donors (10 men

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and 12 women, 18-50 years) and 23 patients (13 men and 10 women, 18-50 years) with non-Hodgkin's lymphomas (NHL) accompanied by severe peripheral blood eosinophilia. The subjects had no clinical or anamnestic signs for exacerbation of chronic inflammatory diseases, hereditary and mental disorders, alcohol and drug abuse. The patients were examined at admission to the hospital before the start of therapy. Venous blood was taken from fasting subjects in the morning. The blood was stabilized with 25 U/ml heparin. Individual morphological forms of leukocytes were counted routinely.

EP granulocytes were isolated in a discontinuous Percoll density gradient (ρ=1.133 g/liter, Amersham Biosciences AB). Peripheral blood EP (2×10^5 cells/well) were cultured in complete nutrient medium containing 90% RPMI 1640, 10% inactivated fetal bovine serum (Sigma), 0.3 mg/ml L-glutamine, 100 μg/ml gentamicin, and 2 mmol/ml HEPES (Flow) at 37°C and 5% CO_2 for 18 h. rIL-3 (10^{-8} g/ml, Biosource), rIL (10^{-8} g/ml, Biosource), and recombinant eotaxin (10^{-8} g/ml, Biosource) were added to some probes to estimate the sensitivity of EP cells to apoptotic modulators. Otherwise, EP were cultured in a serum-free medium. The cells (10^6/ml) were washed with phosphate buffered saline (pH 7.4) and stained in a buffer containing annexin V and FITC (Caltag). After 10 min, these cells were examined on an Epics XL flow cytometer (Beckman Coulter). Green fluorescence was analyzed in the gate of granulocytic cells (FITC, 530 nm). They were detected by small-angle and side light scatter characterizing the size and granularity of cells, respectively. EP apoptosis was recorded by measuring the expression of phosphatidylserine with FITC-conjugated annexin V (Caltag) [14].

Data processing involved methods of statistical description and statistical hypothesis testing. The normality of data distribution was estimated by the Shapiro—Wilk test. The significance of differences between the means was determined by nonparametric Mann—Whitney test (independent samples) and Wilcoxon test (dependent samples). The differences were significant at $p<0.05$ [2].

RESULTS

Hematologists and oncologists often face the problem of interpreting the causes of eosinophilia in patients with neoplastic processes in blood systems that originate from precursor cells of lymphopoiesis and myelopoiesis (acute myeloleukemia, lymphogranulomatosis, NHL, acute lymphoblast leukemia, and EP leukemia) [1,3,13,15]. For example, the relative and absolute number of EP in NHL patients was 15.18±2.66% and 0.69±0.02 G/liter, respectively (*vs.* 2.18±0.06% and 0.10±0.01 G/liter, respectively, in the control).

The development of eosinophilia during hemoblastosis can be mediated by different mechanisms. The direct pathogenetic mechanism of eosinophilia under conditions of malignant process is associated with production of chemotactic factors by cells of several tumors [1,3]. At the same time, the EP reaction is considered as an immune response to antigenic stimulation with tumor tissue [3]. Moreover, the development of eosinophilia during lymphoproliferative diseases of the blood system (*e.g.*, NHL) can be related to increased production of IL-5 by monoclonal population of tumor T cells. IL-5 mediates the increase in EP recruitment from the bone marrow to the blood [1,13]. Moreover, persistent eosinophilia and appearance of EP granulocytes with a long time of recirculation in the blood flow during hemoblastoses can result from inhibition or dysfunction of EP apoptosis system [1].

This is confirmed by decreased level of spontaneous apoptosis in EP granulocytes from NHL patients (by 3.2 times compared to healthy donors, Table 1).

Variations in the concentration of some regulatory cytokines can serve as a signal modulating apoptosis [1,15]. This feature is typical of IL-5 [7, 11]. *In vitro* incubation of EP with rIL-5 significantly inhibited apoptosis of EP from healthy donors (compared to intact culture). While in NHL patients, the number of annexin-positive EP was 45% below the control and did not differ from the basal level (Table 1).

Our results are consistent with published data. Previous studies showed that IL-5 activates peripheral blood EP and increases their survival *in vitro* by inhibiting apoptosis. The specific and nonspe-

TABLE 1. Number of Apoptotic EP in the Blood (%) from Healthy Donors and NHL Patients during *in Vitro* Culturing with Apoptosis Modulators (*M*±*m*)

Experimental conditions	Healthy donors	NHL patients
Spontaneous apoptosis	14.94±1.09	4.34±0.67^{+++}
In vitro modulation of apoptosis		
lack of growth factors	25.81±2.68*	13.09±1.06*$^{+}$
addition of rIL-3	13.29±0.97	7.20±0.09*$^{+++}$
addition of rIL-5	6.16±1.23**	3.38±0.08^{+}
addition of recombinant eotaxin	8.32±1.02*	3.08±0.70^{+}

Note. *$p<0.05$ and **$p<0.01$ compared to spontaneous apoptosis; $^{+}p<0.05$, $^{++}p<0.01$, and $^{+++}p<0.001$ compared to healthy donors.

cific effects of IL-5 on EP are related to receptor-mediated activation of intracellular signaling pathways regulating gene expression. The antiapoptotic effect of IL-5 can be associated with activation of a specific receptor on EP membrane, which is accompanied by phosphorylation of SH2-protein by tyrosine phosphatase-2 [15]. Some authors reported that the involvement of IL-5 in the regulation of apoptosis of EP leukocytes is probably associated with its ability to shift the balance in the Bcl-2 protein family, which includes both antiapoptotic (Bcl-2, Bcl-x_L, Mcl-1, and A1) and proapoptotic molecules (Bcl-xS and Bax) [7,8,11]. Moreover, IL-5 stabilizes the mitochondrial potential of EP [7,8] and inhibits caspase activity [11].

Apart from IL-5, a pan-specific hematopoietin IL-3 plays a key regulatory role in EP proliferation, differentiation, and survival at the early stage of cell development [1,15]. Addition of rIL-3 to cultured EP granulocytes from healthy donors had no effect on the number of apoptotic cells (compared to the basal level). This parameter in NHL patients increased by 1.6 times during incubation with rIL-3. The number of phosphatidylserine-expressing cells decreased by 1.8 times compared to normal (Table 1).

IL-3 is a modulator triggering the endogenous program of cell protection from apoptosis via Bcl-2, Bcl-x_L, *etc*. [11,15]. Study of the pathways for activation of the antiapoptotic program is focused on signal transduction in the system IL-3 receptor/IL-5/GM-CSF. High-affinity receptor (IL-5/GM-CSF/IL-3) expressed on EP granulocytes consists of a common and a unique subunits associated with JAK2 kinase activation with subsequent activation of Stat1 and Stat5, similar to Lyn and Syk of tyrosine kinases and SHPT2 of tyrosine phosphatase with ultimate activation of Ras/ERK kinase pathways, which demonstrates similar mechanisms of the antiapoptotic effects of IL-5 and cytokines IL-3 and GM-CSF [15].

The development of eosinophilia during hemoblastoses can also be mediated via eotaxin, a chemoattractant enhancing EP recruitment. Incubation with recombinant eotaxin was followed by a decrease in the number of annexin-positive EP in the cell culture from healthy donors (by 45% compared to the basal level). After incubation under similar conditions, the count of apoptotic EP in NHL patients did not differ from that in the intact population of cells, but was 2.7-fold below the control (Table 1).

Culturing of EP granulocytes with GM-CSF showed that this modulator is involved in not only EP proliferation and maturation, but also realization of apoptosis due to the absence of the stimulatory effect of chemotaxins [10]. Eotaxin and other chemotactic factors, in turn, do not promote to survival or apoptosis of EP leukocytes [1].

Adequate regulation of cell death under adverse conditions in the macroorganism is a result of interaction of pro- and antiapoptotic regulatory factors [5,12]. After *in vitro* incubation of peripheral blood EP from healthy donors and NHL patients in the absence of growth factors (serum-free medium), the number of annexin-positive EP exceeded that in the intact culture by 1.7 and 3.25 times, respectively. At the same time, after culturing of EP from NHL patients in a serum-free medium, the count of apoptotic cells decreased by 49% compared to the control (Table 1). The deficiency of growth factors is a signal for apoptosis: it induces dephosphorylation of the proapoptotic protein Bad, incorporation of this protein to the outer mitochondrial membrane, release of cytochrome *c*, and activation of caspase-9 [4,5,12].

Thus, *in vitro* culturing of peripheral blood EP from healthy donors with rIL-5 and recombinant eotaxin is accompanied by a decrease in the number of apoptotic EP (compared to spontaneous cell death). In patients with NHL and severe blood eosinophilia, EP leukocytes were insensitive to the antiapoptotic effect of rIL-3, rIL-5, and recombinant eotaxin during incubation with these compounds. It is probably associated with incompetence of EP determined by shortening of cell cycle at the early stage of maturation and increase in the mitotic index. The specified changes result in the appearance of immature EP leukocytes in the blood. These cells are characterized by abnormal reaction to pro- and antiapoptotic stimuli and, therefore, impaired apoptosis in NHL. Low level of spontaneous apoptosis in EP leukocytes in NHL patients, which is mediated by the influence of antiapoptotic factors, allows the cells to realize their effector activity. This activity has a negative effect not only on foreign antigens, but also on cells of the macroorganism.

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