Cytokine-Producing Activity of Bone Marrow Erythrokaryocytes and Its Regulation under Normal Conditions

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We showed that inhibition of cytokine production by bone marrow adherent cells and their soluble products is a mechanism of regulation of cytokine-producing activity of bone marrow erythrokaryocytes under normal conditions.

Key Words: *erythrokaryocytes; bone marrow; cytokines; regulation*

Nucleated erythroid cells (NEC) not only produce erythrocytes, but also perform a variety of immune and immunoregulatory functions [3,5]. Several functions of erythrokaryocytes are, at least in part, mediated by cytokines produced by these cells [6,7]. Hence, erythron, similarly to immunocompetent cells, is one of the major regulatory systems of hemoimmunopoiesis. Previous studies showed that bone marrow erythroid cells of various differentiation stages produce some cytokines, including interleukin-1β (IL-1β), IL-2, IL-4, IL-6, IL-10, transforming growth factor-β (TGF-β), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α) [8]. More comprehensive understanding of immunoregulatory potencies of erythrokaryocytes, mechanisms of their realization, and principles of regulation under normal and pathological conditions will allow us to develop new effective methods of preventive treatment with cytokines, cytokine therapy, and use of cytokines for rehabilitation of patients with bone marrow erythropoiesis dysfunction. This work was designed to compare the spectrum of cytokines produced by mononuclear cells (MNC) and erythrokaryocytes and to evaluate the mechanisms of regulation of cytokine-producing activity of NEC by the bone marrow microenvironment.

MATERIALS AND METHODS

The bone marrow was examined in 22 healthy donors. They were informed about the procedure of bone marrow sampling and goal of the study.

Bone marrow samples (15-20 ml) were obtained by aspiration trepanobiopsy of the posterosuperior iliac spine under anesthesia. For separation of MNC from other cells in the suspension, bone marrow cells were fractionated by centrifugation in a Ficoll-verografin density gradient (1.077 g/liter) at 1200 rpm [1]. Adherent cells were selected from the fraction of MNC by adhesion to plastic [2]. Positive selection of glycophorin A-positive erythroid cells from the fraction of nonadherent cells was performed by the method of indirect panning with specific antibodies against an erythroid marker glycophorin A (Monoclonal Antibodies anti-Red Blood Cell Glycophorin A Antibody, Harlan Sera-Lab).

To obtain conditioned medium, viable cells from all three fractions were cultured separately or together in various quantitative ratios in RPMI-1640

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medium with 10% serum for 24 h. Cell viability was estimated in the erythrosine B exclusion test and control was performed by hemoglobin staining with benzidine [2]. Cytokine concentration was measured by the chemiluminescence method on an ORIGEN Analyzer (IGEN) with biotin and ruthenium-labeled antibodies (R&D System) against human cytokines TNF-α, TGF-β_1, IFN-γ, and IL-6 [9]. Recombinant TNF-α (PharMingen), TGF-β_1 (NIBCS), IFN-γ (Thomae), and IL-6 (R&D System) were used for calibration standard. Cytokine concentration was estimated by the immunofluorescence method (BioPlex, Biorad). The concentrations of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12 (p70), IL-13, IL-17, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage chemoattractant protein (MCP-1), and macrophage inflammatory protein (MIP-1β) in the culture medium were measured using Human 17-Plex (N171A11171) and Human Cytokine Th1/Th2 panels (N171A11081) by the method of flow immunofluorescence. The study was conducted on a dual-beam laser automated analyzer (Bio-plex protein assay system, Bio-Rad) according to manufacturer's instructions.

RESULTS

We confirmed production of IL-1β, IL-2, IL-4, IL-6, IL-10, IFN-γ, and TNF-α by human bone marrow glycophorin A-positive cells [8] and revealed production of IL-8, IL-13, IL-17, G-CSF, GM-CSF, MCP-1, and MIP-1β by these cells (Table 1). IL-5, IL-7, and IL-12 were not detected and we concluded that bone marrow NEC cannot secrete these proteins under specified culturing conditions. Electrochemiluminescence study was performed to evaluate the effect of erythropoietin, the major erythroid growth factor, on the production of IFN-γ, TNF-α, and TGF-β. Erythropoietin significantly stimulated secretion of TNF-α (36.9 *vs*. 21.3 pg/ml prestimulation) and TGF-β_1 (22.5 *vs*. 5.25 pg/ml prestimulation) by erythroid cells ($p<0.05$ compared to spontaneous secretion) and had little effect on IFN-γ production by NEC.

Previous studies showed that human bone marrow NEC produce IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-13, IL-17, IFN-γ, TNF-α, G-CSF, GM-CSF, MCP-1, TGF-β_1, MIP-1β, erythropoietin, and vascular endothelial growth factor under normal conditions [4,8,11]. Therefore, these cells possess high immunoregulatory potentcy. NEC play a regulatory role starting from the initial stage of proliferation and differentiation of stem hematopoietic cells to proliferation and functional activity of other hematopoietic stems.

NEC can stimulate proliferation of early hematopoietic precursors and contribute to colony-stimulating function of the myeloid stem, which is related to production of IL-1β, G-CSF, and GM-CSF by these cells. Erythrokaryocytes regulate chemotaxis of neutrophils and monocytes/macrophages in the bone marrow via production of MCP-1 and IL-8. IL-2 and IFN-γ produced by erythroid cells serve as inductors of the Th1 cell response. IL-4, IL-6, IL-10, IL-13, and TNF-α activate the Th2-mediated humoral immune response. High production of IL-6 suggests that NEC produce a direct effect on B lymphocyte proliferation, plasma cell function, and increase in NEC number (in synergism with CSF and erythropoietin). The ability to produce IL-10, TGF-β_1, and MIP-1β reflects immunosuppressor activity of bone marrow NEC, which is consistent with published data on competition between granulopoiesis, lymphopoiesis, and erythropoiesis. The production of IFN-γ, TNF-α, and TGF-β_1 suggests that erythrokaryocytes have antitumor properties. The data on cytotoxic activity of erythroid cells to tumor cells provide support for this assumption. Autoregulation of NEC was also demonstrated. This is probably related to the fact that NEC express receptors for several cytokines. Erythrokaryocytes produce erythropoietin and GM-CSF thus regulating their own growth and maturation. Erythroid TGF-β_1 is a potential self-stimulator of terminal differentiation and hemoglobin concentration in erythrokaryocytes. Erythropoietin stimulates erythropoiesis antagonists (TGF-β_1 and TNF-α),

TABLE 1. Spontaneous Cytokine Production during 24-h Culturing of Glycophorin A-Positive Erythrokaryocytes from the Bone Marrow of Healthy Donors (*M*±*SE*)

Group	Cytokine	Concentration, pg/ml
Th1 profile	IL-2	7.68±0.95
	IFN-γ	118±9
Th2 profile	IL-4	9.39±0.68
	IL-6	1201±214
	IL-13	434.20±43.00
Proinflammatory cytokines	IL-1β	31.34±5.00
	IL-17	12.96±1.40
	TNF-α	326±31
Antiinflammatory cytokines	IL-10	3.97±0.75
	MIP-1β	795.00±105.95
Growth factors	G-CSF	178.38±25.00
	GM-CSF	55.83±4.10
Chemokines	MCP-1	1680.0±276.7
	IL-8	3031±1093

which reflects the existence of general mechanisms for the positive and negative autocrine regulation of various stages of erythropoiesis [10,12]. Taking into account the fact that cytokines are inducible proteins, it may be suggested that secretion of biologically active substances with opposite activity differs in various subpopulations and depends on induction conditions. For example, the type and amount of NEC-produced cytokines vary under the influence of different erythropoiesis-modulating factors. Therefore, cytokine-producing activity of erythrokaryocytes strongly depends on physiological demands of the organism [6].

The hematopoiesis-inducing microenvironment of the bone marrow plays an important role in the function of hematopoietic stems, including the erythroid stem. We studied cytokine secretion by the entire population of bone marrow MNC from healthy donors (source for the isolation of erythrokaryocytes). As distinct from the peripheral blood, bone marrow MNC isolated on a density gradient of 1.077 g/liter were presented by not only lymphocytes and monocytes, but also NEC and small number of maturing cells of the granulocytic stem (promyelocytes) and blast cells.

Spontaneous production of 9 cytokines by Th1/Th2 MNC from the bone marrow of healthy donors was studied by the method of flow immunofluorometry. After 24-h culturing, the supernatant of bone marrow MNC contained only TNF-α and IL-10 (10.794±5.060 and 9.822±4.770, respectively). Secretion of IL-2, IL-4, IL-12, IL-13, IFN-γ, and GM-CSF was not detected in supernatants of bone marrow MNC.

Glycophorin A-positive cells constitute up to 50% of bone marrow nucleated cells [13]. The percentage of erythroid cells isolated from MNC on a density gradient was 25%. Destruction of several erythroid cells was probably associated with immune hemolysis [1]. NEC constituted a significant part of MNC. However, the majority of cytokines produced by the population of NEC were not found in the conditioned medium with the entire population of bone marrow MNC. NEC entering the fraction of MNC did not exhibit the ability to produce cytokines. We studied the effect of a medium conditioned with MNC, adherent fraction of MNC, and glycophorin A-positive NEC on cytokine production by erythrokaryocytes from normal bone marrow.

MNC, adherent cells, and glycophorin A-positive cells were cocultured after fractionation. For evaluation of the dose dependency of mutual effects of cells during coculturing, glycophorin A-positive cells (10^6 cells, 1 million/ml) were mixed with 200, 300, and 400 μl suspension of MNC and adherent cells at a similar concentration (20, 30, and 40% of erythroid cell number, respectively; Fig. 1). The conditioned medium from the entire population of MNC and population of adherent cells from donors (30% of the total volume of culture medium) was added to a well with NEC. The concentrations of IL-6 and TNF-α were measured in an electrochemiluminescence study after 24-h culturing. Immunofluorescence study showed that these cytokines are produced by both NEC and MNC.

The production of cytokines by glycophorin A-positive NEC far surpassed that in supernatants of

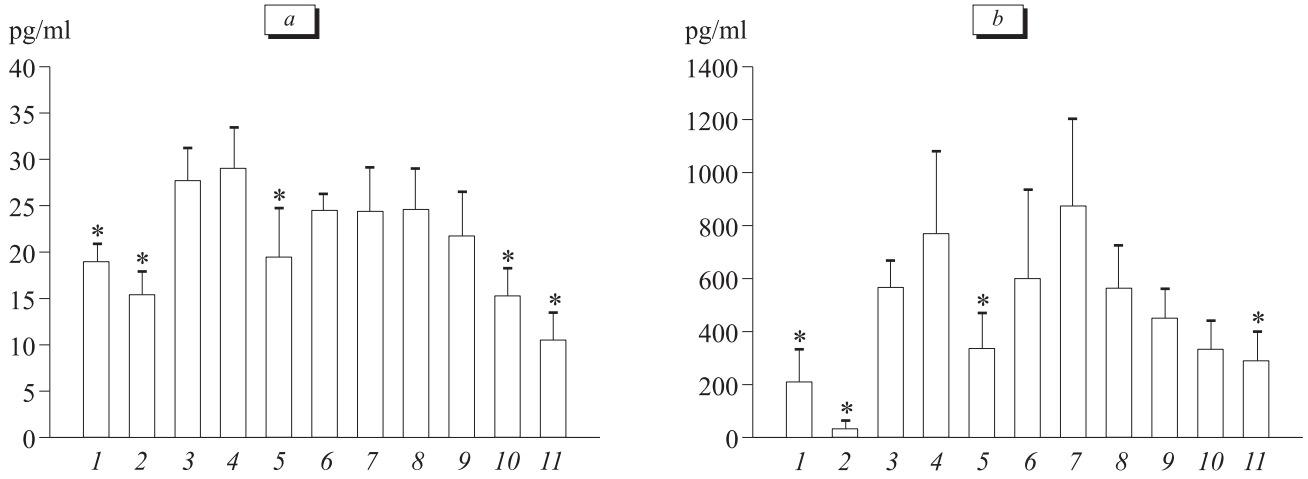

Fig. 1. Cytokine production by glycophorin A-positive cells *in vitro* after 24-h coculturing with MNC, adherent bone marrow MNC, and conditioned media (*M*±*SE*). Production of TNF-α (*a*) and IL-6 (*b*). MNC (*1*), adherent cells (*2*), glycophorin A-positive NEC (*3*), glycophorin A-positive NEC+30% conditioned medium of MNC (*4*), glycophorin A-positive NEC+30% conditioned medium of adherent cells (*5*), glycophorin A-positive NEC+20% MNC (*6*), glycophorin A-positive NEC+30% MNC (*7*), glycophorin A-positive NEC+40% MNC (*8*), glycophorin A-positive NEC+20% adherent cells (*9*), glycophorin A-positive NEC+30% adherent cells (*10*), glycophorin A-positive NEC+40% adherent cells (*11*). **p*<0.05 compared to glycophorin A-positive NEC.

MNC and adherent cells. After addition of the conditioned medium with adherent cells to the culture of glycophorin A-positive NEC, as well as during coculturing of glycophorin A and adherent cells, the concentrations of TNF-α and IL-6 were much lower compared to those observed in spontaneous production of these cytokines by glycophorin A-positive cells. The presence of adherent cells among glycophorin A-positive cells contributed to the dose-dependent decrease in cytokine concentration. Our results indicate that cells from the adherent fraction of MNC inhibit cytokine production by erythrokaryocytes, which is related to the negative regulation of this function of nucleated bone marrow cells.

We conclude that cytokine production should occur under strict regulation. Regulatory activity of bone marrow erythrokaryocytes is limited by the negative regulation with the microenvironment and is manifested only after isolation of erythroid cells from the adherent fraction. These specific features are of pathogenetic importance under pathological conditions accompanied by a decrease in regulatory function of the adherent microenvironment.

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