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Detection of Granulocyte Colony-stimulating Factor Produced by a Newly Established Human Hepatoma Cell Line Using a Simple Bioassay System

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A colony-stimulating factor(CSF)-producing tumor cell line (KX-87) was established from a patient with hepatocellular carcinoma and marked granulocytosis. This cell line formed tumors on nude mice in high frequency and the mice revealed marked granulocytosis. In clonogenic assays of human bone marrow cells, KX-87 conditioned medium (CM) supported the formation of colonies mainly consisting of neutrophilic granulocytes but had no burst-promoting activity. The molecular weight of the colony-stimulating activity (CSA) in KX-87CM was estimated about 25,000 daltons by gel filtration and a new bioassay system. In principle, a subline of murine hemopoietic cell line NFS-60 was cloned which was dependent on KX-87CM. Then the growth of this subline was examined by a rapid and sensitive colorimetric tetrazolium assay. From these results, it was concluded that the CSA which KX-87 cell line produced was G-CSF.

Key words: Hepatoma cell line — Granulocytosis — Colony-stimulating factor — Bioassay system

The stimulation of hemopoiesis by colony-stimulating factors (CSFs) is well known,[1)] and four growth factors have so far been identified as human CSFs, i.e. interleukin-3 (IL-3), granulocyte/macrophage-CSF (GM-CSF), granulocyte-CSF (G-CSF), and macrophage-CSF (M-CSF). Each cDNA has already been cloned[2–6)] and recombinant products are available.

Patients with malignant tumors sometimes reveal varying degrees of leukocytosis.[7)] Some of such cases are caused by unregulated production of CSFs or CSF-like substances by the tumor cells. However, there have been only a few cases from which human CSF-producing cell lines have been established.[8–15)] These cell lines have contributed greatly to the isolation and analysis of CSFs.

In this paper, we report on a newly established CSF-producing tumor cell line and the characterization of the CSF by a new and simple bioassay system using a CSF-dependent cell line and cell growth assay methods.

MATERIALS AND METHODS

Primary culture of the tumor cells The tumor cells were isolated from a 47-year-old Japanese female with an unresectable liver tumor and severe granulocytosis. Her peripheral white blood cell count before chemotherapy was 61,000/μl (95% mature neutrophils) and the neutrophil alkaline phosphatase score was 440. The bone marrow aspirate showed hypercellularity and the chromosomal analysis of bone marrow cells revealed a normal karyotype. The reduction of the tumor size by anticancer chemotherapy was paralleled by an improvement of the peripheral blood granulocytosis, but the white cell count increased after discontinuation of chemotherapy, reaching 110,000/μl shortly before death. The histology of the biopsied and necropsied liver tumor showed a hepatocellular carcinoma (Edmondson's classification: Type IV). HBs antigen was not detected in her serum. Serum α-fetoprotein level was within normal limits.

The tumor cells were isolated from a biopsy sample of the liver before chemotherapy and cultivated in RPMI 1640 medium (Flow Laboratories, UK) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Irvine Scientific, Santa Ana, Calif.), 100 μg/ml of streptomycin and 100 U/ml of penicillin G.

Tumorigenicity in nude mice Nude mice used were four- to eight-week-old female BALB/c strain bred under specific pathogen-free conditions (Shizuoka Laboratory Animal Center, Hamamatsu). Small pieces of the biopsied tumor tissues were transplanted subcutaneously into six of nine mice, and KX-87 cells (about 10^7 cells per mouse) into the remaining three mice. To suppress NK activity, 10 μl of anti-asialo GM_1 antibody solution (Wako Pure Chemicals, Osaka) was injected intraperitoneally into three of the above six mice before transplantation. Mice were observed for one to two months and their peripheral white blood cells were counted.

Surface marker analysis KX-87 cells (1×10^6 cells) were incubated with phosphate-buffered saline supplemented with 1% human AB serum and 5% FBS for 30 min at room temperature in order to avoid nonspecific bind-

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ing, washed and incubated with 10 μl of appropriately diluted solution of anti-My4 (Coulter Corporation, Fla.) and anti-Mo1 (Coulter Corporation) as monocyte-macrophage markers, and L243 (anti-HLA-DR, Becton Dickinson, Calif.) for 30 min at 4°C. After washing, 10 μl of appropriately diluted solution of FITC-labeled goat anti-mouse immunoglobulin antibody (GAM-FITC, Coulter Corporation) was added to each pellet. The cells were incubated for 30 min at 4°C, washed, and observed under an Olympus fluoromicroscope.

Cytogenetic analysis Cultivated cells were treated by the conventional cytogenetic method using colcemid, hypotonic treatment, fixation with methanol-acetic acid and trypsin G-banding.[16]

CFU-GM and BFU-E assays Bone marrow cells were obtained from hematologically normal patients who had given informed consent. The mononuclear cell fraction was isolated by density gradient separation (Ficoll-Paque; Pharmacia Incorporated, Piscataway, N.J.). Non-adherent cells obtained by plating the cells on plastic dishes (Corning #25020, N.Y.) for 60 min were used unless otherwise stated. For CFU-GM assays, these cells were plated at 4×10^4 cells per dish in 0.3% Bacto agar (Difco Laboratories, Detroit, Mich.) containing Iscove's modified Dulbecco's medium (IMDM), 20% FBS and 20% human placental conditioned medium (HPCM) or 10% KX-87 cell conditioned medium (KX-87CM). In one experiment, 20 ng/ml of recombinant human G-CSF (rhG-CSF) or 20 ng/ml of recombinant human GM-CSF (rhGM-CSF) was used. rhG-CSF and rhGM-CSF used in these assays were generously provided by Chugai Pharmaceutical Co. (Tokyo) and Sumitomo Pharmaceutical Co. (Osaka), respectively. The cells were incubated for eight days at 37°C, under 5% CO_2 in humidified air. Colonies with more than fifty cells were enumerated and colony morphology was determined by May-Grünwald-Giemsa and double esterase staining (Mutoh Pure Chemicals, Tokyo). In some experiments, myeloid progenitors were assayed in plasma clot cultures by a modification of the methods of Tepperman *et al.*[17] and evaluated by Giemsa staining. BFU-E were assayed at 1×10^5 cells per dish in 0.88% methylcellulose-IMDM with 1 U/ml of erythropoietin (Connaught, Ontario), 10% bovine serum albumin (Fraction V, Nakarai Chemicals, Kyoto), 30% FBS, and 5×10^{-5} *M* 2-mercaptoethanol with or without 10% KX-87CM. The cells were incubated for fourteen days at 37°C, under 5% CO_2 in humidified air. Colonies with more than one hundred erythroid cells were counted using an inverted microscope.

Establishment of the subline cells from NFS 60 cells which respond to KX-87 conditioned medium NFS60 cell line, a murine IL-3 and murine GM-CSF dependent multilineage hemopoietic cell line,[18,19] was generously provided by Dr. Ihle (National Cancer Institute, Frederick, Md.). This cell line is routinely maintained in RPMI1640 medium supplemented with 200 U/ml of murine GM-CSF, 5×10^{-5} *M* 2-mercaptoethanol and 10% FBS. A subline of NFS60 (NFS60-KX) has been successfully maintained with 20% KX-87CM instead of GM-CSF. The cell growth was evaluated by MTT assay methods.[20] In brief, two-fold dilutions of KX-87CM were prepared in 96-well microplates (Falcon #3072, Becton Dickinson) with RPMI1640 as the dilutant. Each well contained 50 μl. NFS60 cells that had been washed with and resuspended in RPMI1640 supplemented with 10% FBS and 5×10^{-5} *M* 2-mercaptoethanol were added to each well (1×10^4 cells/well). The plate was incubated for 20 h at 37°C, under 5% CO_2 in humidified air. After incubation, 10 μl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide 5 mg/ml in PBS, Sigma, St. Louis, Mo.) was added to each well and then the plate was incubated for an additional 6 h, when 150 μl of acid-isopropanol (0.04 *N* HCl-isopropanol) was added. The resulting solution was read on a Titertech Multiskan at a wavelength of 570 nm. In this assay, the following CSFs were used for comparison: rhIL-3, rhGM-CSF, rhG-CSF, rhM-CSF (Genzyme, Boston, Mass.).

Molecular weight determination of CSF KX-87CM (80 ml) was concentrated to 10 ml using a Minicon B15 (Amicon, Danvers, Mass.) and applied to a 2.5×92 cm column of AcA 54 Ultrogel (IBF Biotechnics, France). Fractions (2.1 ml) were collected and assayed for CSA by MTT bioassay methods using NFS60-KX cells as mentioned above. Molecular weight markers (ribonuclease A, chymotrypsinogen A, ovalbumin, albumin) were obtained from Pharmacia.

RESULTS

Establishment and general characteristics of KX-87 cell line The cultivated tumor cells grew readily under the conditions described in "Materials and Methods," and have continued to grow for more than fifty passages. The cells were cloned by limiting dilution and one of them was named KX-87 cell line. These cells grew adhering to the flask wall, often forming clusters. The nuclear-cytoplasmic ratio was medium to high and the cells had clear nucleoli (Fig. 1). Doubling time was 20–24 h in RPMI 1640 with 15% FBS. Some cells became detached and floated; they could be easily maintained by replating.

Secondary tumors were formed in high frequency on the nude mice into which biopsied tumor tissues or KX-87 cells had been transplanted subcutaneously (Table I). The mean granulocyte count of the peripheral blood of seven tumor-bearing mice was 55,000/μl. In contrast, the mean granulocyte count of the mice which rejected the tumor was 4,000/μl.

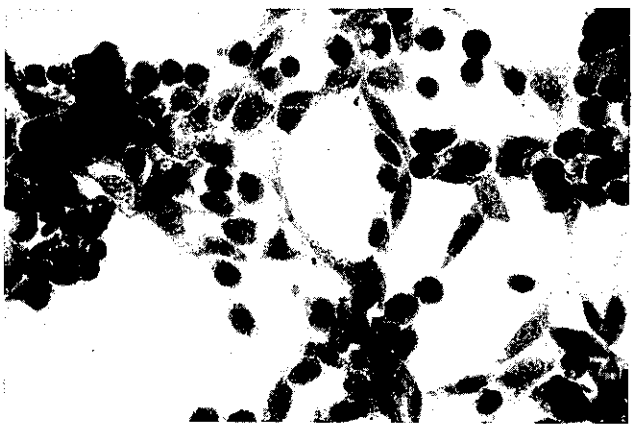

Fig. 1. The morphology of KX-87 cells (May-Grünwald-Giemsa stain, × 200). Some cells tend to form clusters.

Table I. Tumorigenicity in Nude Mice

Implanted material	Anti-asialo GM_1 treatment (+)	(−)	Tumorigenesis (+)/(−)
Biopsied tumor tissues[a]	3		3/0
		3	2/1
KX-87 cells	0	3	2/1

a) Tumor from which the KX-87 cell line originated. The numerals mean the number of mice (nine mice were used in total).

Cytogenetic studies of KX-87 cells revealed tetraploid karyotypes (76–80 chromosomes) with missing X, various deletions and structural abnormalities.

KX-87 cells were examined for reactivity to anti-My4, anti-Mo1 and anti-HLA-DR in order to rule out the possibility that this cell line might have originated from Kupffer cells,[21] and these three markers were found to be negative.

Colony-stimulating activity detected by clonogenic assays of human bone marrow cells Table II shows the colony-stimulating activity (CSA) of various materials including KX-87CM as detected by eight-day human bone marrow cultures. KX-87CM supported CFU-GM colony formation. The colony numbers formed by 10% KX-87CM were comparable to those formed by 20% HPCM or 20 ng/ml of rG-CSF or rGF-CSF. The cell composition of the colonies formed by KX-87CM revealed mainly granulocytic lineage. Macrophage or eosinophil colonies were hardly observed.

The number of BFU-E colonies formed in the presence of 10% KX-87CM was 24±2 per 1×10^5 bone marrow cells as compared with 29±2 per 1×10^5 cells of the control cultures (values are the mean±SE of triplicate experiments; see "Materials and Methods").

Establishment of a bioassay system using NFS60 cell line NFS60 cell line responds to murine G-CSF as well as to murine GM-CSF. Human and murine G-CSF exert cross-species bioactivity, while human and murine GM-CSF do not.[22] We tried to cultivate NFS60 cells in

Table II. Comparison of CSA for Human Bone Marrow Cultures

Case No.	Culture method	Colony stimulator	CFU-GM /4×10⁴ cells	Colony morphology (%)[a] G	GM	M	E
1	Agar	None	0±0[b]	—	—	—	—
		HPCM 20%	123±20	65±8	30±7	4±2	1±1
		KX-87CM 10%	148±29	73±8	26±5	0±0	1±1
		rG-CSF 20 ng/ml	133±18	62±10	31±8	7±3	0±0
		rGM-CSF 20 ng/ml	155±25	20±5	40±11	16±5	24±4
2	Agar	None	0±0	—	—	—	—
		HPCM 20%	46±5	55±10	40±10	5±3	0±0
		KX-87CM 10%	114±26	83±16	14±5	3±3	0±0
3	Plasma clot[c]	None	1±1				
		HPCM 20%	205±6				
		KX-87CM 10%	181±21				
4	Plasma clot[c]	None	3±2				
		HPCM 20%	33±3				
		KX-87CM 10%	30±2				

a) G, >90% granulocytes; M, 90% macrophages; GM, mixed granulocytes and macrophages; E, eosinophils.
b) Values are the mean±SE of triplicate experiments.
c) Evaluation of colony morphologies was difficult and was not done in plasma clot methods.

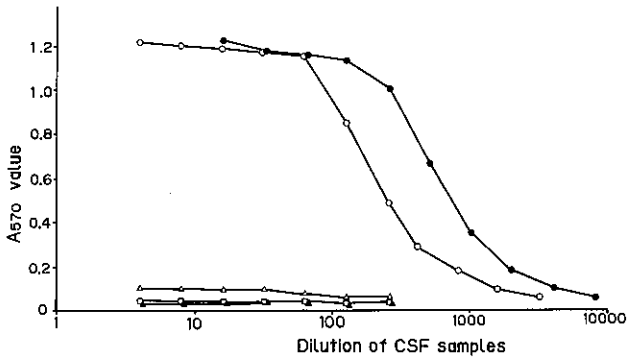

Fig. 2. The response of NFS 60-KX cells stimulated by serial dilutions of several CSF samples and KX-87CM. Cell growth was evaluated in terms of A_{570} values in the MTT assay methods (see "Materials and Methods"). ○, rhG-CSF; △, rhM-CSF; ▲, rhGM-CSF; □, rhIL-3; ●, KX-87CM. The bioactivity of the original solution of each CSF was 5000 U/ml.

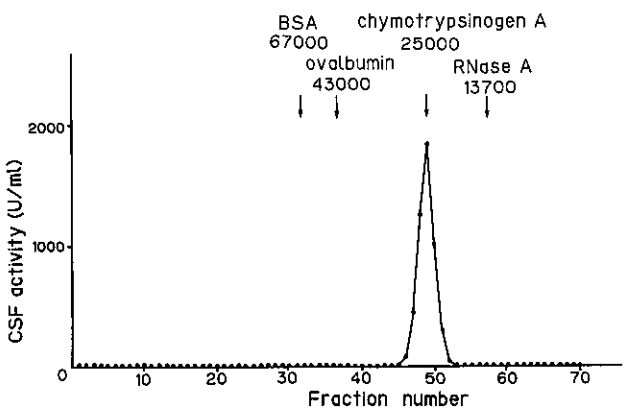

Fig. 3. The bioactivity of each fraction obtained by AcA 54 Ultrogel chromatography of concentrated KX-87CM. The bioactivity was evaluated in terms of CSF calculated from A_{570} values obtained with NFS60-KX cells and the MTT assay methods. The locations of several molecular markers are also shown by arrows.

the presence of 10% KX-87CM in RPMI1640 instead of murine GM-CSF, which was routinely used to maintain the cells. Most of the NFS60 cells died within four days but some survived and began to proliferate. Thus, a KX-87CM-dependent cell line (NFS 60-KX) was established. NFS60-KX cells were morphologically immature blastoid cells, as were the original NFS60 cells. They grew in the presence of KX-87CM but showed no tendency to differentiate into any lineage. Figure 2 shows the dose-response curves of NFS60-KX cells using MTT assay methods. They responded to KX-87CM in a dose-dependent fashion, similarly to rhG-CSF, but not to rhGM-CSF, rhM-CSF or rhIL-3.

Molecular weight determination of CSA contained in KX-87CM Gel chromatography of concentrated KX-87CM on AcA 54 Ultrogel revealed a single peak of CSF activity, with an apparent molecular weight of 25,000 daltons, which almost coincided with that of chymotrypsinogen A (Fig. 3).

DISCUSSION

This new cell line, KX-87, is a CSF-producing cell line of human hepatoma origin, and is the first such line to be established, to our knowledge. Although it is not easy to determine whether KX-87 cell line was actually derived from hepatocytes, the original tumor was diagnosed histologically as a hepatocellular carcinoma. In addition, the results of surface marker studies seem incompatible with a Kupffer cell origin.[21] The possibility of a bile duct cell origin cannot be completely excluded.

The most important character of KX-87 cell line is to produce CSA. The evidence for this function can be summarized as follows. First, the patient bearing the original tumor showed severe granulocytosis. Second, the nude mice which accepted the tumor cells also showed severe granulocytosis. Third, KX-87CM supported CFU-GM colony formation in clonogenic assays of human bone marrow cells.

To determine which type of CSA KX-87 cells produce, it is helpful to examine the morphology of the colony-forming cells. Our results showed that most of the cells formed with KX-87CM were neutrophilic granulocytes (Table II). Therefore, KX-87CM may contain G-CSF activity. As human G-CSF also stimulates murine GM colony formation,[22] the data from the nude mouse study support the conclusion that KX-87 cells produce G-CSF. Although human M-CSF also acts on murine GM colony-forming cells,[22] the results of the clonogenic assays do not fit the pattern expected for M-CSF. The existence of human GM-CSF in KX-87CM instead of G-CSF is highly unlikely, because human GM-CSF is not cross-reactive with murine GM-CSF. Furthermore, burst-promoting activity (BPA), which may or may not be detectable in human GM-CSF,[23-25] was not found in KX-87CM. Therefore, KX-87CM does not contain any detectable GM-CSF activity.

NFS60 cell line has the unique character of responding to several growth factors including G-CSF.[19] We found, however, that the response of NFS60 cells which had been maintained with murine GM-CSF to hG-CSF was not so smooth, and careful cultivation was necessary until some of the cells began to grow. We established a subline of NFS 60, NFS60-KX, which grows in the presence of KX-87CM. This subline was useful for bio-

assays detecting CSF activity by the MTT method. MTT is cleaved in active mitochondria, and so the reduction of MTT occurs only in living cells, resulting in production of formazan, which is measured by the colorimetric assay. This system is preferable to the conventional bone marrow clonogenic assay methods because the former is methodologically much simpler than the latter and needs only two days. A further advantage of MTT assay methods is the lack of any radioisotope, unlike ^{3}H-thymidine incorporation assays. The pattern of responses to KX-87CM was similar to that in the case of hG-CSF, which also supports the idea that KX-87CM contains hG-CSF activity.

To determine the molecular weight of the CSA produced by KX-87 cells, concentrated KX-87CM was applied to a gel filtration column and the CSA of each fraction was checked by MTT bioassay using NFS60-KX cells. This revealed a single peak of CSA with an apparent molecular weight of about 25,000 daltons (Fig. 3). This value seems a little higher than those described in other reports on hG-CSF.[4, 5, 11, 12] This may be because the data obtained by gel chromatography are generally not so accurate, or perhaps because the degree of glycosylation is different, especially in such tumor cells.

Finally, we concluded that the CSA produced by KX-87 cell line is G-CSF. In recent years, CSFs have been clinically applied.[26, 27] Thus, G-CSF derived from KX-87 cell line and the bioassay system using NFS60-KX cells will be of potential use. Further studies will be aimed at the purification of G-CSF from KX-87CM, and analyses of the structure of the G-CSF receptor and of the signal transduction to cell growth or differentiation.

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