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Simultaneous Production of G- and M-CSF by an Oral Cancer Cell Line and the Synergistic Effects on Associated Leucocytosis

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We established a novel cell line, TSU, from an oral cancer patient with marked leucocytosis. The culture supernatant of TSU cells promoted granulocytic colony formation by mouse bone marrow cells, indicating that TSU produced granulocyte-colony stimulating factor (G-CSF). The concentration of G-CSF was 2.45 μg/mg protein, measured by enzyme-linked immunosorbent assay (ELISA). The maximum number of colonies induced by TSU culture supernatant was more than that achieved with recombinant human G-CSF (rhG-CSF) and the size of the colonies induced by TSU supernatant was obviously larger than those achieved with rhG-CSF. The activity of TSU supernatant was completely inhibited by antihuman G-CSF and macrophage-colony stimulating factor (M-CSF) antibodies, but was only partially inhibited by antihuman G- or M-CSF antibody alone. These results indicate that not only G-CSF but also M-CSF, both of which could be produced by TSU cells, are involved in causing leucocytosis; the results suggest that the synergistic production of G- and M-CSF could play an important role in the leucocytosis associated with oral cancer.

Keywords: oral cancer, cell line, leucocytosis, G-CSF, M-CSF

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INTRODUCTION

VARIOUS KINDS of complications often arise in patients with malignant diseases. Leucocytosis, one such complication, occasionally develops in a wide variety of malignancies, including oral cancer [1–4]. Cancer-derived factors are considered to be the main cause of leucocytosis, and the mechanism underlying leucocytosis has been, in part, elucidated by Asano *et al.* [5], who demonstrated that a tumour secreted colony stimulating factor (CSF) that was effective in inducing the bone marrow cell colonies of both humans and mice. Although several CSF-producing cell lines have since been reported [4–11] and soluble factors, such as granulocyte-colony stimulating factor (G-CSF), have been proposed as a cause of the leucocytosis associated with cancer [10], the mechanism responsible for leucocytosis associated with oral cancer remains to be clarified. To address this question, we established a cancer cell line, TSU, from an oral cancer patient who had developed marked leucocytosis; we then investigated the CSF activity in the culture supernatant of these cells, using mouse bone marrow cells as a target.

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MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM) and McCoy's 5A medium were purchased from Sigma (St. Louis, Missouri, U.S.A.) and Gibco (Grand Island, New York, U.S.A.), respectively, and fetal bovine serum (FBS) and horse serum were obtained from Boehringer Mannheim (Mannheim, West Germany) and Whittaker Bioproducts (Walkersville, Massachusetts, U.S.A.), respectively. PF86-1 protein-free medium (PF86-1) was prepared in our laboratory [12]. Naphthol-AS-D-chloroacetate and alpha-naphthyl butyrate were obtained from Sigma. Pokeweed mitogen (PWM) was purchased from Wako (Osaka, Japan). Recombinant human granulocyte-colony stimulating factor (rhG-CSF) was provided by Chugai Pharmaceutical Co. (Tokyo, Japan) and recombinant human macrophage-colony stimulating factor (rhM-CSF) was purchased from Genzyme (Boston, Massachusetts, U.S.A.). Polyclonal rabbit antihuman G- and M-CSF antibody were purchased from Genzyme.

Cell culture

The TSU cell line was established from a patient with gingival squamous cell carcinoma (SCC) who had developed marked leucocytosis, the white blood cell count being more than 80 000/μl at the terminal stage. The cell line was usually maintained with DMEM containing 10% FBS at 37°C in a

humidified atmosphere of 5% CO_2 in air. The TSU cells were spindle-shaped and had loose intercellular connections.

Preparation of culture supernatant

The TSU culture supernatant was prepared as follows: when TSU cells were semiconfluent, the culture medium was replaced with the protein-free medium, PF86-1. The medium was renewed 24 h later and the culture was continued and replaced with fresh PF86-1 medium every 3 days. Although PF86-1 did not support the growth of cells, they remained in good condition for a long period in this medium, no remarkable change being observed in their morphology or cell density. Culture media were collected every 3 days and clarified by centrifugation at 9000 rpm for 30 min at 4°C, then filtered through a Millipore filter. The medium was concentrated about 200-fold with the Minitan (Millipore, Bedford, Massachusetts, U.S.A.) and CENTRIPREP 10 (Amicon, Beverly, Massachusetts, U.S.A.) ultrafiltration systems. It was then sterilised by filtration and stored at −80°C until used as the culture supernatant for the study. The protein content was measured by the Bradford method [13].

Assay of colony stimulating factor

CSF activity in the culture supernatants was assessed by the method originally described by Bradley and Metcalf [14], with some modifications. Briefly, bone marrow cells were isolated from the femurs of 8-week-old C57BL/6N mice. The cells (1×10^5) were cultured in 35 mm Petri dishes, at 37°C in a humidified atmosphere of 5% CO_2 in air, with 1 ml of modified McCoy's 5A medium containing 40% horse serum, 0.3% agar, and the culture supernatants. PF86-1 medium and the culture supernatant of murine spleen cells stimulated with a lectin of pokeweed mitogen, PWM, were used as negative and positive controls, respectively. Furthermore, culture supernatants of four other SCC cell lines, Ca9-22PF, ZAPF, HOC519PF and HOC119PF, which were from patients who had not developed leucocytosis, were also tested for the ability to promote colony formation [12, 15].

All samples were diluted with 1% human serum albumin and 0.01% Tween 20 in McCoy's 5A medium. After 6 days, colonies consisting of more than 50 cells were counted. Using a phase contrast microscope, we divided each colony into three groups morphologically: compact, dispersed, and mixed type, respectively. The colonies were, moreover, characterised histochemically by double-esterase staining for naphthol-AS-D-chloroacetate and alpha-naphthyl butyrate [16].

Quantification of G-CSF in TSU culture supernatant

The amount of G-CSF in the TSU culture supernatant was measured with an ELISA kit purchased from R&D (Minneapolis, Minnesota, U.S.A.).

Neutralisation of CSF activity with anti G- and/or M-CSF antibodies

To eliminate the activity of G- and M-CSF, the samples were pretreated with 10 μg/ml of antihuman G-CSF and/or 5 μg/ml of M-CSF antibody at 37°C for 2 h before they were added to the culture for CSF assay.

Statistical analysis

The statistical significance of the values was analysed by Student's *t*-test. *P* values of $P < 0.05$ were considered significant.

RESULTS

CSF activity in TSU culture supernatant

To ascertain the possibility that the TSU tumour produced might be related to leucocytosis in the patient, we investigated whether the TSU culture supernatant induced haematopoietic cell growth in soft agar medium, using murine bone marrow cells as the target.

The TSU culture supernatant was found to possess an activity capable of promoting colony formation as well as the culture supernatant of murine spleen cells stimulated with PWM [17]. On the other hand, culture supernatants of Ca9-22PF, ZAPF, HOC519PF and HOC119PF exerted no obvious effect on colony formation (Table 1).

As shown in Table 2, the TSU culture supernatant induced

Table 1. CSF activities in the culture supernatant of various cell lines

Cell	TSU	Ca9-22PF	ZAPF	HOC519PF	HOC119PF	PWM-SC	PF86-1 medium
No. of colonies	52.0±7.6	1.6±2.5	2.4±3.4	3.2±1.1	1.8±2.4	34.2±11.2	2.8±1.9

Culture supernatant of PWM-stimulated spleen cells (PWM-SC) and PF86-1 medium were used as positive and negative controls, respectively. Values represent the mean and S.D. colony number per 10^5 cells for five dishes.

Table 2. CSF activities in TSU culture supernatant

	Types of colony			
	Compact	Mixed	Dispersed	Total
TSU	18.3±6.2	15.5±4.2	11.5±3.1	45.3±11.4
PWM-SC	8.25±2.9	5.0±1.8	22.8±4.0	36.0±3.9

Formed colonies were divided into three groups morphologically. The culture supernatant of PWM-stimulated spleen cells (PWM-SC) served as a CSF source. Values represent the mean and S.D. colony number per 10^5 cells for four dishes.

mainly compact and mixed type colonies, while the culture supernatant of PWM-stimulated murine splenocytes predominantly induced colonies of the dispersed type. Further histochemical characterisation of the colonies by double-esterase staining revealed that most colonies formed by the TSU culture supernatant were naphthol-AS-D-chloroacetate esterase-positive (Fig. 1), but alpha-naphthyl butyrate esterase-negative (data not shown). These results suggest that the TSU products expanded the granulocyte lineage.

Dose–response curve

The CSF activity of the TSU supernatant was compared with that of rhG-CSF. As shown in Fig. 2, although both factors stimulated colony formation in a dose-dependent manner, the activity of rhG-CSF plateaued at concentrations greater than 1 ng/ml. However, the TSU culture supernatant induced an approximately 2-fold increase in the maximal number of colonies induced by rhG-CSF. Moreover, the colonies induced by the TSU supernatant were obviously larger than those induced by rhG-CSF (Fig. 3). Since the TSU supernatant induced granulocytic colonies, the amount of G-CSF in the supernatnat was measured. ELISA for human G-CSF revealed that approximately 200-fold concentrated supernatant contained 2.45 μg G-CSF per milligram of protein.

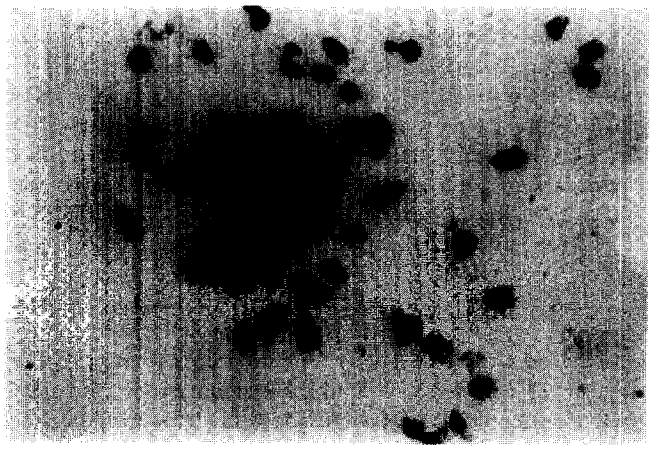

Fig. 1. Double-esterase staining. The majority of colonies induced by TSU culture supernatant were esterase-positive for naphthol-AS-D-chloroacetate. Cells were stained in blue; magnification x 200.

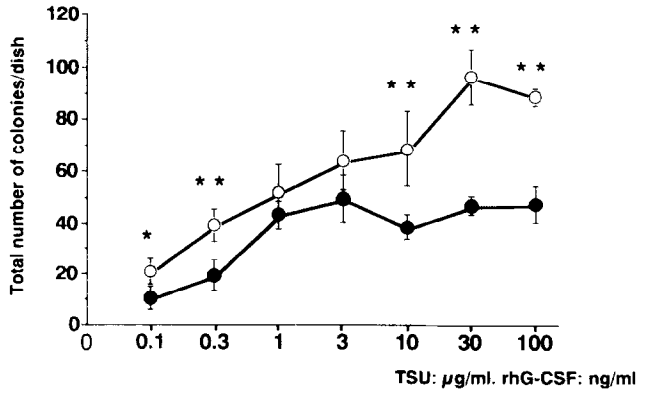

Fig. 2. Dose–response curve of TSU culture supernatant (○) and rhG-CSF (●). Values represent the mean and S.D. for four dishes. *$P<0.05$, **$P<0.01$.

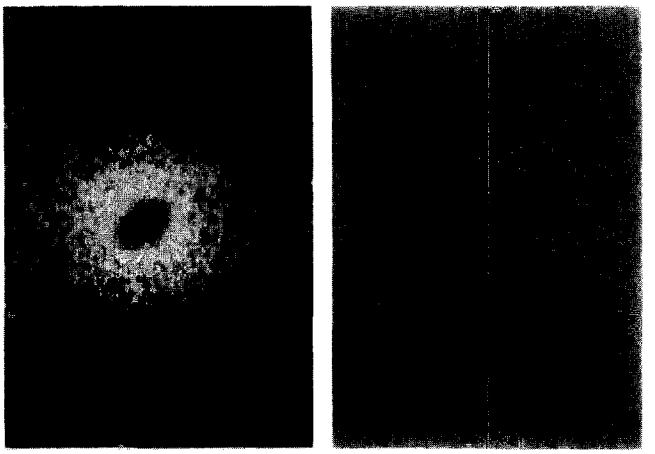

Fig. 3. Phase-contrast micrograph of the colonies induced by TSU supernatant (A) and those induced by rhG-CSF (B). Photographs were taken on day 6 after colony assay in 35 mm Petri dishes; magnification x 40.

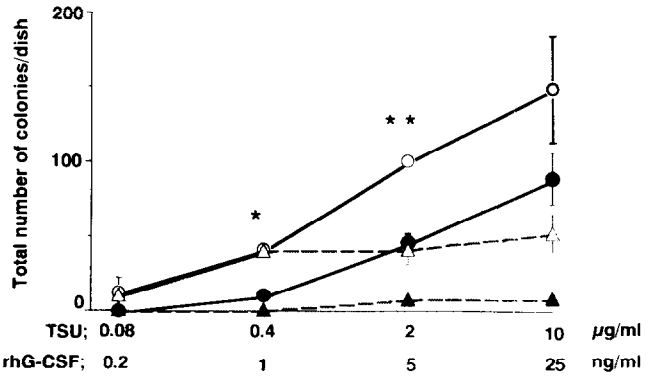

Fig. 4. Neutralisation of CSF activities with an anti hG-CSF antibody. Various doses of the TSU culture supernatant (–) and rhG-CSF (---) were pretreated with (closed symbols) or without (open symbols) 10 μg/ml of anti hG-CSF antibody at 37°C for 2 h before the CSF assay was carried out. The concentrations were exhibited as the final dose in the culture. Values represent the mean and S.D. for two dishes. *$P<0.05$, **$P<0.01$.

Neutralisation of anti G- and/or M-CSF antibodies

To determine whether not only G-CSF but also other CSF were implicated in the CSF activity of the TSU culture supernatant, we carried out a neutralising study, using specific antisera against human G- and M-CSF. Ten micrograms per millilitre of antihuman G-CSF antibody completely inhibited the activity of rhG-CSF, at least up to 250 ng/ml which was finally diluted to one-tenth in culture, but only partially reduced the activity of the TSU culture supernatant by about 40% (Fig. 4). We then examined the participation of M-CSF in the activity of the TSU supernatant. Figure 5 shows that although neither antibody alone completely suppressed the activity of TSU, the activity was abolished by both antibodies acting together.

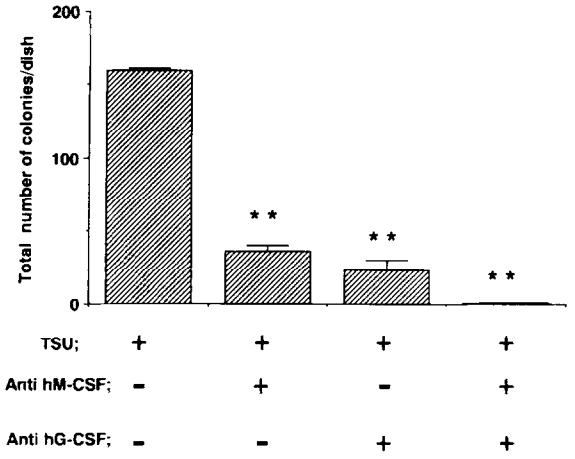

Fig. 5. Effects of anti G- and M-CSF antibodies on the activity of TSU culture supernatant. TSU culture supernatant (100 μg/ml) was pretreated with 10 μg/ml of anti hG-CSF and/or 5 μg/ml of anti hM-CSF antibody at 37°C for 2 h, then added to the culture for the assay. (+), treated with antibody. (−), not treated. Values represent the mean and S.D. for two dishes. **$P<0.01$.

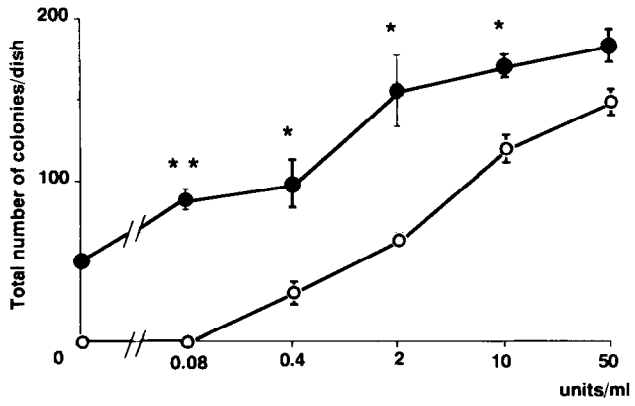

Fig. 6. Effects of various doses of M-CSF on colony formation in the absence (○) or presence (●) of 100 ng/ml of rhG-CSF. Values represent the mean and S.D. for two dishes. *$P<0.05$, **$P<0.01$.

Synergism of G- and M-CSF

The possible synergistic effect of G- and M-CSF on colony formation was also investigated. Figure 6 shows the effects of various doses of human M-CSF on colony formation with or without 100 ng/ml rhG-CSF, where the number of colonies induced by rhG-CSF alone reached a maximum. Human M-CSF stimulated further colony formation in a dose-dependent manner; only 0.08 units/ml of human M-CSF enhanced the activity of rhG-CSF, at which concentration human M-CSF alone had no detectable effect.

DISCUSSION

To study the mechanisms underlying leucocytosis associated with oral cancer, we established a novel cancer cell line, TSU, from a gingival carcinoma patient who had developed marked leucocytosis constituted by mature granulocytes. Few oral cancer cell lines have been established from patients with leucocytosis [4, 10, 11], and it seems that TSU cells will be useful for research on the mechanism responsible for leucocytosis associated with oral cancer. We confirmed that TSU produced factors capable of inducing haematopoietic cell colonies *in vitro* and we demonstrated that TSU products did indeed induce colonies, which were mainly compact and mixed types. To determine the cell type comprising the colonies, further characterisations were carried out histochemically and immunologically. Although the immunophenotyping using antibodies specific for murine granulocytes and macrophages failed because of the interference by agar in culture, the histochemical study revealed that most colonies were naphthol-AS-D-chloroacetate esterase-positive but alpha-naphthyl butyrate esterase-negative, indicating that they were mainly granulocytic. Therefore, CSF produced by TSU was considered to be G-CSF, a potent growth factor specific for granulocytes. Indeed, the quantification by ELISA revealed that TSU supernatant contained a rather high concentration of G-CSF (2.45 μg/mg protein). These results are consistent with previous reports that tumour cell-derived G-CSF leads to leucocytosis in patients with malignant diseases [4–11].

It is of more interest to us, however, that the maximum number of colonies induced by the TSU culture supernatant was greater than that achieved with rhG-CSF and that the size of the colonies induced by TSU was obviously larger than those induced by rhG-CSF. Sato *et al.* [11] have reported that not only G-CSF but also IL-1 contributed to leucocytosis in oral cancer patients and in tumour-bearing mice. Other investigators have reported that tumour necrosis factor (TNF) stimulated the production of G-CSF, suggesting an indirect as well as a direct contributory role of TNF in leucocytosis [18, 19]. Adachi *et al.* [20] have reported that the constitutive production of multiple CSF synergistically increased the neutrophil count in patients with lung cancer. These findings led us to consider that other factors may have been linked to the cause of leucocytosis.

M-CSF, which has been detected in a wide range of human and murine tissues, presenting as a glycosylated homodimer, stimulates macrophage colony formation in both humans and mice [21, 22]. These reports have shown that M-CSF not only directly promotes the proliferation of human monocyte progenitor cells, but that it also enhances the proliferation of human granulocytes indirectly, via augmenting the production of G-CSF and granulocyte-macrophage-CSF by monocytes [23, 24]. Clinical trials of human M-CSF in patients after they had received anticancer chemotherapy resulted in earlier recovery from leucopenia and neutropenia than that seen in the control patients who did not receive human M-CSF infusions, as reported by Motoyoshi *et al.* [25]. Using neutralising antisera, we investigated whether M-CSF was implicated in the CSF activity of TSU. Although each antiserum against G-CSF or M-CSF diminished the activity of TSU, the activity was completely abolished only when both antibodies were added at the same time. These findings indicate that tumour-derived M-CSF, as well as G-CSF, could be a cause of the leucocytosis associated with oral cancer, although the content of M-CSF in TSU culture supernatant was not determined because the kit for measuring the quantity of M-CSF was unavailable. Moreover, our results indicate that G- and M-CSF had a synergistic effect in causing the leucocytosis, i.e. M-CSF enhanced the colony formation induced by rhG-CSF in a dose-dependent manner. These observations strongly suggest that: (1) at least in this patient, leucocytosis could be due to both G-CSF and M-CSF released

by the tumour; and (2) that M-CSF enhanced the action of G-CSF synergistically *in vitro*; this phenomenon may also have occurred *in vivo* in the patient. Similar results were reported by Lee *et al.* [26], who showed that G-CSF and M-CSF were produced constitutively by murine mammary cancer and that these factors appeared to act synergistically in causing leucocytosis in tumour-bearing mice.

Herein, we demonstrated that an oral cancer cell line established from a patient with marked granulocytic leucocytosis, produced not only G-CSF but also M-CSF, and that these factors could function synergistically as a cause of the leucocytosis. However, the biological significance of cancer-producing CSF has not been fully elucidated. Recent studies have demonstrated that neutrophils increased by cancer-producing CSF modulate the host immune system [27] and facilitate cancer metastasis [3, 28]. Indeed, the patient from whom TSU was established had many metastatic lesions. Further investigations of the biological and clinical significance of tumour-derived factors acting as CSF are necessary.

1. Robinson WA. Granulocytosis in neoplasia. *Ann NY Acad Sci* 1974, **230**, 212–218.
2. Mckee LC Jr, Tenn N. Excess leukocytosis (leukemoid reactions) associated with malignant diseases. *S Med J* 1985, **78**, 1475–1482.
3. Shoenfeld Y, Tal A, Berliner S, Pinkhas J. Leukocytosis in non hematological malignancies—a possible tumor-associated marker. *J Cancer Res Clin Oncol* 1986, **111**, 54–58.
4. Yoneda T, Nishikawa N, Nishimura R, Kato I, Sakuda M. Three cases of oral squamous cancer associated with leukocytosis, hypercalcemia, or both. *Oral Surg* 1989, **68**, 604–611.
5. Asano S, Urabe A, Okabe T, *et al.* Demonstration of granulopoietic factor(s) in the plasma of nude mice transplanted with a human lung cancer and in the tumor tissue. *Blood* 1977, **49**, 845–852.
6. Okabe T, Sato N, Kondo Y, *et al.* Establishment and characterization of a human cancer cell line that produces human colony-stimulating factor. *Cancer Res* 1978, **38**, 3910–3917.
7. Kimura N, Niho Y, Ono J, Miyamoto N, Shibuya T, Takai R. An established lung cancer cell line producing colony-stimulating activity. *Proc Jpn Acad* 1978, **54**, 548–552.
8. Hocking W, Goodman J, Golde D. Granulocytosis associated with tumor cell production of colony-stimulating activity. *Blood* 1983, **61**, 600–603.
9. Okabe T, Fujisawa M, Kudo H, Honma H, Ohsawa N, Takaku F. Establishment of a human colony-stimulating-factor-producing cell line from an undifferentiated large cell carcinoma of the lung. *Cancer* 1984, **54**, 1024–1029.
10. Nomura H, Imazaki I, Oheda M, *et al.* Purification and characterization of human granulocyte colony stimulating factor (G-CSF). *EMBO J* 1986, **5**, 871–876.
11. Sato K, Fujii Y, Kasono K, *et al.* Paraneoplastic syndrome of hypercalcemia and leukocytosis caused by squamous carcinoma cells (T3M-1) producing parathyroid hormone-related protein, interleukin 1α, and granulocyte colony-stimulating factor. *Cancer Res* 1989, **49**, 4740–4746.
12. Rikimaru K, Toda H, Tachikawa N, Kamata N, Enomoto S. Growth of the malignant and nonmalignant human squamous cells in a protein-free defined medium. *In Vitro Cell Dev Biol* 1990, **26**, 849–856.
13. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Anal Biochem* 1976, **72**, 248–254.
14. Bradley TR, Metcalf D. The growth of bone marrow cells *in vitro*. *Aust J Exp Biol Med Sci* 1966, **44**, 287–300.
15. Hayashi E, Rikimaru K, Satomura K, *et al.* Establishment and characterization of four human oral cancer cell lines. *J Jpn Stomatol Soc* 1995, **44**, 47–54.
16. Kubota K, Mizoguchi H, Miura Y, Suda T, Takaku F. A new technique for the cytochemical examination of human hemopoietic cells grown in agar gel. *Exp Hematol* 1980, **8**, 339–344.
17. Burgess AW, Metcalf D, Russell SHM, Nicola NA. Granulocyte-, macrophage-, megakaryocyte-, eosinophil- and erythroid-colony-stimulating factors produced by mouse spleen cells. *Biochem J* 1980, **185**, 301–314.
18. Remick DG, Larrick J, Kunkel SL. Tumor necrosis factor-induced alterations in circulating leukocyte populations. *Biochem Biophys Res Comm* 1986, **141**, 818–824.
19. Koeffler HP, Gasson J, Ranyard J, Souza L, Shepard M, Munker R. Recombinant human TNF-α stimulates production of granulocyte colony-stimulating factor. *Blood* 1987, **70**, 55–59.
20. Adachi N, Yamaguchi K, Morikawa T, Suzuki M, Matsuda I, Abe K. Constitutive production of multiple colony-stimulating factors in patients with lung cancer associated with neutrophilia. *Br J Cancer* 1994, **69**, 125–129.
21. Das SK, Stanley ER, Guilbert LJ, Forman LW. Human colony-stimulating factor (CSF-1) radioimmunoassay: resolution of three subclasses of human colony-stimulating factors. *Blood* 1981, **58**, 630–641.
22. Hatake K, Motoyoshi K, Ishizaka Y, Saito M, Takaku F, Miura Y. Purification of human urinary colony-stimulating factor by high performance liquid chromatography. *J Chromatogr* 1985, **334**, 339–344.
23. Ishizaki Y, Motoyoshi K, Hatake K, Saito M, Takaku F, Miura Y. Mode of action of human urinary colony-stimulating factor. *Exp Hematol* 1986, **14**, 1–8.
24. Motoyoshi K, Yoshida K, Hatake K, *et al.* Recombinant and native human urinary colony-stimulating factor directly augments G- and GM-CSF production of human peripheral blood monocytes. *Exp Hematol* 1989, **17**, 68–71.
25. Motoyoshi K, Takaku F, Mackawa T, *et al.* Protective effect of partially purified human urinary colony-stimulating factor on granulocytopenia after chemotherapy. *Exp Hematol* 1986, **14**, 1069–1075.
26. Lee MY, Kaushansky K, Judkins SA, Lottsfeldt JL, Waheed A, Shadduck RK. Mechanisms of tumor-induced neutrophilia: constitutive production of colony-stimulating factors and their synergistic actions. *Blood* 1989, **74**, 115–122.
27. Pallack SB, Lee MY, Cohen E, Lottsfeldt JL, Rosse C. Modulation of murine natural killer cells by a granulocytosis-inducing tumor. *Cancer Res* 1989, **49**, 174–178.
28. Nicoletti G, Lollini P-L, Bagnara GP, *et al.* Are colony-stimulating factor-producing cells facililated in the metastatic process? *Anticancer Res* 1987, **7**, 695–700.

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