Demonstration of Granulopoietic Factor(s) in the Plasma of Nude Mice Transplanted With a Human Lung Cancer and in the Tumor Tissue

By Shigetaka Asano, Akio Urabe, Tetsuro Okabe, Noriharu Sato, Yukio Kondo, Yoshito Ueyama, Shyozo Chiba, Nakaaki Ohsawa, and Kinori Kosaka

A human lung cancer (OTUK-tumor) was transplanted serially to nude mice, which invariably developed a marked neutrophilia. In order to analyze this phenomenon, in vitro agar culture studies were carried out. A three- to fourfold increase of colony-forming units in culture was observed in the femurs of these nude mice. Moreover, the plasma of nude mice bearing this tumor, as well as the tumor extract, had a significantly higher colony-stimulating activity on mouse bone marrow cells than appropriate controls. This activity had the property to stimulate granulocyte and/or mixed cell type colonies rather than mononuclear cells. This activity was also demonstrated, in a dose-dependent way, on normal human bone marrow cells. These findings indicated that the OTUK-tumor produced human granulopoietic colony-stimulating activity, which may have stimulated granulopoiesis in vivo as well.

GRANULOCYTOSIS without any evidence of infection has been observed in some patients with nonhematologic malignancies. The precise mechanism of this phenomenon, however, remains unexplained in most cases.

We investigated a patient with a poorly differentiated squamous cell carcinoma of the lung who developed a mild but persistent neutrophilia. This tumor (OTUK-tumor) was successfully transplanted into nude mice of the BALB/c strain bred under specific pathogen-free conditions.1 These mice developed a marked neutrophilia pari passu with the growth of the transplanted tumor, in marked contrast to those transplanted with other human tumors. The number of peripheral neutrophils returned to normal after removal of the tumor. In view of the known production of various hormones including erythropoietin by human tumors,2 the most plausible explanation for this phenomenon seemed to be the production of granulopoietic factor(s) by this tumor.

The present studies were designed, therefore, to prove their hypothesis with the in vitro agar culture technique. This technique, involving the formation of granulocyte–macrophage colonies, has been widely applied as a quantitative assay of in vitro granulopoiesis in terms of colony-stimulating activity (CSA).3 The present results indicated that the OTUK-tumor produced granulopoietic CSA effective both on mouse and human bone marrow cells, which may stimulate granulopoiesis in vivo as well.
MATERIALS AND METHODS

Test Samples

Nude mice of the BALB/c strain were bred under specific pathogen-free conditions. Human cancer tissues of various origins were maintained by serial subcutaneous transplantations in 6-8-wk-old mice. Blood samples were collected with heparinized syringes by cardiac puncture or axillary bleeding under ether anesthesia. After centrifugation, the plasma was removed and stored at -70°C. Before use, the sample was thawed and filtered through 0.45-μm Millipore membranes. The femoral marrow was also obtained for the estimation of colony-forming units in culture (CFU-C).

Tumor tissues, which had been removed from the nude mice and stored at -70°C, were thawed at room temperature and then homogenized with a Polytron (PT-10) with 5 volumes of cold phosphate-buffered saline. The tumor homogenate was centrifuged at 100,000 g for 60 min at 4°C. The supernatant was dialyzed against 1/60 M phosphate buffer, pH 7.4, for 4 days at 4°C with three changes of buffer. After removal of the precipitate which had formed during dialysis, the supernatant was filtered through 0.45-μm Millipore membranes and stored at -70°C until used.

Agar Culture

The assay of CFU-C was performed by a modification of the method originally described by Bradley and Metcalf. Briefly, 5 x 10^4 bone marrow cells were cultured in 1.0 ml of McCoy's 5A medium (Gibco) supplemented with three times the standard concentration of vitamins and amino acids (Nissui), containing 0.3% purified agar (Difco) and 20% standard conditioned medium from L-cell cultures.

CSA in the plasma or the tumor extract was assayed using mouse and human marrow cells. Mouse bone marrow cells were obtained from the femurs of 6-8-wk-old female mice of C3H/He strain (Doken, Saitama). Human bone marrow cells were obtained from normal volunteers (who gave informed consent) by sternal puncture with heparinized syringes. They were used after washing with culture medium and removal of erythrocytes by hypotonic lysis. A constant number of nucleated cells (7.5 x 10^4 of mouse or 20 x 10^4 of human cells) were cultured in a single layer in 1.0 ml of the supplemented McCoy's 5A medium, containing 0.3% agar, 20% fetal calf serum, and 10% (0.1 ml) of the test sample. For studies of dose-response, dilutions of the original sample in 0.15 ml of McCoy's 5A medium were added as the sample.

After 7-10 days of incubation in a humidified 5% CO₂ atmosphere, discrete colonies containing 40 or more cells were counted with an inverted microscope. The activity on mouse bone marrow cells was standardized using the control culture with an aliquot of L-cell conditioned medium containing a known activity as reference. For morphological analyses of colonies, they were harvested with microhematocrit tubes and stained with 0.6% orcein in 60% acetic acid.

Case Report

A 58-yr-old Japanese male was admitted to the Tokyo University Hospital on March 13, 1974 because of progressive cough and a cervical mass which had increased in size during the pre-

![Fig. 1. Changes in peripheral granulocyte count and tumor weight of nude mice after transplantation and removal of OTUK-tumor. □, granulocyte count; ●, tumor weight.](image)
ceeding 5 mo. Physical examination revealed diminished breath sounds over the left lung field and a fixed mass, 4 × 3.5 cm, in the cervical region. An x-ray film of the chest disclosed multiple discrete shadows in both lung fields. The hemoglobin concentration was 14.3 g/dl, and the white cell count 16 × 10⁹/liter with 78% neutrophils, 4% eosinophils, 3% monocytes, and 15% lymphocytes. Bacteriologic examinations were always negative. The cervical mass was removed and transplanted into nude mice. Histologic examination of this tumor revealed a poorly differentiated squamous cell carcinoma of lung origin. Administration of 500 mg 5-fluorouracil, 200 mg cyclophosphamide, 2 mg mitomycin C, and 0.5 mg Toyomycin twice weekly induced no clinical improvement, and the patient died 1 mo later. Neutrophilia persisted throughout his hospital course.

RESULTS

Granulocytosis and CFU-C in the Femur of Nude Mice Bearing the OTUK-Tumor

A small piece of OTUK-tumor transplanted in nude mice grew out after a latent period of 2-3 wk and reached a weight of about 5 g in 2 mo. Figure 1 shows the changes in the peripheral granulocyte count and the tumor weight after transplantation and removal of the tumor. The granulocyte count increased markedly in parallel with the tumor growth and returned to the normal value 4 days after removal of the tumor. No changes were observed in the other blood cells throughout the observation period. About 8 wk after transplantation, the cellularity in the femur of these mice was about 1.5 times as great as that of normal controls. Granulocytic elements represented 87% of femoral marrow cells in OTUK-tumor-bearing mice, but were 63% of the control. In Fig. 2, the concentration of CFU-C in the femur is compared. A three- to four-fold increase was observed in nude mice bearing the OTUK-tumor. The distribution of morphological types of colonies was also different. As shown in Fig. 3, granulocyte and mixed cell type colonies accounted for 63% of the total in OTUK-tumor-bearing mice, in contrast to 36% in the control.

![Fig. 2](image-url)  
Fig. 2. Numbers of CFU-C in the femurs of OTUK-tumor-bearing and control nude mice. Both groups were assayed concurrently using the same volume of the L-cell conditioned medium. Each point represents the number of colonies per 5 × 10⁶ cells.

![Fig. 3](image-url)  
Fig. 3. Colonies of OTUK-tumor-bearing and normal nude mice studied in Fig. 2 were classified morphologically. Colonies were scored as granulocytic or mononuclear when they contained less than 10% of the other cell type.

![Fig. 4](image-url)  
Fig. 4. Dose-response relationship between number of colonies and amount of plasma of OTUK-tumor-bearing nude mice tested on mouse bone marrow cells. Mean ± SD of colony numbers per 10⁶ nucleated cells for five dishes.
CSA in Plasma on Mouse Bone Marrow Cells

Plasma samples from nude mice bearing the OTUK-tumor were obtained about 2 mo after transplantation. Figure 4 shows the dose–response relationship between the number of colonies and the amount of plasma. A definite CSA was demonstrated with a high degree of linearity up to 0.15 ml of plasma. CSA in these plasma samples was compared with that of plasma samples from the following nude mice, which served as controls: normal nude mice, those bearing other human tumors of comparable size, including Kruckenberg’s tumor and histiocytoma, and those with a wasting syndrome bred under conventional conditions. No peripheral leukocytosis was observed in any of these control mice. As shown in Fig. 5, high activities were detected in all plasma samples from nude mice bearing the OTUK-tumor, while much lower activities were detected in those of normal nude mice and the mice bearing other tumors. The plasma samples of these control mice were also tested at lower doses to exclude false-negative results owing to inhibitory factor(s) operating at high concentrations. Higher activity, however, was never detected with less than 0.1 ml of the plasma. In some of the wasting mice relatively high activities were observed, but most of them were still lower than in the mice bearing the OTUK-tumor.

The distribution of morphological types of colonies was also different. In the case of nude mice bearing the OTUK-tumor, colonies were uniformly dispersed...
Colonies were scored as granulocytic or mononuclear when they contained less than 10% of the other cell type.

GRANULOPOIETIC FACTORS

Fig. 7. Changes in CSA in plasma of nude mice after transplantation and removal of OTUK-tumor tested on mouse bone marrow cells. Each column represents the mean number of colonies stimulated by 0.1 ml of the plasma per 10^5 nucleated cells for five dishes.

and composed of 50-200 granulocytes, and the ratio of granulocyte plus mixed cell type colonies to mononuclear cells was much higher than in the controls, as shown in Fig. 6. These morphological characteristics of colonies stimulated by the plasma of OTUK-tumor-bearing mice were observed even at lower doses down to 0.05 ml. Figure 7 shows the change in CSA in the plasma after transplantation and removal of the tumor. Only the CSA which stimulated granulocyte and/or mixed cell type colonies gradually increased; no remarkable changes were observed in mononuclear colonies. This activity returned to the pretreatment level in 4 days after removal of the tumor.

CSA in Tumor Extract on Mouse Bone Marrow Cells

Ultrasupernatant of the homogenate of the OTUK-tumor per se failed to reveal activity; dialysis was necessary for the supernatant to develop activity. The results are shown in Table 1. Colony formation seemed to be stimulated in a dose-dependent way. The extract stimulated more granulocyte and/or mixed cell type colonies than mononuclear cells, as shown in Table 2, in a similar manner as observed for CSA in the plasma. Another transplantable tumor, Kruck-

<table>
<thead>
<tr>
<th>OTUK-Tumor Extract</th>
<th>CSA* Tested at a Dose (v/v) of:</th>
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<tbody>
<tr>
<td></td>
<td>0%</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>0</td>
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</tbody>
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*Mean ± SD of colony number per 10^5 nucleated cells for five dishes.

Table 2. Distribution of Three Morphological Types of Colonies of Mouse Bone Marrow Cells Stimulated by OTUK-Tumor Extract

<table>
<thead>
<tr>
<th>Number of Colonies Tested</th>
<th>Type of Colonies*</th>
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<tbody>
<tr>
<td></td>
<td>Granulocytic</td>
</tr>
<tr>
<td>120</td>
<td>45 (37.4%)</td>
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</table>

*Colonies were scored as granulocytic or mononuclear when they contained less than 10% of the other cell type.
enberg's tumor, was also examined by the same method, but no definite activity could be detected in its extract.

**CSA in Plasma and Tumor Extract on Normal Human Bone Marrow Cells**

The plasma of nude mice bearing the OTUK-tumor and the tumor extract were examined for CSA using normal human bone marrow cells as the target. Representative results are shown in Fig. 8. Both plasma and tumor extract stimulated colony formation in a dose-dependent manner. With a larger dose, the colonies tended to have a larger size, and the number of clusters consisting of fewer than 40 cells (usually not counted as colonies) increased. These colonies were mostly of granulocytic or mixed cell morphology.

**DISCUSSION**

Nude mice bearing the OTUK-tumor developed a marked neutrophilia in parallel with the tumor growth. The neutrophilia was accompanied by a significant increase in the number of CFU-C in their femurs. Morphological analyses of the colonies that developed during culture showed that the increase in granulocytic and/or mixed cell type colonies was much more striking than that of mononuclear cells as compared with normal mice, further confirming the increase of granulocytosis at the stem cell level.

Since CSA is mandatory for in vitro growth of granulocytic and mononuclear cell colonies in semisolid culture, it has been considered as an important humoral regulator of granulocyte and monocyte formation in vivo. Indeed, evidence has been accumulating, albeit not yet sufficient, to support this view. In this report results have been presented that indicate the presence of CSA in the plasma of mice bearing the OTUK-tumor as well as in the tumor extract.

The plasma from these nude mice revealed significantly higher CSA on mouse bone marrow cells than that of controls. The finding that much lower activities were detected in plasma of nude mice bearing other transplantable human tumors would rule out the possibility that the high level of CSA in plasma might be due to a nonspecific response to the heterotransplant.

CSA in the plasma of nude mice bearing this tumor had the property to stimulate more granulocytic and/or mixed cell type colonies than mononuclear ones through a relatively broad range of doses tested. In contrast, the plasma of con-
control mice, including wasting mice, which showed relatively high activity, stimulated preferentially mononuclear cell type colonies at any doses tested. This characteristic became even clearer in the study in which we followed CSA in the plasma after transplantation of this tumor. Only CSA stimulating granulocytic and/or mixed cell type colonies gradually increased with tumor growth, with little change in CSA stimulating mononuclear cell type colonies.

It is unambiguous that the OTUK-tumor produces CSA. The granulopoietic CSA in the plasma returned to normal after removal of the tumor. Moreover, the CSA with similar characteristics was demonstrated in the ultrasupernatant of the tumor homogenate in an undialyzable form. The possibility that mouse tissues, which have been known to contain some CSA effective on homologous bone marrow cells, might have contaminated the OTUK-tumor grown in nude mice, could be excluded for the following reasons. First, the tumor tissue grown in nude mice was morphologically indistinguishable from the original human tumor except for mild infiltration of mouse neutrophils. Second, and more significantly, not only the tumor extract but also the plasma of nude mice bearing this tumor stimulated colony formation of human bone marrow cells as well. Human CSA can stimulate both human and mouse bone marrow cells, but in general, mouse CSA does not stimulate human bone marrow cells.

The finding that neutrophilia was observed in the patient as well as in nude mice with this CSA-producing tumor provides additional evidence indicating that CSA is an important regulator of granulopoiesis in vivo.

Although a certain kind of granulopoietic factor has been described already in a mammary tumor of mice, the production of such factors by human tumor has not been documented. In view of the known production of CSA by normal lung tissue and the higher incidence of peripheral leukocytosis in patients with lung cancer compared to other neoplasms, the production of granulopoietic factors by the OTUK-tumor of lung origin is intriguing. Further studies on the isolation and characterization of CSA of the OTUK-tumor are being conducted in our laboratory.

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REFERENCES


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