METHODS

Radiation-Induced Hemopoietic Cell Growth Factor: Detection in a Culture

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A simple, rapid, and easily reproducible method was developed for testing activity stimulating the growth of hemopoietic microenvironment in long-term bone marrow culture. It was found that in irradiated mice this activity is produced by bones.

Key Words: *stromal precursor cells; hemopoietic microenvironment; osteogenesis; irradiation; serum from irradiated mouse; long-term bone marrow culture; ectopic hemopoiesis focus*

Ectopic hemopoietic foci forming after implantation of the syngeneic bone marrow under the renal capsule are 2-3-fold larger in irradiated mice than in intact ones [1]. The number of hemopoietic microenvironment "niches", their cellularity, and the total number of early myeloid precursors (splenic colony-forming units) increases. The capacity to form large foci is retained throughout the life in irradiated animals and does not depend on the irradiation dose (4-12 Gy). The serum of irradiated mice stimulated the growth of ectopic hemopoiesis foci in intact mice [1]. The nature of this activity stimulating the growth of hemopoietic microenvironment (MSA) is unknown. It can be a product of a new gene or unknown activity of some growth factor or chemokine involved in the regulation of the hemopoietic system. However *in vivo* testing of individual serum fractions from irradiated mice or products of expression of various genes is virtually impossible; moreover, formation of a hemopoietic focus takes about 6 weeks. The aim of the present study was to develop a simple method for MSA testing and for identification of the organ producing MSA.

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MATERIALS AND METHODS

C57Bl/6 and (C57Bl/6×CBA2)F_1 mice aging 5-6 weeks to 1.5 years and Wistar rats were used in the study. For obtaining MSA-containing serum, the mice were irradiated in a dose of 6-12 Gy (in the latter case they were injected with a protective dose of syngeneic bone marrow cells) and the rats were exposed to a dose of 6 Gy. Blood was collected from the femoral vein no earlier than 1 week postirradiation. After clot retraction, the serum was centrifuged (3000 rpm) and the supernatant was sterilized through 0.22 μ filters.

Long-term bone marrow culture was maintained using T. M. Dexter's method [3] in a medium supplemented with equine serum and ETC (1:2). Bone marrow from one femur was explanted into a culture (1 femur per 25 cm^2 bottom surface, or 1 femur per flask, 2 femurs per 24-well or 96-well plates). The cells were cultured in Fisher medium with glutamine, antibiotics, 10^{-6} M hydrocortisone, and 20% serum ($^1/_3$ ETC and $^2/_3$ equine serum). The volume of the medium was 10 ml/flask, 1 ml/well for a 24-well plate, and 0.2 ml/well for a 96-well plate. Half the medium was replaced once a week. The culture was maintained for 1-3 weeks.

For MSA testing, the number of adhesive cells per well was determied after culturing or the number

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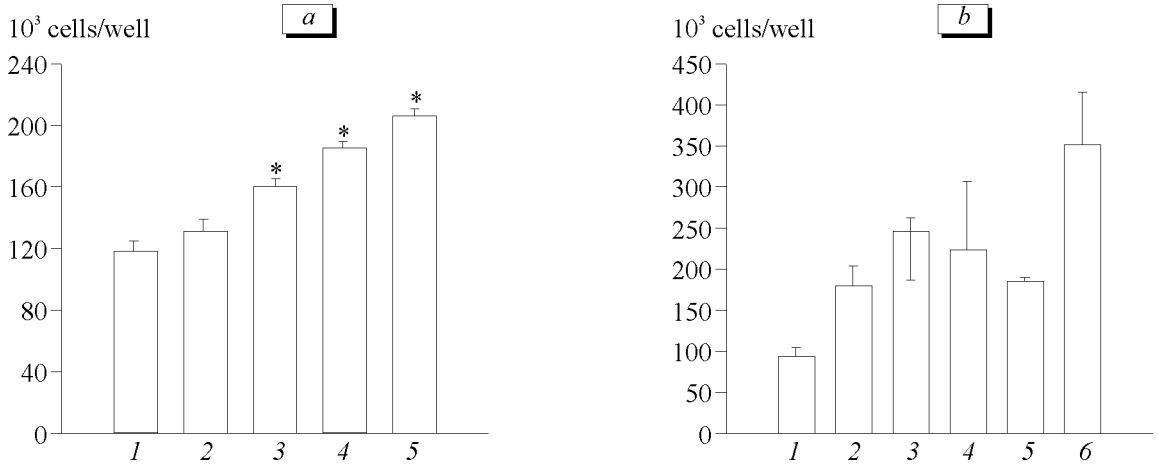

Fig. 1. Effect of serum from irradiated mice (*a*) and rats (*b*) on stromal cell count in long-term bone marrow culture. *1*) control (without serum); *2*) 2% normal mouse serum. *a*) *3-5*: 0.5, 1, and 2% serum from irradiated mouse, respectively; *b*) *3*: 2% serum from irradiated mouse; *4*: normal rat serum; *5-6*: 1 and 2% serum from irradiated mouse. *$p<0.001$ compared to normal mouse serum.

of live cells was measured colorimetrically using a CellTiter 96 Aqueous One Solution Proliferation Assay kit (Promega). For each sample, cells in 4-5 flasks or 6 wells of a 24-well plate were counted or in 6-8 wells of a 96-well plate by the colorimetric method. Before counting, the wells were washed twice with Versain solution and adhesive cells were transformed into a unicellular suspension with trypsin. For colorimetry, the cells were incubated with MTS (a new component of tetrazolium reduced by cells to colored formazan) for 3-4 h [2]. Adsorption was measured at 490 nm.

For detecting the source of MSA, the bone marrow, thymus, bones, liver, and spleen from irradiated mouse were implanted to intact mice subcutaneously or under the renal capsule. Splenocyte suspension was injected intravenously to animals pretreated with heparin (50 U/mouse). Simultaneously, intact bone marrow was implanted under the renal capsule and after 1.5 months the size of ectopic hemopoitic focus was determined by the number of hemopoietic cells [1].

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

RESULTS

Mouse serum added to long-term bone marrow culture always increased the count of stromal sublayer cells. Serum from irradiated mouse produced a more pronounced and dose-dependent effect on the growth of stromal cells (Fig. 1, *a*). Serum from irradiated rat also stimulated the growth of mouse stromal cells (Fig. 1, *b*). Hence, MSA is not species-specific, which is also characteristic of other hemopoietic growth factors. These data indicate that blood serum of both normal and irradiated mice stimulates the growth of stromal cells. Stimulation index of the

TABLE 1. Effect of Organs from Irradiated Mouse on the Growth of Ectopic Hemopoiesis Focus in Recipient Mice (*M*±*m*)

Mice	Organ, site of transplantation	Size of focus, 10^6 cells
Intact	—	9.0±1.7
Irradiated	—	21.1±6.2
Intact	Bone marrow from 6 femurs subcutaneously	8.6±2.3
Intact	5-6 femurs subcutaneously	22.0±4.1
Intact	Thymus under renal capsule	14.8±3.4
Intact	Equivalent of $^1/_2$ spleen intravenously	10.5±1.2
Intact	$^1/_3$ spleen under renal capsule	11.8±3.3
Intact	Spleen subcutaneously	13.1±2.8
Intact	$^1/_3$ liver subcutaneously	14.8±3.2
Intact	Serum from irradiated mouse intravenously	20.1±1.5
Intact	Serum from normal mouse intravenously	14.0±2.8

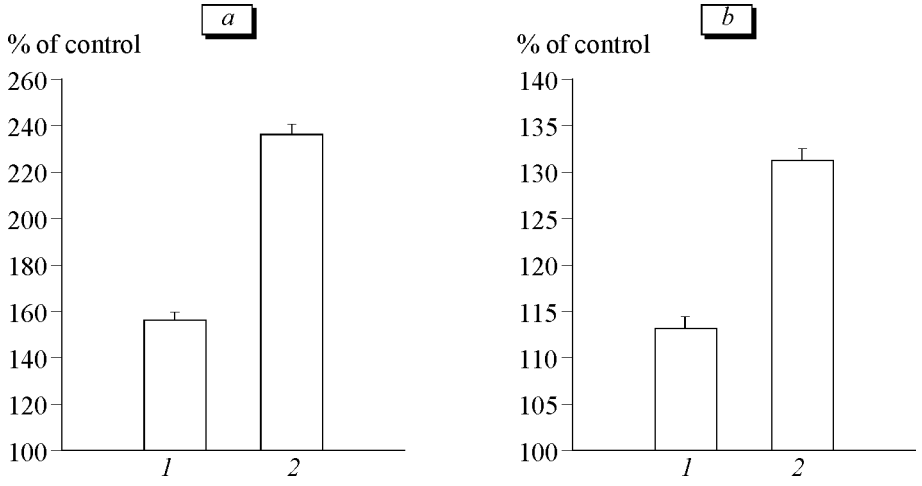

Fig. 2. Cellularity of cultures (*a*; *X*±*s*, *n*=7) and adsorption in colorimetric evaluation of cell count (*b*; *X*±*s*, *n*=8). *1*) 1% normal serum; *2*) 1% irradiated serum.

serum from irradiated mouse far surpassed that of normal serum (Fig. 2, *a*), the irradiated to intact serum activity ratio was 1.65±0.29 ($p<0.02$). All these experiments were carried out on cells cultured in flasks or 24-well plates. For testing more samples, *e. g.* fractions after chromatography, a simpler model is needed, *e.g*. 96-well plate cultures. However it is difficult to count more than 100 wells over a day in such a culture. Colorimetry simplifies this task. In this case the ratio of serum activities of irradiated to intact mice is 1.16±0.11 (Fig. 2, *b*). The stimulation index, measured by this method, is notably lower than in cultures, but the significance of difference is higher ($p<0.001$), because the range of variations becomes narrower with increasing the number of measurements. Hence, the cellularity of the sublayer in long-term bone marrow cultures in 96-well plates is a simple and sufficiently reliable method for MSA testing.

In order to detect the source of MSA in the body, various organs from irradiated mouse were transplanted to intact mice simultaneously with bone marrow implantation under the renal capsule. Intravenous infusion of serum from irradiated mice or subcutaneous transplantation of 5-6 bones stimulated the growth of ectopic hemopoiesis foci (Table 1). Subcutaneous transplantation of 6 bones from intact mice did not stimulate the growth of the focus ($12.2\pm2.3\times10^6$ cells/focus). Hence, MSA is produced in irradiated bones and released into blood serum.

We developed a relatively simple method for testing MSA and found its source, which simplifies purification of MSA from the serum of irradiated mice and search for the gene encoding this activity.

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