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Role of Interferon-γ in Regulation of Antiproliferative Activity of Bone Marrow Nonadherent Cells

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Bone marrow nonadherent cells were cultured for 3 days with supernatants of concanavalin A-stimulated splenocytes containing interferon-γ, whose activity was estimated by the ability to induce NO production. Supernatants with a high inducing activity decreased natural suppressor activity of bone marrow nonadherent cells and production of NO, but the activity of the supernatant of these cells increased. Inhibition of NO production during treatment with these supernatants prevented the decrease in suppressor activity; production of NO then increased. Supernatants with a low inducing activity increased natural suppressor activity of bone marrow nonadherent cells but not NO production.

Key Words: *natural suppressor cells; hemopoiesis; nitric oxide; suppressor factor; interferon-γ*

Stromal microenvironmental cells and immature hemopoietic cells (natural suppressor cells, NSC [7]) of the myeloid, erythroid, and lymphoid lineages [1,3,10] inhibiting cell proliferation are involved in the regulation of hemopoiesis. This effect is realized via secretion of various suppressor factors (NO, transforming growth factor-β, and interleukin-10) [2,10,11]. Interferon-γ (IFN-γ) [2] and granulocyte-macrophage colony-stimulating factor (GM-CSF) [9] stimulate NO production by bone marrow NSC. At the same time, long-term incubation (3 days) of these cells with GM-CSF or interleukin-3 (but not with IFN-γ) stimulates production of transforming growth factor-β [7].

In these experiments, splenocytes stimulated with concanavalin A (Con A) served as the source of GM-CSF, in particular IFN-γ. Recombinant GM-CSF and supernatant of Con A-stimulated splenocytes were used to obtain bone marrow NSC actively producing transforming growth factor-β [8]. The supernatant with IFN-γ, whose presence was confirmed by induction of NO production, was used as a stimulator of NSC.

Here we studied the effects of supernatants containing various concentrations of IFN-γ on natural suppressor activity of bone marrow cells and analyzed the role of NO in this process.

MATERIALS AND METHODS

Experiments were performed on 30 C57B1/6J mice (conventional strain) aging 4 months obtained from the collection of the Laboratory of Experimental Biomedical Modelling (Tomsk Research Center).

Bone marrow cells were washed out from the femur with phosphate buffer. The spleen was minced and washed with phosphate buffer to obtain splenocytes.

The cells were cultured in RPMI-1640 medium (Sigma) containing 10% fetal calf serum, 20 mM

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HEPES, 0.05 mM 2-mercaptoethanol (Sigma), 50 μg/ml gentamicin, and 2 mM L-glutamine (Flow) at 5% CO_2. In some experiments, 0.5 mM N^G-monomethyl-L-arginine (NMMA, Sigma) was added into wells.

The supernatant of splenocytes stimulated with Con A was obtained as described previously [8]: splenocytes (5×10^6 cells/ml) were incubated in the presence of Con A (4 μg/ml) for 24 h, and 10 μg/ml α-methyl-D-mannosite (Sigma) was added into the supernatant.

Freshly isolated bone marrow cells (3×10^6 cells/ml) were cultured with various concentrations of the supernatant for 72 h in 75-ml plastic flasks (Costar). Nonadherent cells were washed out and suspended in the culture medium. These cells (2×10^5 cells/well) were incubated in 96-well plates (U-shaped bottom, Costar) for 36 h to estimate proliferation. ^{3}H-thymidine (1 μCi/well) was added 16 h before the end of culturing. Natural suppressor activity was analyzed by inhibition of proliferation of syngeneic splenocytes. To this end, bone marrow cells in various concentrations or their supernatants (after 24-h culturing of 10^7 cells/ml) diluted $^1/_3$ were incubated with splenocytes (2×10^5 cells/well) in the presence of 4 μg/ml Con A (Sigma) for 72 h in 96-well plates (Costar). ^{3}H-thymidine (1 μCi/well) was added 16 h before the end of culturing.

NO production was evaluated by the content of nitrites using Grace reagent [4]. The reagent (0.1 ml) was mixed with the supernatant (0.1 ml), and absorption at 550 nm was measured. The concentration of nitrites was estimated by the calibration curve constructed using sodium nitrite.

The results were analyzed by Student's t test, the differences were significant at $p<0.05$.

RESULTS

Suppressor activity of bone marrow cells decreased and was nearly undetectable after 3-day culturing with high concentrations of the supernatant (Table 1). The initial activation of NSC during the treatment with this supernatant was mediated by NO, while additional stimulation in the suppressor test (with cytokines of mitogen-stimulated splenocytes) did not increase NO production. Moreover, the minor NO production by bone marrow cells treated with the supernatant in a high concentration considerably decreased.

Suppressor activity of supernatants of bone marrow cells obtained without additional stimulation surpassed that of control cells. Thus, these cells secreted a suppressor factor different from unstable and short-living NO. Secretion of this factor is probably inhibited by splenocyte cytokines, because these cells do not display suppressor activity in the test.

Splenocyte supernatants 1 and 2 with high and low abilities to induce NO production, respectively, were used in further experiments.

These supernatants induced various effects on suppressor activity of bone marrow cells (Table 2). Supernatant 1 stimulated NO production during the treatment of cells; then, suppressor activity and NO production decreased. Inhibition of NO production by the NO synthase inhibitor NMMA during the treatment with supernatant 1 stimulated NO production by all cells independently on culturing conditions. Suppressor activity of bone marrow nonadherent cells increased approaching the activity of control myelocytes. Therefore, the inhibition of natural suppressor activity of bone marrow cells by IFN-γ is probably mediated by NO. This assumption is confirmed by previous studies [6,11] showing that NO is involved in IFN-γ-induced apoptosis of stem cells or their differentiation into mature forms (depending on the stage of maturation), which leads to elimination of hemopoietic precursors. These processes also contribute to the increase in natural suppressor activity induced by NMMA in the absence of the supernatant.

Suppressor activity of bone marrow nonadherent cells treated with supernatant 2 increased (Table 2), while NO production by these cells was below the control level. Therefore, supernatant 2 (by contrast to supernatant 1) caused simultaneous production of NO

TABLE 1. Effects of Splenocyte Supernatant on Natural Suppressor Activity (SA) and Nitrite Concentration (NC) in Bone Marrow Nonadherent Cells ($X\pm m$)

Supernatant concentration, %	Effector/target cell ratio	1:1			0.5:1			SA of supernatants
		SA, %	NC, μM	NT without target cells, μM	SA, %	NC, μM	NT without target cells, μM	
Control	3.3±1.7	97.9±1.1	64.2±0.6	<2	78.4±5.0	37.7±1.0	<2	34.1±2.3
30	12.2±0.5*	50.9±3.8*	29.9±1.8	—	—	—	—	
50	23.2±1.3*	5.6±2.2*	2.9±0.2*	8.7±0.4	-3.2±2.0*	2.9±1.2*	2.8±0.3	76.7±1.3*

Note. *$p<0.05$ compared with the control.

TABLE 2. Role of NO in Effects of 50% Splenocyte Supernatant on Natural Suppressor Activity (SA) of Bone Marrow Nonadherent Cells ($X \pm m$)

Culturing conditions	Effector/target cell ratio	1:1			0.5:1	
		SA, %	NC, μM	NT without target cells, μM	SA, %	NC, μM
Control	3.9±0.5	68.0±3.1	26.6±1.3	<2	31.3±2.4	3.5±1.4
+NMMA	<2	87.2±1.1*	28.6±1.9	<2	40.6±2.7*	6.0±0.6
Supernatant 1	60.9±0.9*	40.0±3.7*	22.3±0.7*	22.1±0.4	2.0±1.5*	2.5±0.0
+NMMA	16.3±0.1**,++		75.9±6.2+	53±0±0.2**,++	37.4±5.9++	12.0±1.0*,++
Supernatant 2	10.7±0.2	88.5±2.2**	0.8±2.2	10.6±2.2	55.6±1.8**	5.0±0.2

Note. *$p<0.05$ and **$p<0.002$ compared with bone marrow cells cultured without supernatants and NMMA; +$p<0.05$ and ++$p<0.002$ compared with culturing in the presence of supernatant 1 without NMMA.

TABLE 3. Effects of 50% Splenocyte Supernatant on Proliferation of Bone Marrow Nonadherent Cells (cpm, $X \pm m$) and Nitrite Concentration (NC, $X \pm m$)

Supernatant	Without NMMA		With NMMA	
	proliferation	NC	proliferation	NC
Control	4087±729	<2	3633±242	<2
No. 1	836±162*	21.5±0.8	2460±500+	5.0±0.6++
No. 2	6105±515*	14.5±0.3	8383±244**,+	<2

Note. *$p<0.01$ and **$p<0.001$ compared with the control; +$p<0.05$ and ++$p<0.001$ compared with culturing without NMMA.

and another suppressor factor. The mechanisms of their production were probably different, because cytokines of mitogen-stimulated splenocytes inhibited production of only one of these factors.

Transforming growth factor-β was assumed to be the suppressor factor induced by supernatant 2. This biologically inactive factor secreted by cells is activated by short-term heating to 100°C [5]. Heating of the supernatant of bone marrow cells treated with supernatant 2 to 100°C for 5 min markedly decreased suppressor activity and increased ^{3}H-thymidine incorporation (from 4218±702 to 9455±1663 cpm *vs.* 14 568±2983 cpm in the control). Thus, neither NO nor transforming growth factor-β played the role of this additional suppressor factor. Even small amounts of IFN-γ probably prevented the production of transforming growth factor-β.

Therefore, both supernatants stimulated natural suppressor activity of bone marrow cells, but the mechanisms responsible for realization of these activities were different. These processes probably play various biological roles, because supernatant 1 induces short-term activation (3 days) changed to inhibition, while supernatant 2 causes relatively stable activation maintained at least for 4-5 days.

Supernatant 1 inhibited proliferation of nonadherent myelocytes, while supernatant 2 stimulated this process probably due to the presence of colony-stimulating factors (Table 3). NO produced after treatment with the supernatant suppressed bone marrow cells, because NMMA stimulated their proliferation.

Thus, the mechanisms of realization of bone marrow suppressor activity are different and depend on the presence or absence of some cytokines and their ratio in the NSC microenvironment. The data suggest that IFN-γ, which activates NSC and modulates the stimulatory effects of cytokines, plays a key role in these processes.

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