Jpn. J. Cancer Res. (Gann), 79, 843–849 ; July, 1988

Proliferative Effect of Human Granulocyte Colony-stimulating Factor on Blast Cells of Acute Promyelocytic Leukemia

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The effect of human granulocyte colony-stimulating factor (G-CSF) on leukemic cells of acute promyelocytic leukemia (APL) was examined. Mononuclear cells obtained from bone marrow cells containing more than 90% blasts from seven APL patients were incubated in the presence of G-CSF using semisolid and liquid culture systems. On day 7, the cells from all the patients produced many clusters consisting of 8–40 cells. These cells appeared to be promyelocyte-like blast cells in four patients and had differentiated to more mature neutrophils in three patients. On day 14, the number of clusters decreased except for two patiens. Blast cells from the two patients showing the increase of blast clusters could proliferate in a liquid culture containing G-CSF. Blast cells cultured for 14 days formed many secondary cultures after replating on a methylcellulose medium. Moreover, chromosomal analyses of blasts cultivated in the presence of G-CSF for 7 days showed t(15;17) in all metaphases in one patient. It appears that the leukemic cells from APL patients could proliferate in the presence of G-CSF.

Key words: Acute promyelocytic leukemia — Granulocyte colony-stimulating factor — Colony assay — Karyotype

We have previously reported that human G-CSF*2 is a lineage-specific factor which acts on granulocyte progenitor cells and not on early erythroid progenitor cells.[1] Recently, recombinant CSFs such as granulocyte-macrophage CSF,[2] G-CSF[3,4] and macrophage-CSF[5] have been used to examine the mechanisms controlling hemopoiesis, and they have also been clinically applied.[6–9] It is important to clarify whether leukemic progenitors can proliferate in the presence of CSF, since it might be administered to acute leukemia patients. Begley *et al.*[10] and Nicola,[11] using cell autoradiographic analysis of the binding of ^{125}I-G-CSF, found that promyelocytes and APL cells had a uniformly higher receptor number than mature neutrophils and the other leukemic cells, respectively. In this study, we examined the effect of G-CSF on leukemic cells from patients with APL.

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*2 Abbreviations: G-CSF, granulocyte colony-stimulating factor; APL, acute promyelocytic leukemia.

MATERIALS AND METHODS

Patients Seven Japanese patients were diagnosed as having APL according to the French-American-British classification of acute leukemia at Jichi Medical School Hospital (Table I). Patient 5 was diagnosed as having an M3 variant by electron microscopic examination. Their bone marrow cells obtained before chemotherapy contained more than 90% blasts. Chromosomal analysis of bone marrow cells showed t(15;17)(q22;q11) in five patients and normal karyotype in two patients. All patients had complications of disseminated intravascular coagulation on admission. Patients 2, 3, 4, 5 and 7 achieved complete remission in response to induction chemotherapies, while patients 1 and 6 responded poorly. A healthy volunteer was also examined as a control.

Bone Marrow Mononuclear Cells (MNCs) Bone marrow cells were separated by centrifugation in Ficoll-Metrizoate (Lymphoprep, Nyegaad, Oslo) after which the interface was harvested and washed twice in Iscove's modified Dulbecco's medium (IMDM, GIBCO, Grand Island, NY).

Colony Assays of Leukemic Cells Colony assays of leukemic progenitors were carried out by methylcellulose culture. A mixture (1 ml) of 30% fresh platelet-poor plasma (PPP), 1% bovine

Table I. Clinical and Hematological Data for Each APL Patient

Patient no.	Age/Sex	Sample	Blasts (%)	Chromosomal analysis	Peripheral blood			Clinical status	
					WBC ($/mm^3$)	Hb (g/dl)	Platelets ($\times 10^4/mm^3$)	Complication of DIC	Response to chemotherapy
1	59/F	BM	98.0	46,XX	2,600	4.8	2.0	+	Failure
2	45/M	BM	96.6	46,XY,t(15;17)	700	6.8	0.7	+	CR
3	46/M	BM	92.6	46,XY,t(15;17)	1,100	5.3	4.7	+	CR
4	21/M	BM	94.8	46,XY,t(15;17)	6,700	5.7	2.0	+	CR
5	44/F	BM	96.6	47,XX,+10,−17, +i(17q),t(15;17)	13,100	13.7	2.8	+	CR
6	66/F	BM	94.8	46,XX	325,000	6.3	1.1	+	Failure
7	50/M	BM	98.0	46,XY,t(6;12),t(15;17)	7,900	9.5	2.1	+	CR

Abbreviations: F, female; M, male; BM, bone marrow; CR, complete remission; DIC, disseminated intravascular coagulation.

serum albumin (Sigma Chemical Co., St Louis), $5 \times 10^{-5} M$ 2-mercaptoethanol (Sigma Chemical Co.), IMDM, 5×10^4 MNCs/ml, and 1, 10, or 100 ng of purified native or recombinant human G-CSF (Chugai Pharmaceutical Company, Tokyo) was incubated at 37° in a hypoxic culture system (5% CO_2, 5% O_2 and 90% nitrogen). G-CSF had a specific activity of $2.5–10.0 \times 10^7$/mg protein.[12, 13] The culture plates were evaluated on days 7 and 14 under an inverted microscope. Groups containing from 8 to 40 cells were counted as clusters and those containing more than 40 cells as colonies. Clusters and colonies were picked up at random from the semisolid media and pooled and suspended in media containing 30% fetal calf serum, and their morphology was determined using May-Grünwald-Giemsa-stained cytospin preparations. Erythroid bursts were formed in the presence of phytohemagglutinin-stimulated leukocyte-conditioned medium (PHA-LCM) and erythropoietin and their number was scored as previously reported.[14]

Liquid Cultures of Leukemic Progenitors A mixture (1 ml) containing 10^5/ml MNCs, 10% PPP, IMDM and 1, 10, or 100 ng/ml G-CSF was incubated in 24-well multiplates (Coster, Cambridge, Mass.) at 37° in the hypoxic culture system. Half-medium change and addition of G-CSF were performed every week. The number of cells and their morphology were examined every week. Blasts supported by this liquid culture were centrifuged, washed and replated into the methylcellulose medium with or without G-CSF.

Chromosomal Analyses of Cultivated Blasts A mixture (10 ml) containing 10^6/ml MNCs, 20% fetal calf serum, IMDM and 20 ng/ml G-CSF was also incubated in 25 cm^2 culture flasks. The medium was changed and G-CSF was added every other day. On day 7, 1 ng/ml colchicine (demecolcine, Sigma) was added to the medium which was further incubated overnight. Air-dried slides were analyzed by Q-banding.

Results

Colony Formation in the Presence of G-CSF On day 7 of culture, the cells from the APL patients produced many clusters and a few colonies in the presence of G-CSF (Table II). Fifty thousand bone marrow cells formed 306–2558 clusters (replating efficiency; 0.61–5.51%) in the presence of 100 ng/ml G-CSF. In the presence of 100 ng/ml G-CSF, normal control BM cells formed more colonies and fewer clusters compared with APL. No clusters were formed by the cells from patients 1, 2, 5, 6 and 7 in the absence of G-CSF. Although we previously showed that the number of normal granulocyte-macrophage colonies reached a plateau at a G-CSF concentration of 20 ng/ml,[1] the number of leukemic clusters increased in proportion to G-CSF concentration except for the cells from patient 1. Figure 1 shows typical clusters; aggregations consisted of round, refractile and medium-sized cells under an inverted microscope. Morphological analyses of cells from pooled colonies and clusters showed promyelocyte-like blasts without differentiation in patients 1, 5, 6 and 7 (Fig. 2), while the cells from patients 2, 3 and 4 were found to be morphologically differentiated into mature neutrophils (Table III).

On day 14 of culture, the number of clusters decreased except in the cases of patients 1

Table II. Colony Formation of Bone Marrow Cells from APL Patients

Patient no.	No. of colonies/clusters per 5×10^4 cells in the presence of G-CSF				Erythroid burst/5×10^4 cells
	(−)	1 ng/ml	10 ng/ml	100 ng/ml	
On day 7 of culture					
1	0/0	0/770±99	0/1397±88	0/996±115	
2	0/0	0/113±11	1±1/222±26	1±1/306±26	
3	0/17±5	0/226±28	1±1/468±49	3±1/682±66	
4	0/124±32	0/363±64	0/696±109	0/1146±154	
5	0/0	0/1105±65	0/1980±30	0/2558±203	
6	0/0	0/0	0/25±3	0/390±25	
7	0/0	0/386±28	0/1435±49	0/1760±58	
Normal BM	0/25±3	6±2/356±12	126±10/239±25	178±28/146±4	
On day 14 of culture					
1	0/0	0/1177±121	0/2974±513	0/1850±339	0
2	0/0	0/180±26	1±1/159±39	3±1/43±1	1±1
3	1±1/29±6	1±1/93±11	2±1/202±5	1±1/299±116	2±1
4	0/64±5	1±1/148±2	0/144±17	1±1/281±30	6±1
5	0/240±20	0/1265±345	5±1/1870±200	65±15/2128±58	0
6	0/48±13	0/93±43	0/573±13	0/2532±133	0
7			ND		
Normal BM	2±1/66±2	35±5/90±10	61±7/92±8	57±5/95±23	64±6

Aggregations that contained more than 40 cells were counted as colonies and those containing from 8 to 40 cells as clusters; values represent the mean±SD of four plates.
ND, not done.

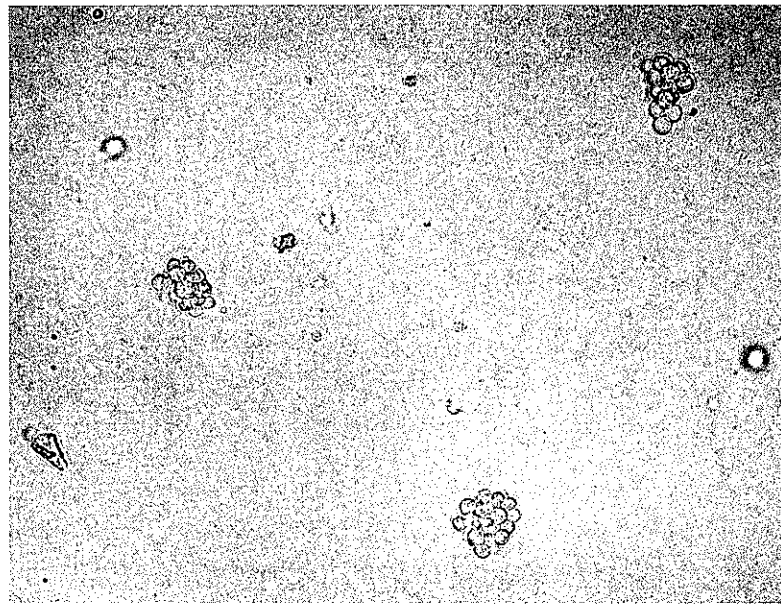

Fig. 1. Blast clusters (from patient 1) under an inverted microscope.

and 6. Morphological analyses of pooled cells showed further differentiation in patients 2, 3, 4 and 5, but not in patients 1 and 6 (Table III). The number of erythroid bursts of APL decreased in comparison with the normal control.

G-CSF Effects in Liquid Culture System In the culture of cells obtained from patient 6, the number of morphologically blast-like cells gradually increased until day 40 in the pres-

Fig. 2. May-Grünwald-Giemsa-stained cytospin preparation of pooled cluster cells from patient 1.

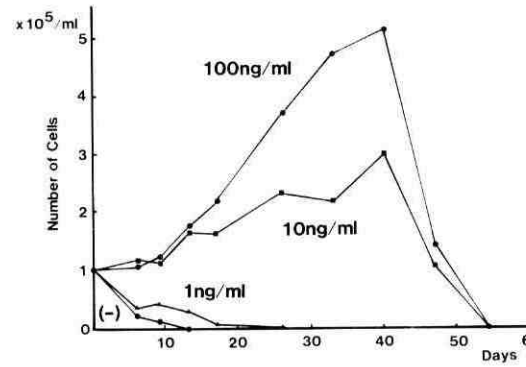

Fig. 3. The effects of various concentrations of G-CSF on bone marrow mononuclear cells from patient 6 in a liquid culture system.

Table III. Morphological Analyses of Pooled Colony and Cluster Cells Formed in the Presence of G-CSF

Patient no.	Differential count[a] (%)							
	MB	Pro	M	Met	Band	Seg	Mϕ	Lym
On day 7 of culture								
1		100						
2		62	19	4		1	14	
3		17	35	36	4	1	7	1
4		56	13	5			24	2
5		85	5	1			8	1
6		99					1	
7		94	5				1	
Normal BM	4	40	10	16	5	6	11	8
On day 14 of culture								
1		99					1	
2		21			3		76	
3		7	36	1			56	
4		33	27	9	3		28	
5		36	39	2		1	1	
6		100						
7				ND				
Normal BM		13	1	3	9	56	18	

Abbreviations: MB, myeloblast: Pro, promyelocyte; M, myelocyte; Met, metamyelocyte; Band, band form neutrophil; Seg, segmented neutrophil; Mϕ, macrophage; Lym, lymphocyte; BM, bone marrow; ND, not done.

a) Differential counts were performed on 100 cells.

Table IV. Colony Assay of Liquid-cultured Cells in the Presence of G-CSF

Patient no.	Duration of liquid culture (days)	In the presence of 10 ng/ml G-CSF	No. of colonies and clusters per 10^4 cells	Differential count of blasts (%)
1	16	+	18±1	97.3
	16	−	0	
6	15	+	205±7	83.3
	15	−	0	
Normal BM	14	+	1±1	0
	14	−	0	

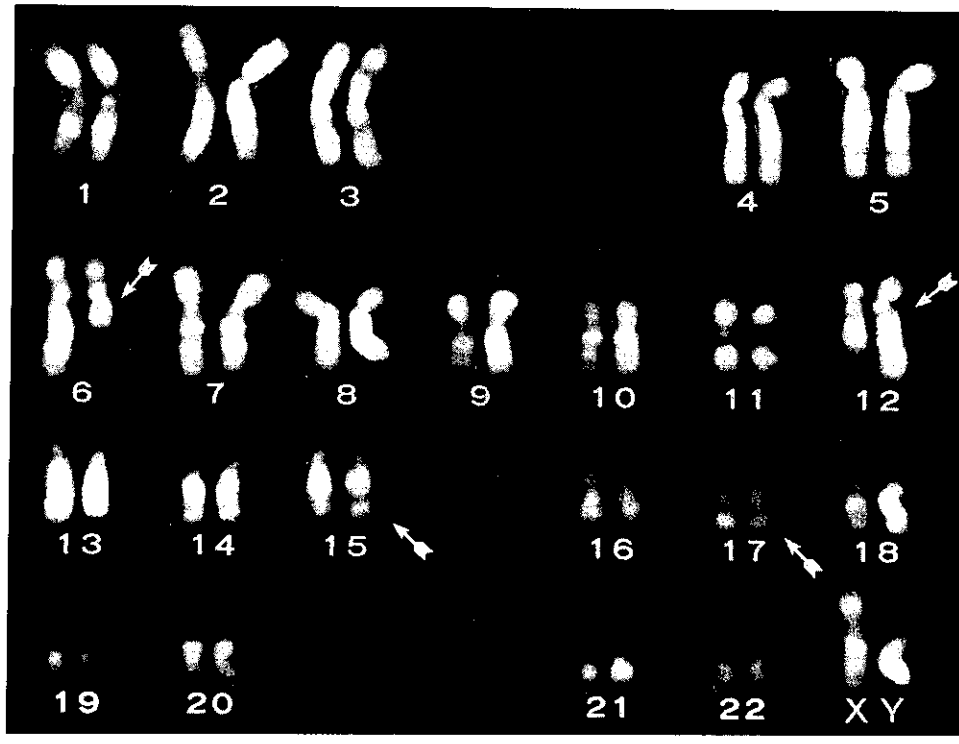

Fig. 4. Q-banded karyotype from 7-day-cultivated cells in patient 7: 46,XY,t(6;12)(q11;q13), t(15;17)(q22;q21).

ence of 10 or 100 ng/ml G-CSF, while it decreased without G-CSF or 1 ng/ml G-CSF as shown in Fig. 3. The same results were obtained for the cells from patient 1, but in the cultures of cells from the other patients the number of cells gradually decreased in spite of the addition of G-CSF.

Blast cells obtained from patients 1 and 6 were cultured for two weeks in a liquid culture system. After replating of these cells in a methylcellulose medium, they produced clusters mainly composed of blasts in the presence but not in the absence of G-CSF (Table IV). The cells from normal controls produced a

very few clusters and colonies in the presence of G-CSF after replating from the liquid cultures and they contained only mature neutrophils.

Chromosomal Analyses of Cultivated Blasts Chromosomal analyses of blastic cells cultured for 7 days in the presence of G-CSF showed 46,XY,t(6;12)(q11;q13),t(15;17)(q22;q21) in 28 (90.3%) and 45,XY,9,t(6;12)(q11;13),t(15;17)(q22;21) in 3 (9.7%) of 31 metaphases in patient 7 (Fig. 4). Differential counting of the cultured cells gave the following results: promyelocytes 89.2%, myelocytes 9.8%, metamyelocytes 0.4%, lymphocytes 0.4% and macrophages 0.2%.

Discussion

In this study, we showed that bone marrow mononuclear cells from APL patients could produce significantly more clusters than those from normal controls in the presence of G-CSF using a methylcellulose culture system. The morphological analyses of the cells from pooled colonies and clusters showed the appearance of APL-like blast cells. In a liquid culture system, the bone marrow mononuclear cells from two patients (patients 1 and 6) could proliferate and survive for more than 40 days in the presence of G-CSF without differentiation but with a high replating efficiency. Moreover, cultured cells were confirmed to be of leukemic origin by chromosomal analysis in patient 7. These data suggested that G-CSF has proliferative activity for APL cells.

G-CSF has been reported to have a differentiation effect on APL cells.[4] According to our morphological analyses of pooled colonies and clusters which were formed in the presence of G-CSF, some of the APL cells seemed to differentiate to more mature neutrophils. Whether the mature cells of a colony or cluster originated from a leukemic progenitor or from a normal residual stem cell may be difficult to determine morphologically, because normal stem cells usually still remained at first diagnosis in acute leukemia patients and were able to form granulocyte-macrophage (GM) colonies in the presence of G-CSF. The normal stem cells can also form erythroid bursts as well as GM colonies in the presence of PHA-LCM and erythropoietin. The finding that very few erythroid bursts formed from APL patients on admission suggests not only a decrease of erythroid precursor cells but also that of myeloid precursor cells. Although we tried to examine the karyotypes from a single colony or pooled colonies in these patients with t(15;17) to determine the origin, we could not obtain any analyzable metaphases. The finding that a large number of clusters were formed by the bone marrow cells from these patients with APL showed that these progenitors are probably not derived from normal stem cells but from APL progenitor cells.

Recently, G-CSF, GM-CSF and M-CSF have all been applied to cancer patients with neutropenia induced by chemotherapy or bone marrow transplantation or patients with myelodysplastic syndrome.[7-9] Our findings on the *in vitro* effect of G-CSF on the proliferation of APL cells are consistent with the results obtained by Begley *et al.*[10] that APL cells exhibited G-CSF receptors. Thus, much attention should be paid not only to the recovery of granulopoiesis but also to the proliferation of leukemic cells after administration of G-CSF to APL patients.

Acknowledgments

We would like thank to Messrs. M. Murai, M. Sonoyama, M. Todoroki, and Ms. Kurokawa for their skillful technical assistance. This work was supported by Grants-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan.

(Received Feb. 16, 1988/Accepted May 27, 1988)

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