Production of Granulocytic Colony-Stimulating Factor in Patients with Chronic Myeloleukemia

M. Ya. Lisovskii, G. Yu. Miterev, N. D. Khoroshko, and V. G. Savchenko

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny,* Vol. 126, No. 7, pp. 94-97, July, 1998
Original article submitted January 17, 1997

After several passages, bone marrow fibroblasts and stromal adherent layers of a long-living bone marrow culture derived from patients with chronic myeloleukemia cannot maintain hemopoiesis in a culture for a long time. Immunofluorescent microscopy and flow cytometry showed that fibroblasts after passing differ from stromal cells of a normal long-living bone marrow culture: they do not produce granulocytic colony-stimulating factor. Adherent layers of bone marrow culture derived from patients with chronic myeloleukemia contain a far lesser number of cells producing granulocytic colony-stimulating factor than normal bone marrow cells.

Key Words: *stromal cells; fibroblasts; granulocytic colony-stimulating factor; hemopoiesis*

Proliferation and differentiation of hemopoietic cells is maintained by their close interactions with hemopoietic microenvironment stromal cells [1]. These cells produce macrophagal colony-stimulating factor (CSF), granulocytic CSF (G-CSF), granulocytic-macrophagal CSF (GM-CSF), stem cell factor, flt3 ligand, thrombopoietin, growth-transforming factor-β, and interleukines (IL) IL-1, IL-3, IL-6, and IL-11 [6,10,11,15].

Stromal cells of a long-living bone marrow culture (LBMC) of an origin common with passed bone marrow fibroblasts (PBMF) maintain stable hemopoiesis in a culture [3] by stimulating the proliferation and differentiation of primitive precursor cells close to stem cells initiating a long-living culture [13]. PBMF can stimulate colony formation by hemopoietic precursors [9] but not maintain stable hemopoiesis in a culture [15]. Stromal adherent layers of LBMC derived from patients with chronic myeloid leukemia cannot maintain hemopoiesis for a long time [11,15].

We checked up the hypothesis about decreased production of growth factors by PBMF and stromal layers of adherent cells of LBMC from patients with chronic myeloleukemia, in comparison with stromal cells of normal LBMC, as the cause of inability of these cells to maintain stable hemopoiesis in a culture.

Hematology Research Center, Russian Academy of Medical Sciences, Moscow

MATERIALS AND METHODS

Bone marrow was obtained from healthy donors during exfusions for bone marrow transplantation and from patients with chronic myeloleukemia. Erythrocytes were precipitated from bone marrow suspension by 0.1% methylcellulose (Sigma). For preparing PBMF, the cells were explanted in 25 cm^2 plastic flasks (Lux), 2×10^7 cells/10 ml α-MEM with 20% fetal calf serum (Vektor) and antibiotics. The medium was replaced twice a week. The cells were analyzed after 3 passages. The resultant PBMF populations contained no hemopoietic CD45-positive hemopoietic cells and CD14-positive macrophages. For preparing LBMC, 20×10^6 bone marrow cells were explanted in T-25 flasks with 10 ml complete nutrient medium containing 75% α-MEM (Flow Labs.), 12.5% fetal calf serum (Vektor), 12.5% equine serum (Vektor) with 4 mmole/liter glutamine, 10^{-4} mole/liter 2-mercaptoethanol, 10^{-6}

0007-4888/98/0007-0724$20.00

mole/liter hydrocortisone (Sigma), and antibiotics. The cultures were incubated in an atmosphere with 5% CO_2 at 37°C for 3-4 days and then at 33°C. Every week half of the culture medium was replaced with fresh medium.

PBMF and adherent cell layer of LBMC were transformed into a one-cell suspension with 0.25% Trypsin (Sigma) and after washing analyzed by indirect immunofluorescence microscopy on slides or by flow cytometry. The following antibodies to growth factors were used: monoclonal murine to IL-1β and IL-6 (Protein Contour), LM-CSF, stem cell factor, and growth-transforming factor-β and rabbit polyclonal to G-CSF (Genzyme). Murine monoclonal antibodies to smooth-muscle α-actin (Sigma) and type IV collagen (BioGenex Labs.) were used.

For microscopic examination the cells were adhered to 8-well slides precoated with poly-D-lysine, fixed in acetone, washed, and incubated with anticytokine antibodies and control IgG. After washing, secondary antispecies antibodies conjugated with ficoerythrin or fluoresceinisothiocyanate (Sigma) were added. The preparations were examined under an Axioplan fluorescence microscope (Zeiss). The expression of growth factors in the cells was assessed semiquantitatively (0—4+) by two researchers. Brightly autofluorescing macrophages were disregarded in analysis of LBMC. Positive control for anticytokine antibodies were U5637 cells and phytohemagglutinin-stimulated lymphocytes. Hemopoietic cells of LBMC stromal layers were the internal negative control.

Flow cytometry was carried out on an EPICS C device (Coulter). The cells were fixed in acetone and stained by antibodies in a suspension. For increasing the sensitivity of analysis the histogram deduction method with a Compare software (Coulter) was used.

RESULTS

The production of IL-1β, IL-6, GM-CSF, G-CSF, stem cell factor, and growth-transforming factor-β by PBMF and LBMC adherent layers was compared in 4 experiments. In all experiments PBMF reacted with antibodies to IL-1β, IL-6, GM-CSF, stem cell factor, growth-transforming factor-β, but not to G-CSF (Table 1). Large stromal cells in adherent LBMC layers were stained by antibodies to the stromal cell markers type IV collagen and smooth-muscle α-actin and to all cytokines, including G-CSF (Table 1). Small hemopoietic cells not reacting with antibodies to type IV collagen and smooth-muscle α-actin were not stained by antibodies to cytokines. Flow cytometric analysis of cytokine expression by PBMF was carried out to verify these results (3 experiments). It showed no fluorescence of PBMF stained with antibodies to G-CSF and positive staining of antibodies to all other cytokines (Fig. 1, *a*). On the other hand, LBMC adherent cells reacted with antibodies to G-CSF and all other cytokines (Fig. 1, b). In order to verify the production of G-CSF by LBMC adherent stromal cells, we labeled the cells simultaneously with antibodies to G-CSF and smooth-muscle α-actin. Two-dimensional cytometry in 2 experiments showed 7 and 9% positive cells, which confirms the production of G-CSF by LBMC adherent fibroblastoid stromal cells.

The production of these cytokines by LBMC adherent cells was studied in 6 patients with chronic myeloleukemia. In all cases a confluent layer of adherent cells with predominating fibroblastoid cells was formed during 3-4 weeks of culturing, which was confirmed by positive reaction of cells to smooth-muscle α-actin. Flow cytometry showed no cells producing G-CSF in 3 out of 6 patients and their low number in 3 other patients. On the other hand,

TABLE 1. Expression of Cytokines in PBMF and Stromal LBMC Cells (Fluorescence Microscopy)

Antibodies to cytokines	PBMF				LBMC stromal cells			
	number of bone marrow samples							
	1	2	5	6	3	4	5	6
Stem cell factor	1+	±	1+	1+	1+	2+	1+	1+
GM-CSF	2+	1.5+	1.5+	1.5+	1+	1+	1.5+	1.5+
G-CSF	0	0	0	0	1+	1+	1+	±
IL-1β	±	1.5+	1+	1.5+	1.5+	1+	1+	1+
IL-6	1+	1.5+	1.5+	1+	±	±	1+	1.5+
GTF-β	2+	2+	1.5+	1.5+	2+	1+	1+	1+

Note. Expression was assessed by intensity of fluorescence expressed semiquantitatively (±—4+). Here and in Table 2: GTF — growth-transforming factor.

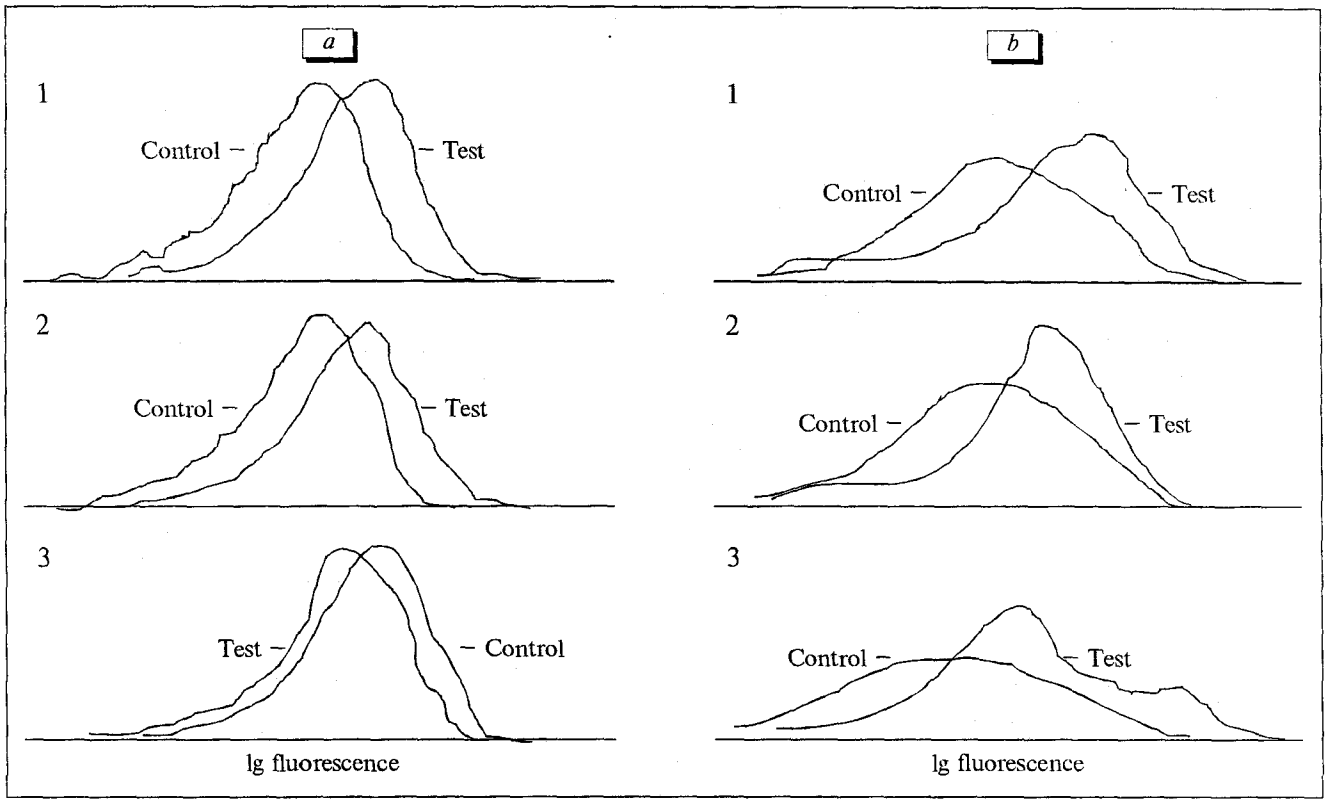

Fig. 1. Production of interleukin-1β (*1*), stem cell factor (2), and granulocytic colony-stimulating factor (*3*) in passed bone marrow fibroblasts (*a*) and long-living bone marrow culture (*b*). Control: isotypical immunoglobulins. The data of a representative experiment are shown. Ordinate: cell number.

G-CSF was detected in normal LBMC adherent cells in all 4 experiments (Table 2).

Thus, PBMF differ from normal LBMC adherent cells by inability to produce G-CSF. Our results are in line with previous study [5] which failed to detect G-CSF mRNA in PBMF. However, other scientists observed the expression of G-CSF mRNA in LBMC adherent cells and secretion of this factor into culture medium [3,8]. It is still unclear what cells produce G-CSF in LBMC adherent layers. Our data indicate that this factor is produced by stromal fibroblastoid cells. Macrophages also produce G-CSF; however, analysis of these cells was hampered by strong autofluorescence.

The production of G-CSF by LBMC stroma from patients with chronic myeloleukemia incapable of maintaining stable hemopoiesis [11,15] decreased, while the production of other cytokines was virtually normal.

A considerable body of evidence points to the significance of G-CSF production by stromal cells. In LBMC, the expression of G-CSF, but not FM-CSF by stromal cells as a result of transfection led to permanent proliferation of primitive hemopoietic precursor cells [7]. LBMC stroma of patients with incompetent bone marrow transplants did not produce G-CSF but secreted GM-CSF [12]. The level of serum endogenous G-CSF produced mainly by the hemopoietic microenvironment correlated with taking in of allogeneic and autologous transplants [2]. Since neither PBMF, nor LBMC stroma of patients with chronic myeloleukemia do not main-

TABLE 2. Expression of Cytokines by LBMC Adherent Cells in Health and Chronic Myeloleukemia (Flow Cytometry, (*M±m*)

LBMC type	Percentage of LBMC adherent cells positively stained by antibodies to cytokines					
	G-CSF	GM-CSF	stem cell factor	IL-1β	IL-6	GTF-β
Norm (*n*=4)	12±3.3	37±7.6	41±7.8	28±6.5	27±8.5	40±9.3
Chronic myeloleukemia (*n*=6)	1.7±0.8*	28±4.3	42±8.9	28±5.3	35±7.9	36±6.2

Note. *$p<0.05$ *vs.* the norm.

tain stable hemopoiesis in a culture, our results may indicate the significance of G-CSF production by bone marrow stromal cells for maintaining myelopoiesis by primitive precursor cells in a culture.

REFERENCE

1. T. D. Allen and T. M. Dexter, in: *Colony Stimulating Factors: Molecular and Cellular Biology,* eds. T. M. Dexter *et al.,* New York-Basle (1990), pp. 1-38.
2. M. S. Cairo, Y. Suen, L. Sender, *et al., Blood,* **79,** 1869-1869 (1992).
3. C. J. Eaves, J. D. Cashman, R. T. Kay, *et al., Ibid.,* **78,** 110-117 (1991).
4. S. Gartner and H. S. Kaplan, *Proc. Natl. Acad. Sci. USA,* **77,** 4756-4759 (1980).
5. S. C. Guba, C. I. Sartor, L. R. Gottschalk, *et al., Blood,* **88,** 1190-1198 (1992).
6. M. C. Heinrich, D. C. Dooley, A. C. Freed, *et al., Ibid.,* **82,** 771-783 (1993).
7. D. E. Hogge, J. D. Cashman, R. K. Humphries, and C. J. Eaves, *Ibid.,* **77,** 493-499 (1991).
8. E. L. Kittler, H. McGrath, D. Temeles, *et al., Ibid.,* **79,** 3168-3178 (1992).
9. M. Lee, G. M. Segal, and G. C. Bagby, *Exp. Hematol.,* **5,** 983-988 (1987).
10. M. Lisovsky, S. E. Braun, Y. Ge, *et al., Leukemia,* **10,** 1012-1018 (1996).
11. M. Lisovsky and V. G. Savchenko, *Leuk. Lymphoma,* **19,** 142-145 (1995).
12. A. R. Magliaccio, G. Magliaccio, G. Johnson, *et al., Blood,* **75,** 305-312 (1990).
13. H. Nagahisa, Y. Nagata, T. Ohnuki, *et al., Ibid.,* **87,** 1309-1316 (1996).
14. J. H. Sutherland, P. M. Lansdorp, D. H. Henkelman, *et al., Proc. Natl. Acad. Sci. USA,* **87,** 3584-3588 (1990).
15. M. Takahashi, A. Keating, and J. Singer, *Exp. Hematol.,* **13,** 926-931 (1985).