The Effect of Bone Marrow Fibroblast and Stromal Cell-Conditioned Media on Hemopoietic Cells in Culture

A. Y. Zaritskey and O. V. Strizhak [1]

Introduction

It is well known that fibroblasts [1] and stromal cells (Sc) are the main components of the bone marrow microenvironment [2–4]. The concept of short-range regulation of hemopoiesis by Sc is widely accepted [2, 4]. Meanwhile bone marrow fibroblasts and Sc are potent producers of long-range factors affecting hemopoiesis: stimulators [5–8], inhibitors, [9] and probably restrictins [1]. Many of the regulatory effects of Sc were shown to be due to the well-known hemopoietic growth factors [10–13]; some of them are anchored to extracellular matrix [14].

Stimulatory activity of bone marrow fibroblasts in semisolid cultures were thoroughly studied, but the results are controversial: absence of any stimulatory activity [7], the necessity of the presence of monocytes for stimulating the granulomonocyte colony formation [8], direct induction of granulomonocyte colony formation [5, 6]. It was also reported that fibroblasts but not their conditioned media (CM) could be potent inhibitors of hemopoietic cell proliferation [9].

In this paper we tried to study the effect of bone marrow fibroblast- and stromal cell-conditioned media (FCM, ScCM) in different hemopoietic disorders on proliferation of hemopoietic cells and cell lines in culture.

[1] 1st Pavlov Medical Institute, Leningrad, USSR.

Materials and Methods

Patients. Healthy individuals, patients with acute nonlymphocytic leukemia (ANLL), acute lymphocytic leukemia (ALL), chronic myelocytic leukemia (CML), myelodysplastic syndrome (MDS), and leukemia-unrelated neutropenias were studied for their bone marrow FCM granulocyte-macrophage colony-stimulating (GMCSA) and inhibiting activity (Tables 1, 2) and for their bone marrow FCM and ScCM effects on blast cell growth (Fig. 1). Experiments were started before drug administration.

Target Cells. Nonadherent bone marrow cells were used as targets in semisolid bone marrow cultures. K-562 and HL-60 line cells and peripheral blood cells of patients with ANLL were used for [^{3}H]thymidine uptake.

Bone Marrow Fibroblasts. Bone marrow cells (5-IO $\times 10^6$) were cultured in Carrel flasks using medium 199 and human serum (20%). The medium was changed twice a week. Two-to-four-week cultures were treated by trypsine (0.125%) and recultured. FCM were collected from secondary confluent cultures, frozen, and then tested.

Bone Marrow Sc. Unprocessed bone marrow cells were cultured in 25-ml T-flasks containing McCoy's 5A medium, 12.5% of fetal calf serum (FCS) 12.5% of horse serum and 10^{-7} *M* hydrocortisone (Upjohn).

Assay for Stimulators in Agar Culture. FCM (0.75 ml) was incorporated in the

Haematology and Blood Transfusion Vol. 35
Modern Trends in Human Leukemia IX
R. Neth et al. (Eds.)

bottom layer of bilayer agar culture. Previously this system was used to study human blood serum colony stimulating activity (CSA) [15]. Human cord blood serum was used instead of FCS. Colonies were scored after 7 days of incubation at 37°C in humidified CO_2 (7%) atmosphere.

Assay for Inhibitors in Agar Culture. FCM activity was studied in three kinds of experiments: (a) addition of FCM to leucocyte feeder, (b) addition of FCM to leucocyte-conditioned medium in the bottom layer, and (c) comparison of stimulatory activity of FCM when nonadherent (NA) or nonfractioned (NF) bone marrow cells were used as targets. The amount of FCM incorporated was 0.75 ml. Optimal concentrations of leucocyte feeder cells and leucocyte-conditioned medium were used.

The Effect of FCM and ScCM on Hemopoietic Cell [^{3}H]Thymidine Uptake. Liquid culture was used in an experiment to asses the effect of the CM on hemopoietic cell [^{3}H]thymidine uptake. HL-60, K-562 line cells, Ficoll-Hypaque fractionated peripheral blood cells of patients with acute myelocytic leukemia (AML), and CML-BC (more than 95% of blasts) were cultured in RPMI medium (10% FCS) in 96-microwell plates. Cell concentration was $1 \times IO^4$ per well. [^{3}H]thymidine (1 μCi/ml) was added to the peripheral blood cells at the initiation of culture for 12 h, and to the cell line cells after 48 h of serum deprivation (2%).

Results and Discussion

FCM of healthy individuals appeared to stimulate GM colony formation by nonadherent bone marrow cells (Table 1).

Our success in revealing stimulatory activity may be due to the large amount of CM added to the bilayer agar culture. Another possibility is the influence of human serum on the production of hemopoietic growth factors. It was recently shown that serum compounds dramatically affect the secretion of regulatory molecules by the cells of bone marrow stroma [11].

FCM CSA varied on a large scale in different hemopoietic disorders. This may be the result either of the presence of hemopoietic cells in fibroblast culture or of the different number of fibroblast cells per culture. These possibilities were studied. Comparative cytogenetic studies were performed in bone marrow hemopoietic cells and in cultured fibroblasts in four CML patients. All mitotic hemopoietic cells were Ph-positive, whereas all fibroblast metaphases were Ph-negative (data not shown), although admixture of nondividing macrophages cannot be precluded.

Preliminary data have shown that confluent cultures in different hemopoietic disorders have nearly equal numbers of fibroblastic cells. Rough correlation is seen between FCM CSA and the amount of myeloid tissue. FCM CSA is much higher in CML or megaloblastic anemia than in AA.

The results of fibroblast inhibitory activity are shown in Table 2. Addition of CML or MDS FCM to LCM resulted in inhibition of colony formation. The effect seems to be due to the direct inhibition of granulomonocyte progenitor cells. Inhibition of colony formation was also noted when FCM was added to the feeder layer. MDS FCM revealed the most significant effect. By contrast, CML fibroblasts failed to reveal an inhibitory effect in this kind of experiment.

Lower colony-forming ability stimulated by feeder plus FCM in comparison to feeder alone may result from the depression of CSA release.

The calculated ratio of NF/NA bone marrow CFA stimulated by FCM shows that NA CFA is higher than NF CFA stimulated by the same conditioned media. There was no significant difference in NF and NA CFA stimulated by LCM. This effect of FCM could be explained neither by the direct inhibition of GM CFC nor by the depression of

Table 1. FCM CSA in different hemopoietic disorders (±SD)

Healthy individuals	ALL	AML	CML	AA	Megaloblastic anemia and hypersplenism
18±8	4±3*	28±15	29*±6	5±5*	25±15
n 17	*n* 9	*n* 11	*n* 12	*n* 4	*n* 5

n, number of patients studied; AA, aplastic anemia.
* Difference in FCM CSA between healthy individuals and patients; $p < 0.05$.

Table 2. Inhibitory activity of FCM in bilayer agar culture

Source of FCM	Assay method		
	$\frac{\text{FCM+Feeder}}{\text{Feeder}}$ (%)	$\frac{\text{FCM+LCM}}{\text{LCM}}$ (%)	$\frac{\text{NF+FCM}}{\text{NA+FCM}}$ (%)
Healthy individuals	120±29.8 ($n=9$)	90±20.8 ($n=17$)	61.7±4.2** ($n=7$)
CML	130.0±3 ($n=8$)	74.6±9.8* ($n=7$)	12.5±2.1** ++ (n=7)
MDS	16.0±4.0** ++	62.3±2.1*	44.2±1.1** +
Absence of FCM	100±5.1 ($n=42$)	100±4.1 ($n=35$)	–
Absence of FCM+LCM	–	–	107±12.1 ($n=13$)

*, ** Differences in the data when FCM is Present or absent: ($p<0.05$; $p<0.01$)
+, ++ Differences between FCM inhibitory activity of healthy individuals and patients with hemopoietic disorders: ($p<0.05$; $p<0.01$).

CSA release. Really, fibroblasts of healthy individuals did not express any inhibitory activity in the previous experiments (Table 2). The most reasonable explanation is the production of inhibitory molecules by mononuclear cells induced by FCM.

We further studied the effect of ScCM and FCM on [^{3}H]thymidine uptake by leukemic cells (Fig. 1). All kinds of ScCM and FCM appeared to inhibit the growth of AML and CML-BC blast cells. ScCM of healthy individuals and AML patients were potent inhibitors of [^{3}H]thymidine uptake by HL-cells whereas CML and CML-BC ScCM stimulated their proliferation. AML ScCM tended to be superior in their inhibitory activity towards all target cells in comparison to ScCM of the other groups of patients.

The effect of ScCM and FCM coincided in all groups studied with more or less quantitative differences. This is in agreement with the data on the common nature of fibroblasts and stromal cells [16]. In healthy individuals and in patients with CML, ScCM was as a rule comparable or inferior to that of FCM, with the exception of healthy individuals' effect of ScCM towards K-562 cells. In AML cells ScCM tended to be more active than FCM.

The difference between FCM and ScCM could not be explained by the presence of hydrocortisone in ScCM. It was shown that hydrocortisone in concentrations equal to its amount in the CM has an insignificant influence on target cell proliferation (less than 12%).

The problem of the heterogeneity of FCM and ScCM activities in different hemopoietic disorders arises. It is probably related to the heterogeneity of fibroblast and stromal cells. In fact, cell

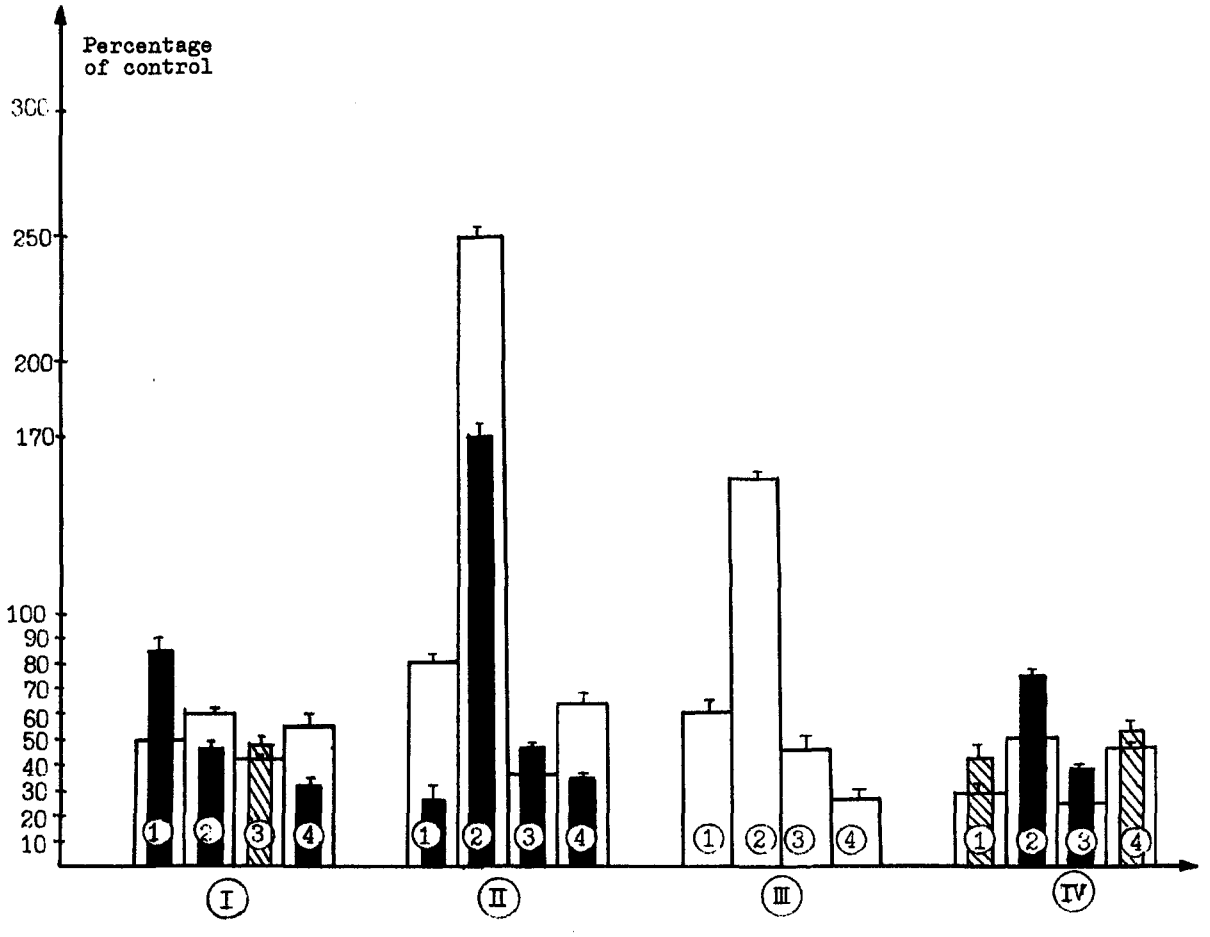

Fig. 1. Inhibiting activity of stromal cell- and fibroblast-conditioned media toward hemopoietic cells in liquid culture. *I–IV*, FCM and ScCM: *I*, healthy individuals ($n = 7$); *II*, patients with CML ($n = 5$); *III*, patients with CML-BC (myeloid) ($n = 5$); and *IV*, patients with AML ($n = 6$). *1–4*, target cells: *1*, K-562; *2*, HL-60; *3*, AML; *4*, CML-BD (myeloid). *Wide columns*, ScCM activity (± SD). *Narrow black and shaded columns*, FCM activity (± SD). *Black narrow columns*, FCM activity is statistically different from ScCM when the same target cells and the source of CM are used

lines derived from stromal cells are very diverse in their functions [17]; moreover, bone marrow fibroblasts in hemopoietic disorders differ in the amounts of receptors to glucocorticoids [18]. Therefore, differences in stimulating or inhibiting activity of FCM or ScCM may be due to the predominant proliferation of different types of stromal cells.

Finally, we have revealed significant variations in FCM and ScCM in the ability to stimulate and inhibit proliferation of hemopoietic cells in patients with hemopoietic disorders. When derived from a nonleukemic clone [19, 20], these cells probably modulate proliferation of normal and leukemic cells in order to prevent stroma-independent leukemic cell growth.

References

1. Zipori D (1989) Stromal cells from the bone marrow: evidence for a restricting activity role in regulation of hemopoiesis. Eur J Haematol 42:225–232
2. Dexter TM, Allen TD, Lajtha LG (1977) Conditions controlling the proliferation of haemopoietic stem cells in vitro. J Cell Physiol 91:335–344
3. Dexter TM, Moore MAS (1977) In vitro duplication and cure of haemopoietic defects in genetically anaemic mice. Nature 269:412–414
4. Dexter TM, Testa NG, Allen TO, Rutherford T, Scolnick E (1981) Molecular and cell biological aspects of erythropoiesis in long term bone marrow culture. Blood 58:699–707
5. Hotta T, Moore MAS (1977) In vitro duplication and cure of haemopoietic de-

fects in genetically anaemic mice. Nature 269:412–414
6. Ben-Ishay Z, Barak V, Shosham S, Prindull G (1989) Bone marrow deficiency in murine acute leukemia: study of marrow macrophages and fibroblasts. Exp Hematol 17:530
7. Castro-Malaspina, Ebell W, Wang S (1984) Human bone marrow fibroblast colony forming units. In: Myelofibrosis and the biology of connective tissue. Liss, New York, pp 209–236
8. Nagao T, Yamauchi K, Schimizi M, Noguchi K (1986) Regulatory role of human bone marrow fibroblasts. Exp Hematol 14:696–701
9. Zipori D, Sasson T (1980) Adherent cells from mouse bone marrow inhibit the formation of colony stimulating factor induced myeloid colonies. Exp Hematol 8:816–817
10. Brockbank KGM, de Jong JP, Piersma AH, Voersman JSA (1986) Haemopoiesis on purified bone-marrow-derived reticular fibroblasts in vitro. Exp Hematol 14:386–391
11. Cashman JD, Eaves AC, Raines EW, Ross R, Eaves CJ (1990) Mechanisms that regulate the cell cycle status of very primitive haemopoietic cells in long-term human marrow cultures. I. Stimulatory role of a variety of mesenchymal cell activators and inhibitory role of TGF. Blood 75:96–101
12. Sieffe CA, Niemeyer CN, Faller DV, Jang Y-C, Clark SC, Nathan DG (1987) Two monokines, II-l and TNF regulate production of haemopoietic growth factors by mesenchymal cells. Br J Haematol 66:571–578
13. Yan Z-J, Wang O-R, McNiece IK, Wolf NS (1990) Dissecting haematopoietic microenvironment. VII. The production of an autostimulatory factor as well as a CSF by unstimulated murine marrow fibroblasts. Exp Hematol 18:348–354
14. Gordon MY, Riley GP, Greaves MF (1987) Compartmentalization of a haemopoietic growth factor (GM-CSF) by glycosaminoglycans in the bone marrow microenvironment. Nature 326:403–405
15. Mintz U, Sachs L (1973) Differences in inducing activity for human bone marrow colonies in normal serum and serum from patients with leukemia. Blood 42:331–339
16. Perkins S, Fleischman RA (1990) Stromal cell progeny of murine bone marrow fibroblast colony-forming units are clonal endothelial-like cells that express collagen IV and laminin. Blood 75:620–625
17. Anderson RW, Mann SL, Crouse DA, Sharp JG (1981) Modulation of one of three murine stromal cell lines to adipose cells by serum and insulin. Cell Recogn JSS 16:734–741
18. Juneja HS, Gardner FH, Minguell JJ, Helmer RE (1984) Abnormal marrow fibroblasts in aplastic anemia. Exp Hematol 12:221–230
19. Singer JW, Kwating A, Cuttner J, Gown A, Jacobson R, Killen PD, Moohr JW, Najfeld V, Powell J, Sanders J, Striker GE, Phialkow PY (1974) Evidence for a stem cell common to hematopoiesis and its in vitro microenvironment: studies of patients with clonal haemopoietic neoplasia. Leuk Res 8:535–545
20. Raskind WH, Singer JW, Morgan CA, Fialkow PJ (1988) Host origin of marrow stromal cells obtained from transplant recipients and transformed in vitro by Simian virus-40. Exp Hematol 16:827–830