Differential Induction of Natural Suppressor Activity of Bone Marrow Cells *In Vitro* by Different Types of Tumors

Yu. P. Bel'skii, N. V. Zemlyanskaya, S. A. Kusmartsev, and I. M. Agranovich*

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The suppressor activity of bone marrow cells not adhering to plastic is shown to appreciably increase after their 48-hour joint preincubation with mastocytoma P-815 supernatant or WEHI-3 myelomonocytic strain and to be unchanged after incubation with supernatants of K-2 erythroleukemic strain or Ehrlich's ascitic carcinoma. P-815, WEHI-3, and K-2 tumors are shown to produce factors characterized by colony-stimulating activity.

Key Words: *suppressor cells; tumor; colony-stimulating activity*

The so-called natural suppressor cells (NSC) in the bone marrow (BM) are known to inhibit the proliferation of immunocompetent and tumor cells *in vitro* by releasing a soluble suppressive factor [5-7,10] and *in vivo* [3,6]. NSC do not possess markers of mature immunocompetent cells and belong to the BM fraction not adhering to plastic [1]. Experiments with different tumor growth models have shown that the development of a malignant process goes along with an appreciable activation of bone marrow NSC [3,4,6,9,13] and their appearance in the spleen [10,13]. Activation of these cells is associated with the direct influence of colony-stimulating factors produced by tumors of many types [14]. Co-incubation of granulocyte-macrophagal colony-stimulating factor (GM-CSF) or interleukin-3 with normal murine BM cells sharply increases the activity of NSC [12,14]. Tumors are known to vary in their ability to activate bone marrow NSC *in vitro*. For example, cocultivation of Lewis' pulmonary adenocarcinoma supernatant and normal BM cells is conducive to a marked increase of bone marrow NSC activity [15], whereas treatment of normal BM with supernatant of Ehrlich's carcinoma somewhat reduces the suppressor activity of BM [2].

The purpose of this work was to study the suppressor-inducing and colony-stimulating activities of the factors produced *in vitro* by different types of tumor cells.

MATERIALS AND METHODS

Experiments were carried out with BALB/c and DBA/2 mice aged 2 to 3 months bred at the Research Laboratory for Experimental Biomedical Simulation, Tomsk Research Center, Russian Academy of Medical Sciences. Ehrlich's adenocarcinoma (in BALB/c mice) and P-815 mastocytoma (in DBA/2 mice) were induced by an intraperitoneal graft of 2×10^6 tumor cells. K-2 and WEHI-3 cell lines were maintained *in vitro* in complete culture medium.

Cells were cultured in RPMI-1640 medium containing 2 mM HEPES buffer, 2 mM L-glutamine, 10% fetal calf serum (Flow), 5×10^{-5} M 2-mercaptoethanol (Fluka), 100 U/ml penicillin, and 100 μg/ml streptomycin.

Research Laboratory for Experimental Biomedical Simulation, Tomsk Research Center, Siberian Division of the Russian Academy of Medical Sciences; *Research Institute of Carcinogenesis, Cancer Research Center, Russian Academy of Medical Sciences, Moscow (Presented by N. V. Vasil'ev, Member of the Russian Academy of Medical Sciences)

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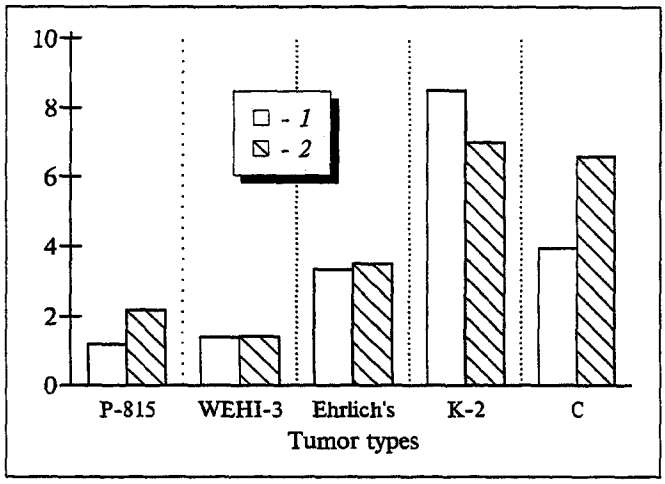

Fig. 1. Level of ^3H-thymidine incorporation by bone marrow (BM) cells after their treatment with tumor supernatant. Ordinate: absolute values of ^3H-thymidine incorporation. *C*: level of isotope incorporation in BM cells preincubated in culture medium without supernatant. Proliferation 4×10^5 (*1*) and 2×10^5 (*2*) BM cells.

Ehrlich's and P-815 tumor cells were removed from the abdominal cavity on days 6-7 after transplantation and washed twice in medium 199 (Vektor, Novosibirsk). For preparation of the tumor cell supernatants, 2×10^6 tumor cells were placed in 24-well Costar plates and incubated 48 h at 37°C (5% CO_2), after which they were centrifuged at 3000 rpm for 5 min and the resultant supernatant was added in a 1:2 dilution to intact BM cells (4×10^6) in 24-well plates. After 48-hour cocultivation of BM cells and tumor supernatants at 37°C in an atmosphere with 5% CO_2, the BM cells were washed three times and their suppressor activity was assessed.

Natural suppressor activity of BM cells was assessed by inhibition of P-815 mastocytoma proliferation during cocultivation [11]. For this purpose, 2×10^4/well P-815 mastocytoma cells and 2×10^5 (or 4×10^5) BM cells were put in 96-well round-bottom plates and incubated for 16 h under the above-mentioned conditions. Four hours before the end of culturing 1 μCi ^3H-thymidine was added to each well and its incorporation routinely measured. The suppressor activity (SA) was expressed in percent and calculated according to the formula:

SA=[1-number of counts in a well(effector cells+target cells)/number of counts in a well with target cells]×100%.

Colony-stimulating activity of tumor supernatants was tested by the capacity to stimulate BM colony growth in methyl cellulose. For this purpose 100 μl of tumor cell supernatant or culture medium were placed in 96-well plates, then 100 μl of intact murine BM cell suspension (10^5/ml) were added in a culture medium of the following composition: 40% RPMI medium, 40% HAM's F-10 medium (Serva), 19% inactivated fetal calf serum, 5×10^{-5} 2-mercaptoethanol, 50 μg/ml gentamicin, 2 mM L-glutamine, and 1% methyl cellulose. After a 7-day culturing at 37°C, 5% CO_2, and 100% air humidity the number of colonies grown per 10^5 myelokaryocytes was counted. Erythropoiesis-stimulating activity of tumor supernatants was assessed as described previously [1].

In some experiments in order to remove the adherent BM cells 4 to 6×10^6 BM cells were incubated 90 min at 37°C in culture medium in petri dishes or 6-well plates.

The results were statistically processed using Student's *t* test.

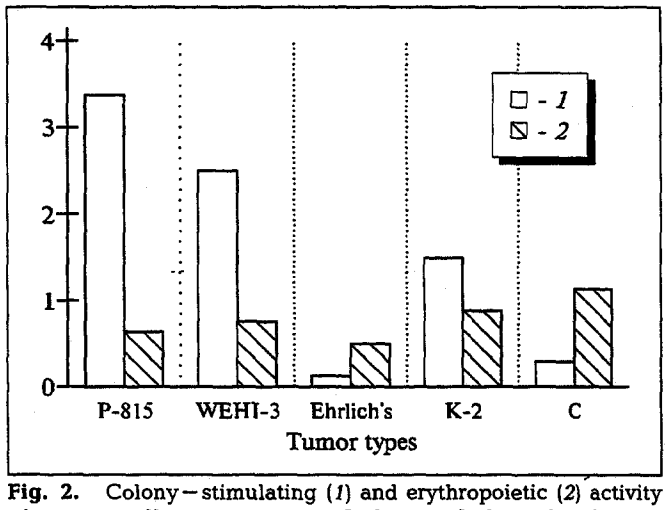

Fig. 2. Colony-stimulating (*1*) and erythropoietic (*2*) activity of tumor cell supernatants. Ordinate: Index of colony-formation stimulation, *C*: control: number of colonies grown without supernatants per 10^5 myelokaryocytes.

RESULTS

Table 1 shows that normal BM cells incubated for 2 days in ordinary culture medium without factors produced by tumors had a marked inhibitory effect on P-815 mastocytoma proliferation *in vitro* (44.1% in a 10:1 ratio and 91.5% in a 20:1 ratio).

Preliminary 48-hour cocultivation of BM cells with WEHI-3 and P-815 mastocytoma supernatants appreciably increased the suppressor activity of BM cells. Addition of BM cells treated with these supernatants to wells with tumor cells in a 10:1 ratio led to 91.9 and 70.6% inhibition of tumor target cell proliferation, respectively, and in a 20:1 ratio to 96.7 and 96.2% inhibition, respectively. It is noteworthy that pretreatment of BM cells with WEHI-3 and P-815 supernatants followed by culturing under the above-mentioned conditions in the absence of tumor target cells led to a drop of the ^3H-thymidine level in comparison with the control (Fig. 1).

TABLE 1. Effects of Tumor Cell Supernatants on Suppressor Activity of BM Cells ($M \pm m$)

Number of BM cells and P-815 tumor cells per well	Proliferation of P-815 mastocytoma upon addition of BM cells preincubated with tumor supernatant, cpm origin of supernatant				
	control (medium)	WEHI-3	Ehrlich's tumor	K-2	P-815
2×10^5 BM+2×10^4 P−815	16 422±2 597	2 384±440	20 302±4 190	15 352±5 614	8 646±566
% suppression	44.1	91.9*	31.0	47.8	70.6*
4×10^5 BM+2×10^4 P−815	2 497±1 795	956±618	5 630±1 066	12 373±3 700	1 105±640
% suppression	91.5	96.7	80.8*	57.9*	96.2

Note. Level of ^{3}H−thymidine incorporation of wells with P−815 cells (without BM) was 29,394±3200 per well. Asterisk shows $p<0.05$ *vs.* control.

The results of studies of the suppressor activity of BM cells preincubated with Ehrlich's tumor and K-2 erythroleukemia supernatants were somewhat different. The level of proliferation of P-815 target cells in the presence of BM cells treated with the above supernatants virtually did not differ from the control values for the 10:1 ratio (Table 1), whereas at a higher concentration of BM cells (20:1), their suppressor activity was reliably decreased (for K-2 $p<0.01$, for Ehrlich's tumor $p<0.05$). It is worth noting that the level of proliferation of BM cells cultured under similar conditions but in the absence of target cells was considerably higher than that of BM cells treated with WEHI-3 or P-815 supernatants.

When analyzing the results, we should remember that the test we used to assess the level of ^{3}H-thymidine incorporation by BM cells is suitable for a comparative assessment of the level of BM cell proliferation and suppressor activity under specific conditions, but that it cannot be used for adequate assessment of colony-stimulating activity [8]. The colony-stimulating capacity of tumor supernatants was tested by the ability to stimulate the growth of BM colonies in methyl cellulose. The data presented in Fig. 2 show that P-815 mastocytoma, WEHI-3 myelomonocytic strain, and K-2 erythroleukemia supernatants reliably stimulated the growth of colonies. Ehrlich's ascitic carcinoma supernatants showed no such ability. None of the studied supernatants possessed erythropoietic activity.

Natural suppressor cells are known to have receptors for interleukin-3 [12], and direct exposure to multi-CSF (or GM-CSF) leads to an appreciable activation of NSC [12,14]. Our data indicate that the suppressor activity of BM cells increased after treatment with supernatant of WEHI-3 cells constitutively producing interleukin-3. The same effect was attained by BM treatment with P-815 mastocytoma supernatant characterized by colony-stimulating activity. On the other hand, K-2 cells producing the factor stimulating colony formation by BM cells somewhat reduced the activity of bone marrow NSC. Hence, since K-2 supernatant did not possess erythropoietic activity, did not stimulate NSC activity, but did exhibit colony-stimulating activity, this activity is probably related to the production of granulocytic or macrophagal CSF incapable of inducing suppressor activity of non-adherent BM cells *in vitro*.

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REFERENCES

1. V. I. Agafonov, A. M. Dygai, V. P. Shakhov, *et al.*, *Radiobiologiya*, **34**, № 1, 111-117 (1994).
2. Yu. P. Bel'skii, I. M. Agranovich, and S. A. Kusmartsev, *Byull. Eksp. Biol. Med.*, **117**, № 5, 514-517 (1994).
3. S. A. Kusmartsev and V. I. Ogreba, *Eksp. Onkol.*, № 5, 23-25 (1989).
4. V. I. Ogreba, S. A. Kusmartsev, and N. V. Vasil'ev, *Vopr. Onkol.*, № 8, 952-955 (1988).
5. I. G. Sidorovich, V. V. Lyakhov, A. A. Vlasov, *et al.*, *Immunologiya*, № 4, 67-69 (1987).
6. R. M. Khaitov, in: *Advances in Science and Technology, Ser. Oncology*, [in Russian], Vol. 13, Moscow (1984).
7. A. K. Duwe and S. K. Singhal, *Cell. Immunol.*, **39**, 79-86 (1978).
8. C. J. Kovach, D. A. Emma, M. G. Evans, *et al.*, *Cell Tiss. Kinet.*, № 18, 235-246 (1985).
9. Y. Oghiso, Y. Yamad, K. Ando, *et al.*, *J. Leukoc. Biol.*, **53**, 86-93 (1993).
10. J. L. Subiza, G. Vinuela, R. Rodrigues, *et al.*, *Int. J. Cancer*, **44**, 307-314 (1989).
11. K. Sugiura, M. Inaba, H. Ogata, *et al.*, *Cancer Res.*, **50**, 2582-2586 (1990).
12. K. Sugiura, S. Ikehara, M. Inaba, *et al.*, *Exp. Hemat.*, **20**, 256-260 (1992).
13. M. R. Young, M. Newby, and H. T. Wepsic, *Cancer Res.*, **47**, 100-105 (1987).
14. M. Young, M. E. Young, and M. A. Wright, *Exp. Hemat.*, **18**, 806-813 (1990).
15. M. Young, M. Wright, and M. Young, *Cancer Immunol. Immunother.*, **33**, 146-153 (1991).