Nude Mice Bearing Human CSF-Producing Tumor: Analysis of Hemopoietic Factor(s) Acting on Primitive Stem Cells

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ABSTRACT. Previously, we serially transplanted tumors that produced colony-stimulating factor (CSF) into nude mice, who developed marked granulocytosis along with tumor growth; their leukocyte counts reaching approximately one million per cu mm. The numbers of CFU-GM, CFU-E, CFU-Meg, CFU-S and BFU-E were increased in nude mice bearing CSF-producing tumor.

We here report that tumor-conditioned medium (TCM) derived from the CSF-producing tumors had colony-stimulating activity (CSA) and burst-promoting activity (BPA) when normal murine spleen cells as well as normal human bone marrow cells were the target cells. The activity of TCM supported multilineage colony formation in 5-fluorouracil (5-FU)-treated mouse spleen cells, in which only the primitive population of stem cells was reserved. No interleukin-3 (IL-3) activity was detected in TCM when assayed using the IL-3 dependent cell lines. We conclude that the factor in TCM acts on pluripotent stem cells and on the early progenitor stage of various cell lineages. It is distinct from IL-3.

We previously reported that a patient with squamous cell carcinoma of the lung developed marked neutrophilic granulocytosis (10). This tumor has been serially transplanted in nude mice for three years, and the mice have developed marked granulocytosis along with tumor growth, their leukocyte counts reaching approximately one million per cu mm. We also found that the number of granulocyte-macrophage colony-forming units (CFU-GM) erythroid burst-forming units (BFU-E), erythroid colony-forming units (CFU-E), megakaryocyte colony-forming units (CFU-Meg) and colony-forming units in the spleen (CFU-S) all increased in number in the spleens of tumor-bearing nude mice as compared with the numbers in spleens of control mice (5).

To determine whether the factor derived from the tumor acts on the primitive population of hemopoietic stem cells, we examined its effects on 5-FU-treated mouse spleen cells. In this system, primitive stem cells (day 12 CFU-S and CFU-GEMM)
are well preserved, whereas more differentiated hemopoietic precursor cells such as
day 8 CFU-S, CFU-GM, BFU-E, CFU-E and CFU-mast are markedly depleted
(2, 12). We also studied the effects of tumor-conditioned medium (TCM) on normal
human bone marrow cells and on normal murine spleen cells.

MATERIALS AND METHODS

Tumor and tumor cell-conditioned medium. As described in previous papers (5, 10), tumor
tissue that had been removed from a 59-year-old female with lung cancer and prominent
granulocytosis (peripheral blood leucocytes: 90,000/mm³) was serially transplanted into
nude mice (nu/nu BALB/cA, 6–8 weeks old, CLEA Japan Inc., Tokyo). Ten weeks after
transplantation all the mice showed marked granulocytosis of more than 6×10⁵/mm³
(average: one million per cu mm.) that consisted mainly of mature neutrophils. We de-
determined that the conditioned medium from the tumor cells and tumor tissue homogenate
contained a colony-stimulating factor (CSF) capable of acting on human and mouse bone
marrow cells. We defined this tumor as a CSF-producing tumor. TCM was prepared as
described elsewhere (10).

As the starting material, 100 ml of TCM was concentrated to about 5 ml in cellulose
tubing with polyethylene glycol (PEG) 6000. A 2×60 cm column of Sephadex G-150
(Pharmacia Fine Chemicals, Uppsala, Sweden) was equilibrated with 0.01 M Tris-HCl,
pH 7.4 containing PEG (500 μg/ml), and the optical density at 280 nm was used to monitor
the protein concentration of the column effluents. All the fractions obtained were passed
through a 0.45 m millipore membrane (Millipore Corp. USA) then assayed for colony-
stimulating activity (CSA) and burst-promoting activity (BPA) using 5-FU-treated murine
spleen cells.

Cells. For the human hemopoietic colony assay, bone marrow cells from healthy
volunteers who gave informed consent were aspirated from the posterior iliac crest. A mono-
nuclear cell suspension was obtained by density gradient centrifugation over Ficoll-Hypaque
(Lymphoprep Nyegaard & Co., Oslo, Norway). These cells were washed twice in alpha-
medium.

For the murine colony assay, 10- to 15-week-old female BDF₁ mice were obtained from
Shizuoka Experimental Animal Center (Shizuoka, Japan). Spleen cells were prepared by
mashing the spleen between the slide glasses in alpha-medium, after which they were pipetted
off and passed through a #100 gauze wire mesh. Cells were washed twice in alpha-medium.

For the murine pluripotent stem cell assay, 5-fluorouracil (5-FU F. Hoffmann-La Roche
& Co. Ltd Basel, Switzerland) was administered intravenously through the tail veins of the
mice at a dosage of 150 mg/kg. Spleen cells were harvested 4 days after this 5-FU inection.

For the assay of murine mast cell growth activity, spleen cells from 10- to 15-week-old
BDF₁ mice were isolated as above, then cultured with methyl-cellulose. On day 15 of this
culture, individual colonies were lifted from the methylcellulose medium under direct
microscopic inspection with a 3 μ l Eppendorf pipette then resuspended in alpha-medium.
These cells were maintained in a solution of alpha-medium, 30% fetal calf serum (FCS) and
10% serum-free pokeweed mitogen spleen cell-conditioned media (PWM-SCM).

Assay for colony-stimulating activity (CSA) and burst-promoting activity (BPA). For
human bone marrow cells, methylcellulose culture was done by a modification of the
technique previously described (1). Routinely, 1 ml of culture medium contained 5×10⁴
human bone marrow cells, 30% FCS, 10% bovine serum albumin (BSA) and 5×10⁻⁵ M 2-
mercaptoethanol (2-ME). Either 5% TCM was added to this solution, or phytohemagglutinin
human leucocyte conditioned medium (PHA-LCM) (positive control), Iscove’s modified Dulbecco’s medium (IMDM), with or without 1 U/ml erythropoietin (EP); 1.2% 1500 centipoise methyl-cellulose was used. Dishes were incubated at 37°C in humidified air flushed with 5% CO₂.

For the murine spleen cells, methylcellulose culture was done with a modification of the technique previously reported (9). Either 10% TCM or 10% PWM-SCM (positive control) was added to one milliliter of culture medium contained 2×10⁵ murine spleen cells or 1.2×10⁶ 5-FU-treated murine spleen cells with 30% FCS, 10% BSA, 1×10⁻⁴ M 2ME and 1.2% of 1500 centipoise methyl-cellulose. Dishes were incubated as described above. On days 7, 10 and 13, colonies and bursts that contained more than 40 cells each were counted under an inverted light microscope.

**Assay of IL-3 activity.** The amount of IL-3 in TCM was assayed by the method of Mosmann (7). Double dilutions of PWM-SCM and WEHI-3-conditioned medium were prepared in 96-well trays with 1640 medium as the dilutant. Each well contained 50 μl of the sample. The FDCP-2 cells that had been washed with RPMI (IL-3 free) then resuspended with RPMI and supplemented with 10% FCS were added to each well. The plate was incubated for 20 to 24 h at 37°C in 5% CO₂ and humidified air. After incubation, 10 microliters of MTT (2,5-diphenyl tetrazolium bromide 5 mg/ml dissolved in PBS) was added to each well. The plate was then incubated for an additional 5 to 6 h and 150 microliters of acid-isopropanol (0.04 N HCl isopropanol) was added. After being left for a few minutes at room temperature, the plates were read on a Multiskan (Titertech Co. Ltd.) at a test wavelength of 590 nm.

**RESULTS**

**Effect of TCM on BFU-E or CFU-GM formed by human bone marrow cells.** Initially, we studied the effect of TCM on the BFU-E and CFU-GM formed by cultured human bone marrow cells (Table 1). The number of colonies formed was similar to the number formed by the addition of PHA-LCM to the positive control. On the addition of TCM and EP more erythroid bursts were formed than on the addition of EP alone, whereas TCM or PHA-LCM alone produced no erythroid burst formation. This result suggests that TCM had both CSA and BPA for human bone marrow cells.

Little amount of EP was detected by radioimmunoassay. Values in the range of 0 to 10 mU/ml are near the lower limit of sensitivity for this assay.

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>GM colonies</th>
<th>erythroid bursts</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>15±3</td>
<td>0</td>
</tr>
<tr>
<td>TCM</td>
<td>122±6</td>
<td>0</td>
</tr>
<tr>
<td>TCM + EP</td>
<td>122±10</td>
<td>30±1</td>
</tr>
<tr>
<td>EP</td>
<td>12±1</td>
<td>15±2</td>
</tr>
<tr>
<td>PHA-LCM</td>
<td>116±7</td>
<td>0</td>
</tr>
<tr>
<td>PHA-LCM + EP</td>
<td>97±5</td>
<td>56±1</td>
</tr>
</tbody>
</table>

a) number of granulocyte-macrophage colonies per 5×10⁴ nucleated cells, counted on day 14, mean±SD

b) number of erythroid bursts per 5×10⁴ nucleated cells, counted on day 14, mean±SD
Effect of TCM on the BFU-E or CFU-GM formed by murine spleen cells. We next investigated whether TCM had CSA and BPA for murine spleen cells (Table 2). With the addition of TCM, a significant number of GM colonies were formed. The addition of both TCM and EP produced more erythroid bursts than the addition of EP alone, therefore, it appears that TCM had both GM-CSA and BPA for murine spleen cells, as well as for human bone marrow cells.

Effect of TCM on 5-FU-treated mouse spleen cells. To determine whether the factor in TCM acts directly on pluripotent stem cells, we examined the CSA in TCM for 5-FU-treated murine spleen cells. CSA was eluted in a fraction (Fr. 70) with a molecular weight 24,000 (Fig. 1). This fraction also showed human-active BPA and GM-CSA (data not shown). The activity of this fraction was similar to that detected on the addition of 10% PWM-SCM (positive control) (Table 3). Various types of colonies could be seen by the addition of this fraction (Fig. 2). These colonies were lifted with an Eppendorf pipette, then cytocentrifuged individually and stained by

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>GM colonies</th>
<th>erythroid bursts</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1 ± 1(a)</td>
<td>0</td>
</tr>
<tr>
<td>TCM</td>
<td>40 ± 4</td>
<td>0</td>
</tr>
<tr>
<td>TCM + EP</td>
<td>25 ± 0</td>
<td>24 ± 1(b)</td>
</tr>
<tr>
<td>EP</td>
<td>6 ± 4</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>PWM-SCM</td>
<td>70 ± 6</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>PWM-SCM + EP</td>
<td>42 ± 0</td>
<td>43 ± 3</td>
</tr>
</tbody>
</table>

(a) number of granulocyte-macrophage colonies per $4 \times 10^4$ murine spleen cells, counted on day 7, mean ± SD.
(b) number of erythroid bursts per $4 \times 10^4$ murine spleen cells, counted on day 7, mean ± SD.

Fig. 1. Sephadex G-150 column fractions of TCM. Open circles represent the numbers of total colonies formed by 5-FU-treated murine spleen cells. Dots indicate optical density. Marker substances; Vo, dextran blue; BSA, bovine serum albumin; TRY, trypsinogen; RF, riboflavin.
May-Grunwald-Giemsa reagent. Of the 60 mixed colonies observed, 48 consisted of three or more lineages (Table 4). Neutrophils, macrophages, a mast cell and a megakaryocyte are shown in a multi-lineage colony in Fig. 3.

**Murine IL-3 activity in TCM.** Mouse IL-3 stimulates the growth of multipotent hemopoietic stem cells (13), and proliferation of the IL-3 dependent cell line (3). We tested IL-3 activity in TCM with the IL-3 dependent cell line FDCP-2. Proliferation of FDCP-2 was not stimulated by TCM (Fig. 4), whereas PWM-SCM and WEHI-3 conditioned media stimulated proliferation in a concentration-dependent manner. Consequently, it appears that TCM does not contain IL-3.

**DISCUSSION**

In a previous study (5), we reported a significant increase in all classes of hemopoietic progenitors in CFU-GM, CFU-E, CFU-Meg and CFU-S in tumor-bearing nude mice. There are two possible explanations for the increased CFU-S; tumor CSF

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**TABLE 3. CSA IN TCM (FR. 70) FOR 5-FU-TREATED MURINE SPLEEN CELLS**

<table>
<thead>
<tr>
<th>Stimulator</th>
<th>total colonies$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>TCM (F. 70)</td>
<td>14 ± 3</td>
</tr>
<tr>
<td>PWM-SCM</td>
<td>15 ± 0</td>
</tr>
</tbody>
</table>

$^a)$ number of colonies per $1.2 \times 10^6$ 5-FU-treated murine spleen cells, counted on day 13, mean ± SD.

**TABLE 4. CYTOLOGICAL ANALYSIS OF MIXED COLONIES DERIVED FROM 5-FU-TREATED MURINE SPLEEN CELLS SUPPORTED BY TCM**

<table>
<thead>
<tr>
<th>Lineages</th>
<th>Number of Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>n m e mast E M</td>
<td>1</td>
</tr>
<tr>
<td>n m e mast E</td>
<td>2</td>
</tr>
<tr>
<td>n m mast E M</td>
<td>5</td>
</tr>
<tr>
<td>n m mast E M</td>
<td>1</td>
</tr>
<tr>
<td>n m mast M</td>
<td>2</td>
</tr>
<tr>
<td>n m mast E</td>
<td>16</td>
</tr>
<tr>
<td>n mast M</td>
<td>2</td>
</tr>
<tr>
<td>n mast E</td>
<td>1</td>
</tr>
<tr>
<td>n m E</td>
<td>14</td>
</tr>
<tr>
<td>n m mast</td>
<td>4</td>
</tr>
<tr>
<td>n E</td>
<td>3</td>
</tr>
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<td>n mast</td>
<td>2</td>
</tr>
<tr>
<td>n m M</td>
<td>3</td>
</tr>
<tr>
<td>mast</td>
<td>1</td>
</tr>
<tr>
<td>n</td>
<td>1</td>
</tr>
<tr>
<td>could not be evaluated</td>
<td>1</td>
</tr>
</tbody>
</table>

total 60
mobilized CFU-GM thus producing the increased CFU-S, or the increased CFU-S was produced by proliferation of very primitive hemopoietic progenitors directly formed by other hemopoietic factor(s) produced by the tumor. We found that the factors in TCM acted on 5-FU-treated murine spleen cells which preserve the primitive population of stem cells and in which late stage progenitors are depleted. The
colonies formed by 5-FU-treated murine spleen cells were composed of various lineages; neutrophils, macrophages, eosinophils, mast cells, megakaryocytes and erythroblasts.

In addition, TCM had both BPA and CSA for murine spleen cells. The possibility that the TCM-derived human lung cancer contained IL-3 was excluded because TCM did not support the proliferation of an IL-3 dependent cell line. The growth factors in our TCM appear to contain a multi-CSF distinct from IL-3.

Hemopoietic growth factors which induce pluripotent hemopoietic differentiation and stimulate the proliferation of very primitive stem cells have not yet been purified from human subjects. Welte et al. (14) reported that a human "pluripotent" hemato-poietic colony-stimulating factor was derived from a human bladder cell carcinoma cell line 5637. Whether this factor is identical to the factor in our TCM must now be determined.

Recently, the cDNA of hG-CSF was cloned from a human squamous cell carcinoma cell line and from bladder carcinoma cell line 5637, which made recombinant hG-CSF available (6, 8). Our previous studies showed that hG-CSF supports the murine GM colony formation directly and multilineage colony formation indirectly (11). Human recombinant GM-CSF does not support colony formation of murine hemopoietic cells (4).

Further study is required to determine whether the factors are single molecules that have supporting activity for very primitive murine stem cells and have CSA and BPA for human bone marrow cells.

REFERENCES

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