Suppressive Activity of Bone Marrow Cells from Patients with Stomach Cancer. Effect of Prostaglandins, Transforming Growth Factor-β, and Nitric Oxide

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It is shown that nonadhesive bone marrow cells from patients with stomach cancer suppress phytohemagglutinin-stimulated proliferation of peripheral blood lymphocytes of healthy donors and proliferation of Molt-4 human lymphoma cells *in vitro*. Suppressive activity of bone marrow cells from cancer patients is not mediated through prostaglandin secretion, since indomethacin has no effect on it. Addition of neutralizing monoclonal antibodies to transforming growth factor-β_1, β_2, and β_3 partially reduces this suppressive effect. Suppressive effect of bone marrow cells from patients with stomach cancer is partially mediated through production of nitric oxide, since the inhibitor of its synthesis N^9-monomethyl-L-arginine diminishes the inhibiting effect of bone marrow cells from cancer patients on phytohemagglutinin-stimulated proliferation of peripheral blood T cells from healthy donors.

Key Words: *stomach cancer; bone marrow; suppressor cells*

Numerous clinical and experimental studies show that malignant tumor growth is accompanied by gradual reduction of immune response and immuno- and hemopoietic imbalance in cancer patients. In particular, in patients with stomach cancer stages I-IV, tumor growth is attended by complex rearrangement of immune homeostasis [3], a progressive decrease in the number of peripheral blood T cells [4], considerable suppression of spontaneous and phytohemagglutinin (PHA) induced lymphocyte blast-transformation [14], and inhibition of interleukin-2 production by peripheral blood mononuclears [8]. Activity of lymphokine-activated killer cells is below the normal level [7]. Activity of natural killer cells in cancer patients is also decreased [5]. Some authors attribute immunosuppression in cancer patients to activation of suppressor cells, primarily T suppressors and monocytes [11]. On the other hand, bone marrow can be a source of suppressor cells in tumor process [1,2,10,12]. Bone marrow-derived suppressor cells, immature myeloid precursors have been found in the spleen of cancer patients and even in the tumor [13]. Elimination of these suppressor cells promoted generation of tumor cytolytic T cells, restored proliferative response of T cells to interleukin-2, and reduced the number of metastases.

Recent investigations have shown that considerable suppression of T cell response in autologous mixed lymphocyte culture observed in patients with stomach cancer does not depend on the stage of tumor process and is mediated by non-T suppressor cells occurring both in the spleen and peripheral blood [6].

Unfortunately, there are practically no clinical studies of immunosuppressor cells in cancer patients. However, the above data suggest that bone marrow is a potential source of immunosuppressor cells in these patients.

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The aim of the present study was to evaluate immunosuppressor properties of nonadhesive bone marrow cells in patients with stomach cancer.

MATERIALS AND METHODS

A total of 40 patients with stomach cancer (stages I-III) and 9 noncancer patients with peptic ulcer were examined. Bone marrow sternal punctures were performed with patient's consent. Freshly isolated bone marrow cells were washed with medium 199, erythrocytes were removed by hypotonic lysis, and the cells were resuspended in RPMI-1640 medium containing 10% calf embryonic serum, 2 mM glutamine (Flow Lab.), 2 mM HEPES buffer (Serva), 50 μM 2-mercaptoethanol (Fluka), and 40 μg/ml gentamicin. Adherent cells were removed by incubating the cell suspension in plastic Petri dishes at 37°C for 2 h; this decreased bone marrow cellularity by approximately 10%. Cell viability assessed by 0.1% Trypan Blue exclusion was no less than 90%. In some experiments cells were fractionated by centrifugation in Ficoll gradient. Peripheral blood mononuclear cells (MNC) were isolated from the blood of healthy donors by centrifugation in Ficoll-Paque gradient (Pharmacia) and washed with medium 199.

Suppressor activity of bone marrow cells from patients with stomach cancer was evaluated by suppression of PHA-stimulated proliferation of peripheral blood MNC obtained from healthy donors. To this end, test cells in different proportions were added to 3×10^5 MNC per well in the presence of 20 μg/ml PHA and incubated in 96-well round-bottom plates for 72 h at 37°C in a humidified atmosphere with 5% CO_2. ^{3}H-Thymidine (1 μCi) was added to each well 18 h prior to the end of incubation. The content of each well was transferred with a harvester (Scatron) to fiberglass filters, and radioactivity was measured in a Mark-III β-counter. The degree of suppression was calculated from the formula: $(1-E/C)\times 100\%$, where E and C are cpm in wells containing MNC with and without bone marrow cells, respectively.

The supernatant of bone marrow cells was obtained by incubating 3×10^6 cells/ml in 6-well plates for 48 h at 37°C followed by centrifugation at 1000 rpm for 5 min. Cell pellet was removed, while the supernatant in different dilutions was added to 3×10^5 MNC per well in the presence of PHA or to 2×10^4 Molt-4 human T lymphoma cells and incubated for 72 and 24 h, respectively. ^{3}H-Thymidine (1 μCi) was added to each well 18 and 6 h before the end of incubation. Indomethacin in a final concentration of 10 μM was used to inhibit prostaglandin synthesis.

In some experiments, monoclonal anti-TGF-β_1, β_2, β_3 antibodies (Genzyme) kindly provided by Dr. V. I. Seledtsov (Institute of Clinical Immunology, Siberian Division of the Russian Academy of Medical Sciences, Novosibirsk) were added to the culture for neutralization of transforming growth factor-β (TGF-β).

Production of nitric oxide was blocked with N^9-monomethyl-L-arginine; inactive analog by N^9-monomethyl-D-arginine was used as the control. Both preparations were kindly provided by Dr. J. L. Subiza (San Carlos University, Madrid).

Production of nitric oxide by bone marrow cells and peripheral blood MNC in cell supernatants was measured using Griess reagent in Beckman spectrophotometer (550 nm).

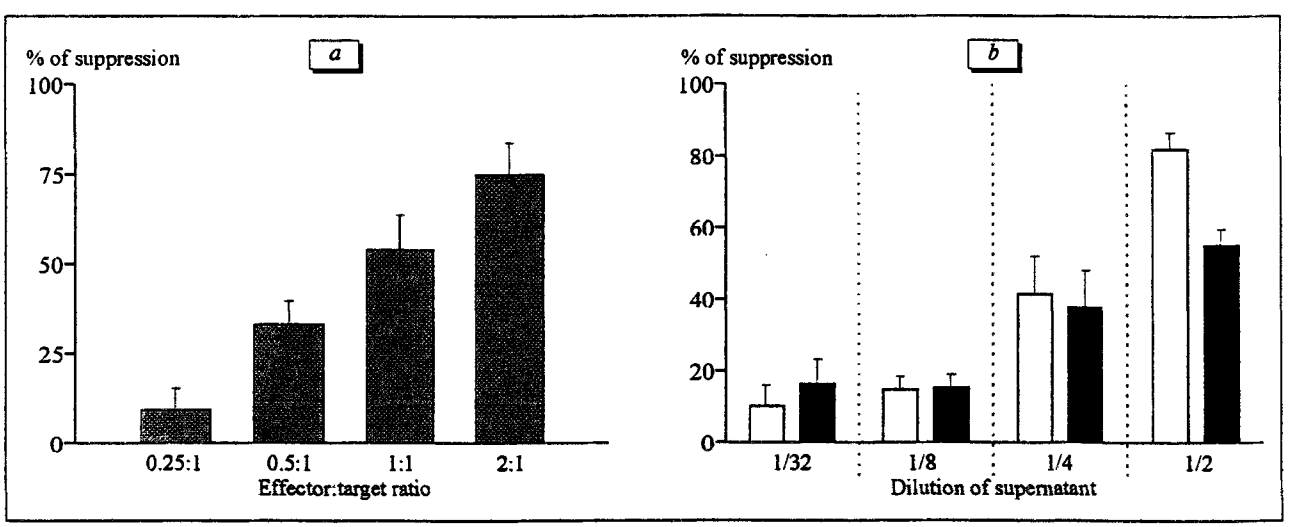

Fig. 1. Effect of bone marrow cells (*a*) and their supernatant (*b*) from patients with stomach cancer on phytohemagglutinin-induced proliferation of peripheral blood mononuclear cells from healthy donors and Molt-4 human lymphoma cells. *b*) Light bars: phytohemagglutinin-stimulated mononuclears; shaded bars: Molt-4 human lymphoma cells.

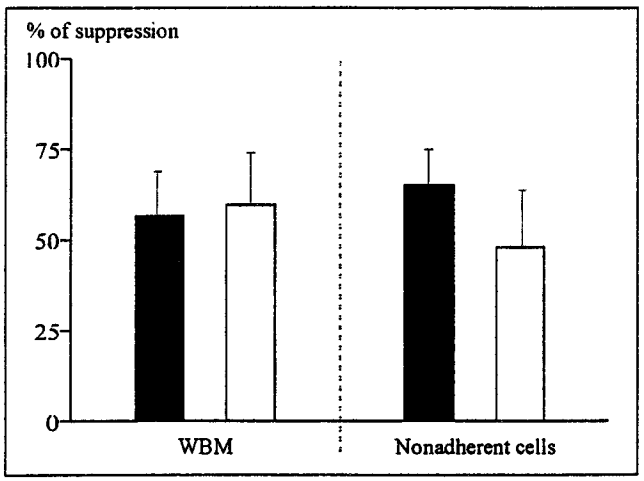

Fig. 2. Suppressor activity of bone marrow cells from patients with stomach cancer in the presence (light bars) and absence (shaded bars) of indomethacin and after removal of adherent cells. WBM: whole bone marrow after erythrocyte lysis.

The data were processed statistically using nonparametric Mann-Whitney test.

RESULTS

Data on suppressor activity of bone marrow cells from patients with stomach cancer evaluated by their ability to inhibit PHA-stimulated proliferation of MNC from healthy donors are presented in Fig. 1, *a*. Inhibition depended on the number of bone marrow cells added to MNC culture. For instance, addition of 1.5×10^5 and 6×10^5 bone marrow cells to 3×10^5 MNC inhibited ^{3}H-thymidine incorporation into PHA-stimulated peripheral blood lymphocytes by 35 and 75%, respectively.

The supernatant of bone marrow cells from cancer patients collected after a 48-h incubation in the absence of other cells or mitogens exerted pronounced suppressive effect on PHA-stimulated MNC from healthy donors (Fig. 1, *a*). It also inhibited *in vitro* proliferation of Molt-4 leukemia cells (Fig. 1, *b*). The observed inhibitory effect was dose-dependent and peaked at 50% dilution of the bone marrow supernatant.

It has been established that prostaglandins play an essential role in immunosuppressor effects observed in cancer patients [9]. In order to find out whether suppressive effect of bone marrow cells from patients with stomach cancer is related to prostaglandin production, incubation was carried out in the presence of 10^{-6} M (Fig. 2) indomethacin (inhibitor of prostaglandin synthesis). As seen from Fig. 2, indomethacin did not change suppressive effect of bone marrow cells from cancer patients.

Moreover, removal of adherent cells (primarily, macrophages) from the bone marrow suspension also had no effect on the level of suppression (Fig. 2). Thus, our findings suggest that suppressive effect of bone marrow cells from patients with stomach cancer is realized through a soluble factor (not prostaglandin) produced by nonadherent bone marrow cells.

Proliferation of both immunocompetent and tumor cells is inhibited among other factors by TGF-β produced by bone marrow cells [13]. For evaluation of the role of TGF-β in the immunosuppressor effect of bone marrow cells we used neutralizing monoclonal antibodies to TGF-β_1, β_2, β_3. The presence of anti-TGF-β antibodies throughout the incubation period had no effect on the suppressive effect of bone marrow cells (Fig. 3). However, taking into account that TGF-β is often secreted in a latent form, nega-

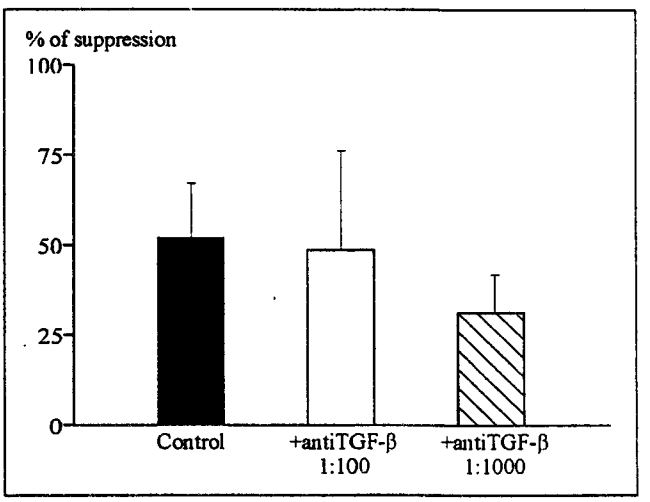

Fig. 3. Effect of neutralizing monoclonal antibodies against transforming growth factor-β (TGF-β) on suppressor activity of bone marrow cells from patients with stomach cancer.

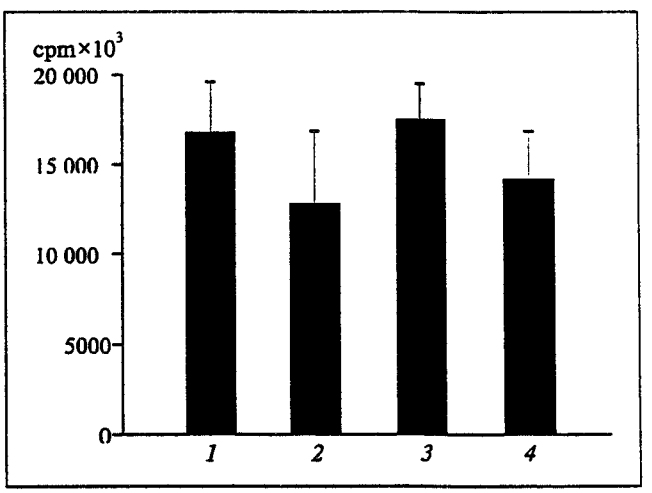

Fig. 4. Effect of inhibitor of nitric oxide synthesis N^9-monomethyl-L-arginine on suppressor activity of bone marrow cells from patients with stomach cancer. *1*) ^{3}H-thymidine incorporation by phytohemagglutinin-stimulated mononuclears; *2*) addition of bone marrow cells from patients with stomach cancer; *3*) N^9-monomethyl-L-arginine; *4*) N^9-monomethyl-D-arginine.

tive *in vitro* test with neutralizing antibodies is insufficient to exclude the contribution of this cytokine to the studied suppressive effect.

Figure 4 illustrates the effect of the inhibitor of nitric oxide production N^9-monomethyl-L-arginine and its inactive analog N^9-monomethyl-D-arginine on the suppressive effect of nonadhesive bone marrow cells from patients with stomach cancer (1:1 effector:target ratio). This effect considerably decreased in the presence of nitric oxide ($p<0.05$).

Thus, immunosuppressor cells of nonadhesive bone marrow fraction from patients with stomach cancer inhibit PHA-induced proliferation of MNC through a non-prostaglandin factor. This suppressive effect is partially mediated through nitric oxide production and TGF-β.

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