Jpn. J. Cancer Res. **93**, 667–676, June 2002

# Establishment and Characterization of a New Lung Cancer Cell Line (MI-4) Producing High Levels of Granulocyte Colony Stimulating Factor

Ryoji Harada,[1] Yoshiki Uemura,[1,4] Makoto Kobayashi,[1] Adriana Zamecnikova,[1] Hideshi Nakata,[1] Takahiro Taguchi,[2] Mutsuo Furihata,[3] Yuji Otsuki[3] and Hirokuni Taguchi[1]

[1]*Department of Internal Medicine,* [2]*Department of Anatomy,* [3]*Department of Pathology, Kochi Medical School, Kohasu, Okocho, Nankoku, Kochi 783-8505*

**We established a human lung cancer cell line, MI-4 from the pleural effusion of a 69-year-old male with advanced large cell undifferentiated carcinoma of the lung complicated by leukocytosis. The culture supernatant of MI-4 contained high levels of granulocyte colony stimulating factor (G-CSF). The intracellular localization of the G-CSF was identified by immunocytochemistry. Reverse transcription-polymerase chain reaction (RT-PCR) revealed G-CSF mRNA expression in this cell line. The cell line was successfully transplanted into nude mice. The transplanted nude mice also showed leukocytosis with a high serum G-CSF level. Southern blot analysis did not show amplification or rearrangement of the *G-CSF* gene in MI-4 cells. Spectral karyotyping (SKY) and fluorescence *in situ* hybridization (FISH) analyses revealed that this cell line has an additional chromosome 17 attached to a segment of chromosome 10 besides two intact chromosomes 17, and that each of these three chromosomes 17 has a *G-CSF* gene on chromosome 17q. Inflammatory cytokines, tumor necrosis factor (TNF)-α and interleukin (IL)-1β, significantly enhanced G-CSF expression at both the protein and mRNA levels in MI-4. However, these cytokines did not stimulate the growth of MI-4 cells, regardless of abundant G-CSF production. TNF-α rather suppressed it, in a dose-dependent manner. Exogenous recombinant human G-CSF and anti-G-CSF antibody did not promote or inhibit the growth of MI-4 cells at any concentration examined. In addition, RT-PCR analysis did not show G-CSF receptor mRNA expression. These results suggest that this cell line does not have an autocrine growth loop for G-CSF. This cell line should be very useful for understanding the biological activity of G-CSF in G-CSF-overproducing lung cancer.**

Key words: Lung cancer — G-CSF — Cell line — MI-4

Some cancer patients manifest leukocytosis without overt inflammation. The production of granulocyte colony-stimulating factor (G-CSF) by the tumor itself is thought to be responsible for this paraneoplastc syndrome.[1–3] At least 80 cases of G-CSF-producing tumors have been reported up to the present. Although many types of G-CSF-producing tumors such as carcinoma of the thyroid,[4,5] kidney,[6] oral cavity,[7] bladder,[8] gallbladder,[9] stomach,[10] hepatoma[11] and melanoma[12] have been reported, carcinoma of the lung[13–17] is predominant. Large cell carcinoma is the overwhelmingly dominant histologic type in lung cancer.[18,19] However, there have been few reports confirming the establishment of a G-CSF-producing cell line; to our knowledge, 10 G-CSF-producing lung cancer cell lines have been reported. Four of them are derived from large cell carcinoma.[14,20–22] Recently, we studied a patient with large cell lung carcinoma who exhibited leukocytosis, and we established a new cell line *in vitro* from this patient. This cell line produces a high level of G-CSF in the medium. In this paper, we describe the establishment and characterization of this new lung cancer cell line.

[4] To whom correspondence and reprint requests should be addressed.
E-mail: uemuray@kochi-ms.ac.jp

## MATERIALS AND METHODS

**Patient** The patient was a 69-year-old male with large cell carcinoma of the lung. He underwent left upper lobectomy. Histologically, the surgical specimen of the lung tumor showed large cell undifferentiated carcinoma. However, the tumor metastasized to Virchow's node after 4 years. Chemotherapy and radiotherapy afforded only temporary control and the tumor grew rapidly. As the disease progressed, his peripheral blood white blood cell (WBC) count increased to 92 000/mm$^3$ with 95% mature neutrophils, without evidence of infection. The serum level of G-CSF was 141 pg/ml (normal <30 pg/ml). The patient gradually deteriorated with cachexia, and died 9 months after recurrence. At autopsy, metastatic tumors were found in the bilateral lungs, anterior mediastinum, right adrenal gland and retroperitoneal lymphnodes, besides Virchow's node. Furthermore, he had a large quantity of malignant pleural effusion and ascites.

**Cell culture** Tumor cells were obtained from the pleural effusion and cultured for the establishment of a cell line.

The cells were cultured in RPMI-1640 medium containing 10% fetal calf serum (FCS) at 37°C in an incubator, under a 100% humidified 5% $CO_2$ atmosphere. Subcultures were carried out twice weekly by removing the cells from 75 $cm^2$ tissue flasks with 0.025% trypsin in 0.02% EDTA and splitting them 1:3. After 30 passages, a cell line, named MI-4, was established. The cells were cultured under RPMI-1640 medium containing 5% FCS for the following *in vitro* experiments.

**Morphological study** The morphological study of MI-4 cells was done under a phase-contrast microscope. Immunocytochemical staining of MI-4 cells was performed using the following monoclonal antibodies: AE1/AE3 (Becton Dickinson, Mountain View, CA), Cytokeratin 8 (Enzo Biochem, New York, NY), Cytokeratin 18 and Cytokeratin 19 (ScyTek, Logan, UT) for keratin, MUC-1 for KL-6 (gift from Dr. Nobuoki Kono), EMA (Dakopatts, Glostrup, Denmark) for epithelial cell membrane antigen, CEA (Bio-Science, Emmenbrücke, Switzerland) for carcinoembryonic antigen, PE10 (Dakopatts) for surfactant apoprotein A, G-CSF (Genzyme-TECHNE Research Products, Minneapolis, MN) for granulocyte colony stimulating factor and a polyclonal antibody: Hup-1(Dakopatts) for human urine protein-1.

**Transplantation of cells to nude mice** The MI-4 cells were inoculated subcutaneously to the dorsal side of 5 BALB/c nude mice at 4 weeks of age at the concentration of $10^7$ cells in 0.2 ml of RPMI-1640 medium. Immediately before death, the peripheral leukocytes count was determined and the serum G-CSF concentration of mice was measured by enzyme-linked immunosorbent assay (ELISA) (Genzyme-TECHNE Research Products). Histological sections were prepared from tumors and pertinent organs such as the liver, spleen, lungs, kidneys and bone marrow. The sections were stained with hematoxylin and eosin.

**DNA extraction and Southern blot analysis** The MI-4 cells were treated with proteinase K and extracted with phenol and chloroform. DNA of peripheral blood mononuclear cells (PBMCs) provided by a healthy donor was extracted as a control DNA. Purified DNA (10 $\mu$g) was digested with *Eco*RI, *Hin*dIII, *Rsa*I, *Pvu*II and *Bgl*I (Toyobo, Tokyo), electrophoresed through 1% agarose gels, and transferred onto nylon membranes (Bio-Rad, Richmond, CA). The nylon membranes were hybridized to $^{32}$P-labeled human G-CSF cDNA probe, pBRV2 (kindly provided by Chugai Pharmaceutical Co., Tokyo). Hybridization was performed for 16 h at 65°C in 7.5% sodium dodecyl sulfate (SDS)/0.25 *M* $Na_2HPO_4$. Blots were washed twice for 10 min in 5% SDS/0.02 *M* $Na_2HPO_4$ at 65°C. Bands were visualized by autoradiography.

**Chromosome analysis** Chromosome analyses of the MI-4 cells were performed using the Giemsa banding technique. In addition, spectral karyotyping (SKY) analysis was performed to identify precisely the chromosomal rearrangements and the marker chromosomes, as previously described.[23] We also performed fluorescence *in situ* hybridization (FISH) to detect *G-CSF* gene on chromosome 17. However, the size of the human G-CSF cDNA probe, pBRV2 was too small to exhibit clear signals in usual FISH analysis. The gene encoding for G-CSF has been mapped to the region 17q11.2–21 by *in situ* hybridization.[24] Therefore, we used a digoxigenin-labeled centromere-specific probe for chromosome 17 (Oncor, Inc., Gaithersburg, MD) and PML SpectrumOrange/RARA SpectrumGreen dual color translocation probe (Vysis, Inc., Downers Grove, IL) to search for the *G-CSF* gene. The retinoic acid receptor α (*RARA*) gene is localized at 17q21, which contains one of the breakpoints in the 15;17 chromosome translocation specific for acute promyelocytic leukemia (APL). *In situ* hybridization has demonstrated that the G-CSF coding region is proximal to the breakpoint on chromosome 17 in APL and is located between the centromere and the *RARA* gene.[25] The centromere of chromosome 17 and the *RARA* gene were detected as red and green FISH signals respectively.

**Detection of G-CSF, GM-CSF, M-CSF, IL-β and TNF-α** G-CSF content in the culture medium or serum was determined using an ELISA kit (Genzyme-TECHNE Research Products) according to the supplier's instructions. The sensitivity of this kit is 0.4 pg/ml. Contents of granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage colony-stimulating factor (M-CSF), IL-β and TNF-α in the culture medium were determined using an ELISA kit (R & D Systems, Minneapolis, MN). The sensitivities of the GM-CSF, M-CSF, IL-β and TNF-α kits are 3, 9, 1 and 4.4 pg/ml, respectively.

**Quantitative reverse transcription-polymerase chain reaction (RT-PCR)** Total RNA was extracted from the cultured cells by the guanidine-thiocyanate method using ISOGEN solution (Nippon Gene Co., Tokyo). OKa-C-1 cells and U937 cells were used as positive controls for detection of G-CSF and G-CSF receptor mRNA expression, respectively. BALL-2 cells were used as a negative control for G-CSF and G-CSF receptor mRNA expression. One microgram of the RNA was converted to cDNA with Molony murine leukemia virus reverse transcriptase in 20 $\mu$l of reaction mixture. For quantification, 1 $\mu$l aliquots of cDNA samples were subjected to PCR in 50 $\mu$l of reaction solution containing 0.5 $\mu M$ of each specific primer, 10 m*M* Tris-HCl, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 0.2 m*M* dNTP, and 1 unit of *Taq* polymerase. Primers used for G-CSF were 5′-TAGAGCAAGTGAGGAAGATCCAGG-3′ for sense and 5′-AGTTCTTCCATCTGCTGCCAGATG-3′ for antisense, giving a 328 bp fragment. Primers used for G-CSF receptor were 5′-TGGACTGCAGCTGGTTTCAGGAAC-3′ for sense and 5′-GGTCTGACAGTTGCCCCGGCTC-3′ for antisense, giving a 668 bp fragment. Primers

used for β-actin were 5′-ACCTTCAACACCCCAGCC-ATG-3′ for sense and 5′-GGCCATCTCTTGCTCGAA-GTC-3′, giving a 309 bp fragment. Reaction was performed for 30 cycles for G-CSF, 35 cycles for G-CSF receptor and 20 cycles for β-actin in a DNA thermal cycler (TaKaRa, Ohtsu). The PCR steps for G-CSF and β-actin included denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and polymerization at 72°C for 1 min. The PCR steps for G-CSF receptor included denaturation at 95°C for 55 s, annealing at 72°C for 70 s, polymerization at 65°C for 95 s, and one cycle of 435 s at 72°C. Amplification cycle numbers were optimized for each sequence using the serial dilution method to achieve a dose-dependent amplification.[26] One microliter of PCR product was electrophoresed on 1% NuSieve GTG agarose gel and stained with ethidium bromide. The intensity of the bands was evaluated using a UV-light box imaging system (Atto, Tokyo).

**Effects of TNF-α and IL-1β on G-CSF production and propagation of MI-4 cells** For the evaluation of growth sensitivity to TNF-α and IL-1β, cell proliferation assay was performed using the "CellTiter 96" $AQ_{ueous}$ One Solution Cell Proliferation Assay kit (Promega Corp., Madison, WI). Briefly, MI-4 cells were plated in 96-well plates and exposed for 48 h to the media containing 1% FCS and 0.1 to 10 ng/ml of TNF-α and IL-1β. Then a solution of tetrazolium salt MTS was added directly and the mixture was incubated for 4 h at 37°C. The absorbance of the solution was measured at 490 nm in a spectrophotometer. To examine the effects of TNF-α and IL-1β on G-CSF production in MI-4 cells, cells were exposed to 0.1 to 100 ng/ml of these cytokines for 48 h, or to examine G-CSF mRNA expression, for 24 h. Then G-CSF in cultured medium and mRNA expression in harvested cells were analyzed by quantitative ELISA and RT-PCR respectively. The values of G-CSF in the conditioned medium were normalized with respect to the number of cells using values obtained in the cell proliferation assay. The experiment was done in triplicate, and the mean and standard deviation were calculated.

**Effect of exogenous G-CSF and anti-G-CSF antibody on propagation of MI-4 cells** We examined whether administration of a specific anti-human G-CSF monoclonal antibody (Genzyme-TECHNE Research Products) and exogenous recombinant G-CSF (kindly provided by Chugai Pharmaceutical Co., Tokyo) would inhibit and promote the growth of tumor cells, respectively. The proliferating activity of the cultured cells was measured by the cell proliferation assay described above. Anti-G-CSF antibody (500 ng/ml) was diluted 512-, 256-, 128-, 64-, 32-, and 16-fold with phosphate-buffered saline (PBS) and added to the culture to make 10%. Serial concentrations (0.1–100 ng/ml) of recombinant G-CSF were also added to the culture. NFS-60 cells (ATCC CRL-1838), which proliferate in response to G-CSF in a dose-dependent manner, were used as a control. The experiment was done in triplicate, and the mean and standard deviation were calculated.

## RESULTS

**Morphological features of the cultured cells (MI-4)** MI-4 cells adhered weakly to the culture dish and were easily detached (Fig. 1). The doubling time was 96 h. The adherent cells were morphologically polygonal and epithelial-like. The cells contain granules in the cytoplasm.

**Immunocytochemical analysis** Immunocytochemical staining showed that MI-4 cells were positive to AE1/AE3, Cytokeratin 8, Cytokeratin 18, Cytokeratin 19, EMA, G-CSF and MUC-1, but negative to Hup-1, CEA, and PE10 (Table I).

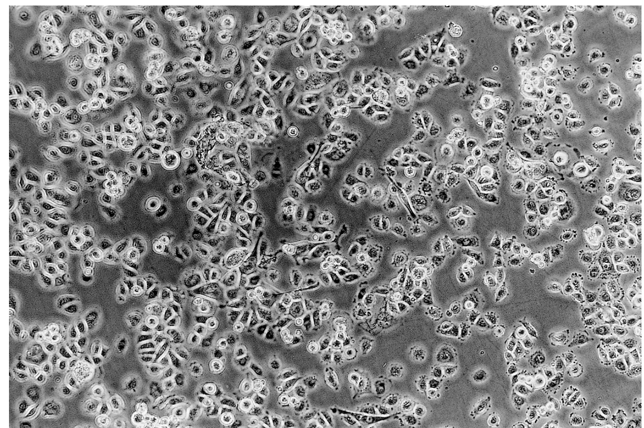


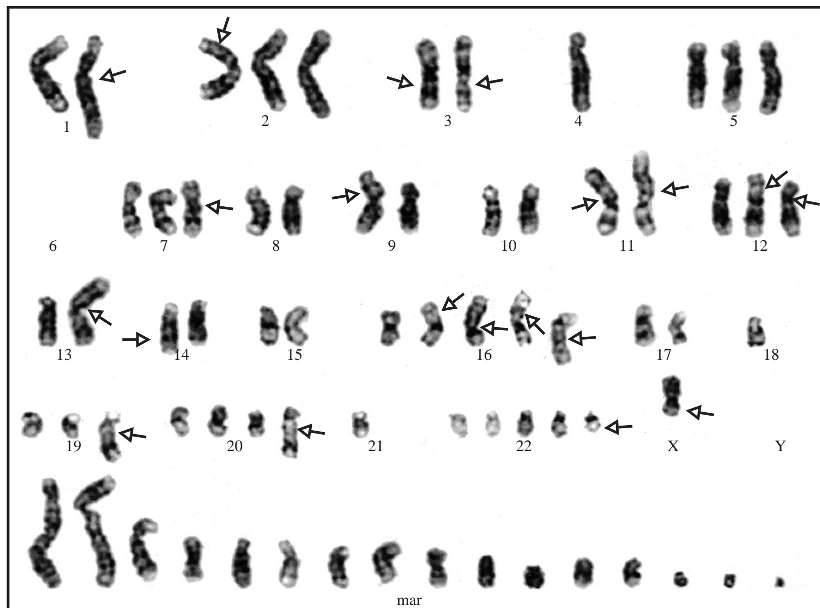
Fig. 1. Morphological features of cultured MI-4 cells by phase-contrast microscopy (×200).

Table I. Immunohistochemical Staining of MI-4 Cell Line

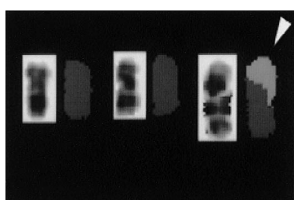
| Antibody | Specificity | MI-4 |
|---|---|---|
| AE1/AE3 | Epithelial cells | ++ |
| Cytokeratin 8 | Epithelial cells | + |
| Cytokeratin 18 | Epithelial cells | + |
| Cytokeratin 19 | Epithelial cells | + |
| Epithelial membrane antigen | Epithelial cells, plasma cells and some lymphoma cells | + |
| MUC-1 | KL-6 | + |
| Hup-1 | Human urinary protein-1 | − |
| CEA3 | Carcinoembryonic antigen | − |
| PE10 | Surfactant apoprotein A | − |
| G-CSF | G-CSF-producing cells | + |

**Chromosome analysis** Although the patient's original tumor cells were not examined, MI-4 cells at the 40th passage had a modal chromosome number of 69 with complex structural abnormalities (Fig. 2A). G-banding analysis showed only an intact pair of chromosome 17 and sixteen marker chromosomes. SKY analysis demonstrated another

A

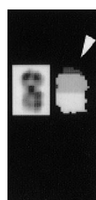



B

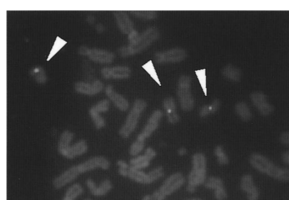


Chromosome 17
Chromosome 16
Chromosome 7
Chromosome 21

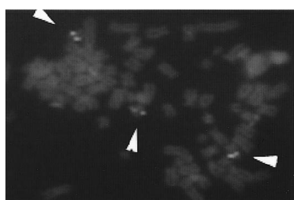
C



D

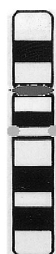


E



F

alpha satellite DNA (centromere)
G-CSF (q11.2-12)
RARA (q21.1)



Chromosome 17

Fig. 2. Chromosome analysis of MI-4 cell. A typical karyotype of MI-4 cells (69, add(X)(q11), add(1)(q11), +add(2)(p21), add(3)(q11), add(3)(q21), −4, +5, −6, −6, +add(7)(q11), add(9)(p11), add(11)(p11), add(11)(p11), +add(12)(p11), add(12)(p11), add(13)(p11), add(14)(q32), +add(16)(p11), +add(16)(p11), +add(16)(p11), add(16)(p13), −18, +add(19)(q13), +20, +add(20)(q13), −21, +22, +22, +del(22)(q13), +16mar) (A). SKY analysis of metaphase chromosome. The G-banded karyotype is on the left and the classified SKY images on the right. The colors of chromosomes of origin in the classified image are listed. There are two intact chromosomes 17 and another with a segment of chromosome 16 (B). A marker chromosome with a segment of chromosome 17 (C). FISH analysis of metaphase chromosomes. The α satellite DNA of chromosome 17 was detected as a red signal (D). The *RARA* gene was detected as a green signal (E). An illustration of *RARA* gene, *G-CSF* gene and α satellite DNA mapped on chromosome 17 (F).

chromosome 17 with a segment of chromosome 16 (Fig. 2B) and a marker chromosome with a small segment of chromosome 17 (Fig. 2C). FISH analysis showed three red signals for the centromere of chromosome 17 (Fig. 2D) and three green signals for the *RARA* gene in metaphase cells (Fig. 2E). Each locus of the *RARA* gene, *G-CSF* gene and centromere on chromosome 17 is illustrated (Fig. 2F). These results indicate that the three chromosomes 17, except the marker chromosome, might have *G-CSF* genes.

**Southern blot analysis of the *G-CSF* gene** In order to define if the constitutive expression of G-CSF by the tumor cells is due to the changes in genomic structures, we carried out Southern blot analysis. DNA (10 $\mu$g) isolated from MI-4 tumor cells and normal PBMCs was digested with *Eco*RI, *Hin*dIII, *Rsa*I, *Pvu*II or *Bgl*I and subsequently electrophoresed on 1% agarose gel. They were transferred to nylon membrane, then hybridized to G-CSF cDNA probe. The size of bands detected with MI-4 DNA were identical to that in the case of PBMC DNA, regardless of digestion with the different restriction enzymes (Fig. 3). This result is compatible with the reported restriction maps of the *G-CSF* gene. Moreover, there was little difference in the signal intensities between MI-4 and PBMC DNA. Thus, the *G-CSF* gene of MI-4 cells showed no rearranged configuration or amplification.

**Transplantation to nude mice** Approximately $10^7$ MI-4 cells were inoculated subcutaneously into five mice. A tuberous tumor was formed in 1 to 2 weeks at the site of inoculation in all 5 nude mice and grew gradually. The animals died of the tumor in about 2 months, with massive metastasis. Immediately before death, the mean WBC count of tumor-bearing mice was 350 000/mm$^3$ with 96% mature neutrophils and the mean serum G-CSF level was 850 pg/ml. A histological study of the transplanted tumor tissue showed a morphology similar to that of the original tumor tissue (Fig. 4A). The cells contain granules in the cytoplasm with obscure cytoplasmic outline. Nuclei were lobulated with multiple, enlarged and irregular nucleoli. The lungs of tumor-bearing mouse showed marked capillary leukostasis and alveolar septae packed with mature neutrophils in all cases (Fig. 4B).

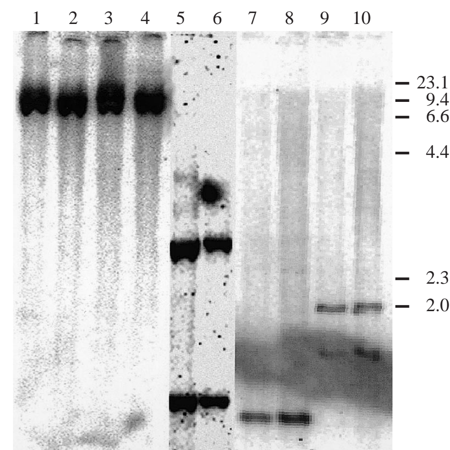



Fig. 3. Southern blot analysis of human G-CSF in MI-4 cells and PBMCs from a healthy volunteer. DNA was hybridized with the G-CSF probe. Lanes 1, 3, 5, 7 and 9, DNA from normal PBMCs; lanes 2, 4, 6, 8 and 10, DNA from MI-4 cells; 10 $\mu$g of DNA was digested with *Eco*RI (lanes 1, 2), *Hin*dIII (lanes 3, 4), *Rsa*I (lanes 5, 6), *Pvu*II (lanes 7, 8) or *Bgl*I (lanes 9, 10).

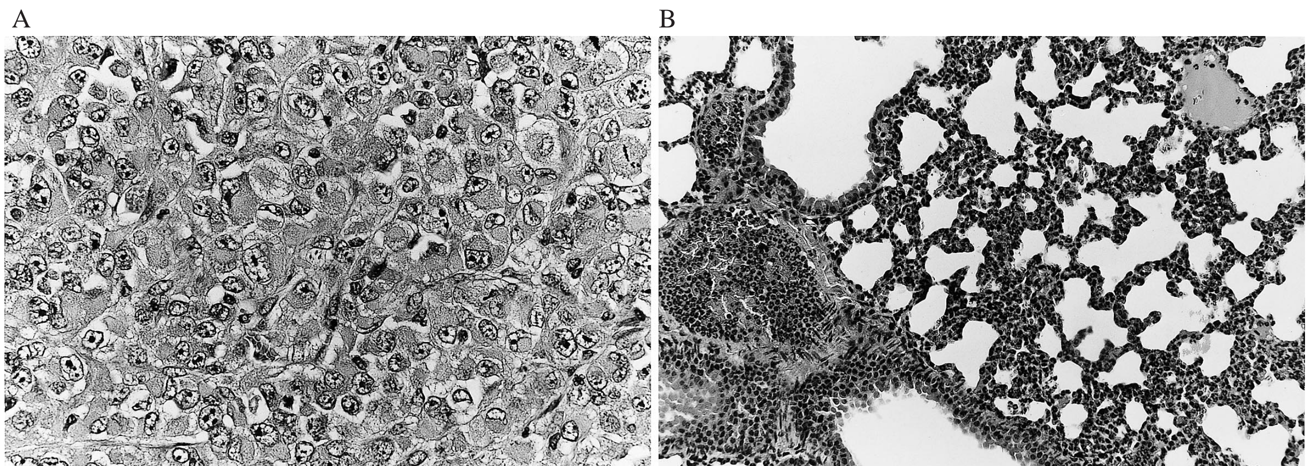



Fig. 4. Histology of nude mouse tumor produced by transplantation of MI-4 cells (A). The neoplastic cells showed round lobulated nuclei with multiple, enlarged and irregular nucleoli and cytoplasm with an obscure cytoplasmic outline. Hematoxylin and eosin staining (×200). Lung of nude mouse transplanted with MI-4 cells showing capillary leukostasis and alveolar septae packed with mature neutrophils (B). Hematoxylin and eosin staining (×100).

**Detection of human G-CSF and G-CSF receptor mRNA in MI-4 cells** RT-PCR analysis showed the presence of G-CSF transcript (Fig. 5A), but not G-CSF receptor (Fig. 5B) in this cell line.

**Detection of G-CSF, GM-CSF, M-CSF, IL-β and TNF-α in the medium** MI-4 cells were plated in 96-well plates and cultured in the media containing 5% FCS for 48 h. Then the medium was collected for ELISA. All cytokines in the fresh medium containing 5% FCS were present, if at all, at levels below the sensitivity of the ELISA kits. Contents of G-CSF, GM-CSF and M-CSF in the medium were 2230±40, 140±19 and 160±23 pg/ml respectively while TNF-α and IL-1β were undetectable by the ELISA kits.

**Effect of TNF-α and IL-1β** IL-1β and TNF-α stimulated G-CSF production by MI-4 cells in a dose-dependent manner (Fig. 6A). Maximal G-CSF production by IL-1β and TNF-α was observed at concentrations of 10 ng/ml and 100 ng/ml respectively. Maximal G-CSF levels induced by these cytokines were about ten times the control. IL-1β and TNF-α (0.1 ng/ml) enhanced production and mRNA expression of G-CSF (Fig. 6B). However, these cytokines did not promote the growth of MI-4 cells at any concentration (Fig. 6C). TNF-α rather suppressed cell growth in a dose-dependent manner.

**Effect of anti-G-CSF antibody and recombinant G-CSF** Exogenous recombinant G-CSF (Fig. 7A) and anti-G-CSF antibody (Fig. 7B) did not promote and inhibit the growth of MI-4 cells compared with NFS-60 cells at any concentration, respectively.

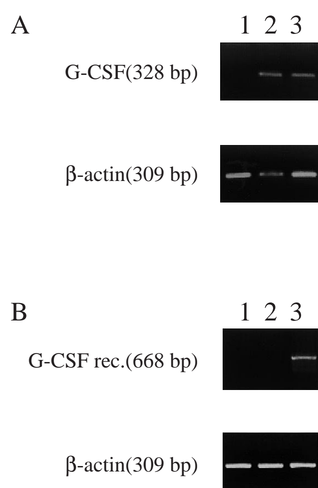



Fig. 5. Detection of G-CSF and G-CSF receptor mRNAs by RT-PCR. G-CSF mRNA expression (A). Negative control BALL-2 (lane 1), MI-4 (lane 2) and positive control OKa-C-1 (lane 3). G-CSF receptor mRNA expression (B). Negative control BALL-2 (lane 1), MI-4 (lane 2) and positive control U937 (lane 3).

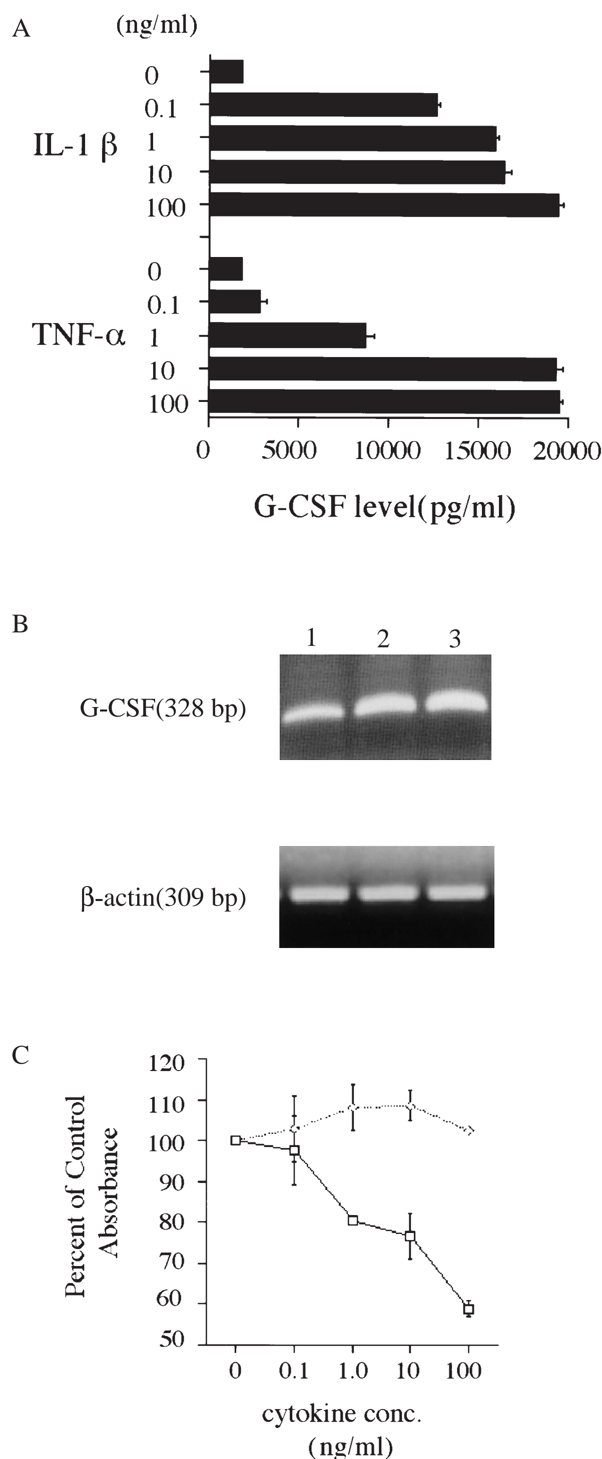



Fig. 6. G-CSF production by MI-4 cells at various concentrations (0–100 ng/ml) of TNF-α and IL-1β (A). G-CSF mRNA expression at 0.1 ng/ml of TNF-α and IL-1β (B). Control (lane 1), 0.1 ng/ml of TNF-α (lane 2) and 0.1 ng/ml of IL-1β (lane 3). Proliferation of MI-4 cells in the presence of various concentrations (0–100 ng/ml) of TNF-α and IL-1β. ◇ IL-1β; □ TNF-α (C). The data represent the mean±SD of three experiments.

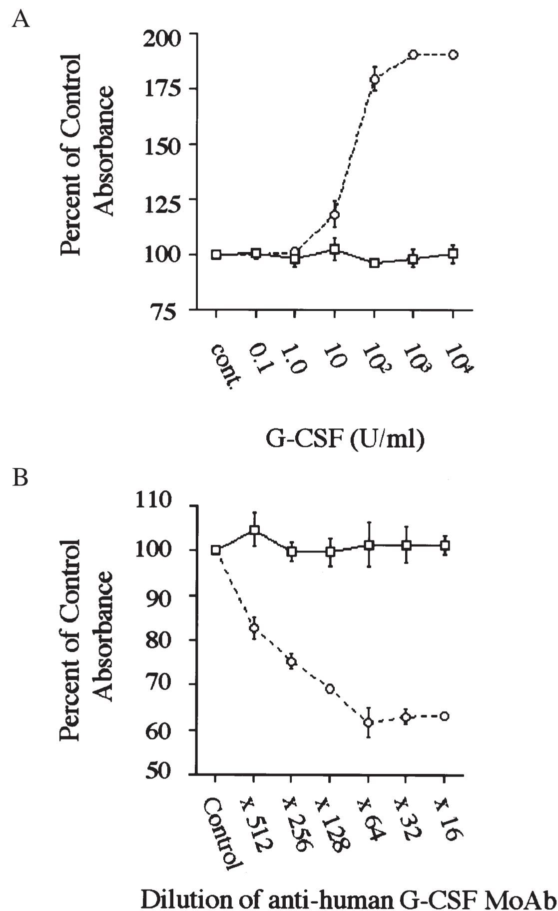



Fig. 7. Proliferation of MI-4 cells in the presence of various concentrations of exogenous recombinant G-CSF (A) and anti-G-CSF antibody (B). MI-4 cells and control murine NFS-60 cells were grown for 48 h in serum-free medium in the presence of increasing concentrations of G-CSF ($0.1-10^4$ U/ml) and anti-G-CSF antibody (dilution by 16- to 512-fold). The data represent the mean±SD of three experiments.

**DISCUSSION**

In this study, we established a new cell line (MI-4) which produces G-CSF from the pleural effusion of a patient with large cell undifferentiated carcinoma of the lung with marked leukocytosis, without any evidence of infection. Culture medium of MI-4 cells also contained a high level of G-CSF. Immunocytochemistry showed cytoplasmic staining for human G-CSF in MI-4 cells. Moreover, RT-PCR analysis revealed G-CSF mRNA expression in this cell line. Nude mice transplanted with MI-4 cells showed marked leukocytosis with a high serum level of G-CSF. We thought that this cell line would be a useful tool for understanding the cellular and molecular basis for G-CSF production in lung cancer.

As the causes of G-CSF over-production in tumors, trans-acting regulatory mechanisms, amplification and rearrangements of the *G-CSF* gene and co-amplification of oncogenes such as c-*myc* and c-ki-*ras* have been suggested.[27–30] In MI-4 cells, Southern blot analysis did not show any amplification or rearrangement of the *G-CSF* gene. We also studied whether chromosomal aberrations specific for chromosome 17 could be a cause of G-CSF over-production in the MI-4 line. G-banding analysis showed only two intact chromosomes 17, whereas SKY analysis revealed an additional chromosome 17 with a segment of chromosome 16 and one marker chromosome containing a part of chromosome 17. To examine whether these chromosomes 17 have *G-CSF* genes, we performed FISH analysis using G-CSF cDNA probe. However, the size of the G-CSF probe we used was too small to clearly demonstrate the FISH signal. Therefore, we used DNA probes for the centromere of chromosome 17 and the *RARA* gene instead of the *G-CSF* gene. The *RARA* gene is localized at 17q21, proximal to the *G-CSF* gene. The *G-CSF* gene should be localized between the centromere and the *RARA* gene on chromosome 17q. The results of FISH analyses suggested that the *G-CSF* gene might not be disturbed in the three chromosomes 17. The *G-CSF* gene was not detected in any marker chromosome. Previously we have established a squamous cell lung cancer cell line OKa-C-1, which abundantly produces G-CSF.[16] In OKa-C-1 cells, Southern blot analysis did not show any amplification or rearrangement of the *G-CSF* gene. SKY analyses showed another chromosome 17 with a segment of chromosome 10 besides three intact ones and eight marker chromosomes without any segment of chromosome 17 (data not shown). The FISH analysis suggested the existence of the *G-CSF* gene on the four chromosomes 17 in OKa-C-1 (data not shown). Thus, it is unlikely that overexpression of G-CSF would be caused by amplification and rearrangement of the *G-CSF* gene or specific translocation of chromosome 17 in G-CSF-producing lung cancer. The increased number of chromosomes having the *G-CSF* gene was observed in MI-4 and OKa-C-1 cell lines. However, numerical changes of chromosomes have been frequently revealed in lung cancer cell lines, regardless of cytokine production.[31, 32] Therefore, it is not likely that the increased number of chromosomes would directly correlate with G-CSF production in these cell lines. Further approaches will be needed to define the mechanisms of G-CSF production by lung tumor cells.

It has been suggested that in a certain kind of hematopoietic malignant cell, such as acute myeloid leukemia cells with G-CSF receptor, an autocrine mechanism for aberrant secretion of G-CSF may exist and may lead to abnormal cell growth.[33] Several reports have also demonstrated the presence of an autocrine growth loop for G-CSF in non-hematopoietic tumor cells.[10, 34, 35] However, in

MI-4 cells, we could not detect the expression of G-CSF receptor mRNA by RT-PCR. In addition, exogenous recombinant human G-CSF and anti-G-CSF monoclonal antibody had no influence on the growth of MI-4 cells. It has been reported that IL-1β and TNF-α stimulate G-CSF production from various cell types, for example, astrocytoma cell line, medulloblastoma cell line, and bone marrow stromal cells.[36–38] We have previously demonstrated that IL-1β and TNF-α significantly stimulate G-CSF production by the tumor cell line OKa-C-1.[39] In this study, we showed that both cytokines also significantly enhanced G-CSF production by MI-4 cells. MI-4 cells produced neither TNF-α nor IL-1β themselves. However, these cytokines did not promote the growth of MI-4 cells regardless of abundant G-CSF production. TNF-α rather inhibited the growth of both cell lines in a dose-dependent manner. We could not detect the presence of an autocrine growth loop for G-CSF in OKa-C-1 cell line (data not shown). Thus, our molecular biological study could not demonstrate a crucial role for G-CSF in mediating a growth advantage for these lung cancer cell lines. To our knowledge, there has been no report about autocrine growth control by G-CSF in lung cancers. Some studies have reported that several tumor cell types simultaneously secrete GM-CSF and M-CSF, in addition to G-CSF.[40–42] We also detected that MI-4 cells secreted some GM-CSF and M-CSF, besides a large amount of G-CSF. Although the presence of receptor for GM-CSF or M-CSF was not examined in this study, we can not rule out the possibility that these cytokines could support the growth of MI-4 cells. Further studies will be needed to elucidate the biological activity of G-CSF in lung cancer with G-CSF over-production. It has been reported that the prognosis is significantly worse in non-small cell lung cancer showing *G-CSF* gene expression.[43] Regulation of G-CSF production by tumor cells might be an approach to improve the prognosis of patients with G-CSF-producing lung cancer.

*(Received December 4, 2001/Revised March 6, 2002/2nd Revised March 20, 2002/Accepted March 28, 2002)*

**REFERENCES**


1) Asano, S., Urabe, A., Okabe, T., Sato, N. and Kondo, Y. Demonstration of granulopoietic factor(s) in the plasma of nude mice transplanted with a human lung cancer and in the tumor tissue. *Blood*, **49**, 845–852 (1977).
2) Okabe, T., Sato, N., Kondo, Y., Asano, S., Ohsawa, N., Kosaka, K. and Ueyama, Y. Establishment and characterization of a human cancer cell line that produces human colony-stimulating factor. *Cancer Res.*, **38**, 3910–3917 (1978).
3) Ueyama, Y. and Tamaoki, N. Leukocytosis in nude mice into which human tumors are transplanted—*in vivo* screening system for hematopoietic growth factors in human tumors. *In* "Hematopoietic Growth Factors: Molecular Biology to Clinical Applications of rG-CSF," ed. P. J. Quesenberry, S. Asano and S. Saito, pp. 27–53 (1991). Excerpta Medica, Tokyo.
4) Okabe, T., Nomura, H. and Ohsawa, N. Establishment and characterization of a human colony-stimulating factor-producing cell line from a squamous cell carcinoma of the thyroid gland. *J. Natl. Cancer Inst.*, **69**, 1235–1243 (1982).
5) Baba, M., Kaito, M., Hasegawa, H., Nakayabu, M., Yoshida, Y., Uda, Y., Shima, T., Suzuki, S. and Shimizu, N. Establishment and characterization of a new human thyroid anaplastic cancer cell line that produces human colony-stimulating factor. *Mie Med. J.*, **41**, 155 (1991).
6) Higaki, I., Hirohashi, K., Fukushima, S., Wanibuchi, H., Seike, N., Yamane, T., Kubo, S., Tanaka, H., Shuto, T., Yamamoto, T. and Kinoshita, H. Renal pelvic carcinoma producing granulocyte colony-stimulating factor: report of a case. *Surg. Today*, **31**, 266–268 (2001).
7) Akatsuka, A., Shimamura, K., Katoh, Y., Takekoshi, S., Nakamura, M., Nomura, H., Hasegawa, M., Ueyama, Y. and Tamaoki, N. Electron microscopic identification of the intracellular secretion pathway of human G-CSF in a human tumor cell line: a comparative study with a Chinese hamster ovary cell line (IA1-7) transfected with human G-CSF cDNA. *Exp. Hematol.*, **19**, 768–772 (1991).
8) Ito, N., Matsuda, T., Kakehi, Y., Takeuchi, E., Takahashi, T. and Yoshida, O. Bladder cancer producing granulocyte colony-stimulating factor. *N. Engl. J. Med.*, **323**, 1709–1710 (1990).
9) Takeda, T., Ichiyanagi, A., Sano, K., Yoshida, J., Tsutsumi, Y. and Miyaji, T. A case of gallbladder cancer producing granulocyte-colony-stimulating factor. *Gastroenterol. Jpn.*, **25**, 762–767 (1990).
10) Baba, M., Hasegawa, H., Nakayabu, M., Shimizu, N., Suzuki, S., Kamada, N. and Tani, K. Establishment and characteristics of a gastric cancer cell line (HuGC-OOHIRA) producing high levels of G-CSF, GM-CSF, and IL-6: the presence of autocrine growth control by G-CSF. *Am. J. Hematol.*, **49**, 207–215 (1995).
11) Tohyama, K., Yoshida, Y., Kubo, A., Sudo, T., Moriyama, M., Sato, H. and Uchino, H. Detection of granulocyte colony-stimulating factor produced by a newly established human hepatoma cell line using a simple bioassay system. *Jpn. J. Cancer Res.*, **80**, 335–340 (1989).
12) Lilly, M. B., Devlin, P. E., Devlin, J. J. and Rado, T. A. Production of granulocyte colony-stimulating factor by a human melanoma cell line. *Exp. Hematol.*, **15**, 966–971 (1987).
13) Fahey, R. J. Unusual leukocyte responses in primary carcinoma of the lung. *Cancer*, **4**, 930 (1951).
14) Shijubo, N., Inoue, Y., Hirasawa, M., Igarashi, T., Mori, M., Matsuura, A., Uede, T. and Suzuki, A. Granulocyte

colony-stimulating factor-producing large cell undifferentiated carcinoma of the lung. *Intern. Med.*, **31**, 277–280 (1992).

15) Teramachi, M., Miyamoto, N., Yamamoto, Y., Sasaka, T., Nakamura, T. and Kitamura, F. A case of large cell carcinoma of the lung which is suspected of producing granulocyte colony-stimulating factor. *J. Jpn. Respir. Soc.*, **30**, 1327–1332 (1992).
16) Asahi, Y., Kubonishi, I., Imamura, J., Kamioka, M., Matsushita, H., Furihata, M., Ohtsuki, Y. and Miyoshi, I. Establishment of a clonal cell line producing granulocyte colony-stimulating factor and parathyroid hormone-related protein from a lung cancer patient with leukocytosis and hypercalcemia. *Jpn. J. Cancer Res.*, **87**, 451–458 (1996).
17) Watanabe, M., Ono, K., Ozeki, Y., Tanaka, S., Aida, S. and Okuno, Y. Production of granulocyte-macrophage colony-stimulating factor in a patient with metastatic chest wall large cell carcinoma. *Jpn. J. Clin. Oncol.*, **28**, 559–562 (1998).
18) Takeuchi, R., Kasagi, K., Ohta, H. and Konishi, J. Diffuse bony uptake of thallium-201-chloride in the granulocyte colony-stimulating factor-producing lung carcinoma. *J. Nucl. Med.*, **39**, 241–243 (1998).
19) Kasuga, I., Yonemaru, M., Minemura, K., Utsumi, K., Torii, Y., Ichinose, Y., Toyama, K. and Ebihara, Y. A case of large cell carcinoma of the lung producing granulocyte colony-stimulating factor. *J. Jpn. Respir. Soc.*, **32**, 73–77 (1994).
20) Shibuya, T. Establishment of a G-CSF and GM-CSF producing cell line from human large cell carcinoma of the lung. *Eur. J. Haematol.*, **43**, 182–183 (1989).
21) Suzuki, A., Takahashi, T., Okuno, Y., Nakamura, K., Tashiro, H., Fukumoto, M., Konaka, Y. and Imura, H. Analysis of abnormal expression of g-csf gene in a novel tumor cell line (KHC 287) elaborating G-CSF, IL-1 and IL-6 with co-amplification of c-myc and c-ki-ras. *Int. J. Cancer*, **48**, 428–433 (1991).
22) Inoue, M., Minami, M., Fujii, Y., Matsuda, H., Shirakura, R. and Kido, T. Granulocyte colony-stimulating factor and interleukin-6-producing lung cancer cell line, LCAM. *J. Surg. Oncol.*, **64**, 347–350 (1997).
23) Schrock, E., Mnoir, S., Veldman, B., Schoell, B. and Wienberg, J. Multicolor spectral karyotyping of human chromosomes. *Science*, **273**, 494–497 (1996).
24) Simmers, R. N., Smith, J., Shannon, M. F., Wong, G., Lopez, A. F., Baker, E., Sutherland, G. R. and Vadas, M. A. Localization of the human G-CSF gene to the region of a breakpoint in the translocation typical of acute promyelocytic leukemia. *Hum. Genet.*, **78**, 134–136 (1988).
25) Simmers, R. N., Webber, L. M., Shannon, M. F., Garson, O. M., Wong, G., Vadas, M. A. and Sutherland, G. R. Localization of the G-CSF gene on chromosome 17 proximal to the breakpoint in the t(15;17) in acute promyelocytic leukemia. *Blood*, **70**, 330–332 (1987).
26) Kinoshita, T., Imamura, J., Nagai, H. and Shimotohno, K. Quantification of gene expression over a wide range by the polymerase chain reaction. *Anal. Biochem.*, **206**, 231–235 (1992).
27) Alt, F. W., Kellems, R. E., Bertino, J. R. and Schimke, R. T. Selective multiplication of dihydrofolate reductase genes in methotrexate-resistant variants of cultured murine cells. *J. Biol. Chem.*, **5**, 1357–1370 (1978).
28) Schwab, M., Alitalo, K., Varmus, H. E., Bishop, J. M. and George, D. A cellular oncogene (c-Ki-ras) is amplified, overexpressed, and located within karyotypic abnormalities in mouse adrenocortical tumour cells. *Nature*, **303**, 497–501 (1983).
29) Bishop, J. M. The molecular genetics of cancer. *Science*, **235**, 305–311 (1987).
30) Ymer, S., Tucker, W. Q., Sanderson, C. J., Hapel, A. J., Campbell, H. D. and Young, I. G. Constitutive synthesis of interleukin-3 by leukaemia cell line WEHI-3B is due to retroviral insertion near the gene. *Nature*, **317**, 255–258 (1985).
31) Leibovitz, A., Wymer, J., Massey, K., Thompson, F. H. and Nelson, M. A. Establishment of a new lung sarcoma cell line from a human lung carcinosarcoma. *Cancer Lett.*, **82**, 145–152 (1994).
32) Virmani, A. K., Tonk, V. S. and Gazdar, A. F. Comparison between fluorescence *in situ* hybridization and classical cytogenetics in human tumors. *Anticancer Res.*, **18**, 1351–1356 (1998).
33) Metcalf, D. The roles of stem cell self-renewal and autocrine growth factor production in the biology of myeloid leukemia. *Cancer Res.*, **49**, 2305–2311 (1989).
34) Tachibana, M., Miyakawa, A., Tazaki, H., Nakamura, K., Kubo, A., Hata, J., Nishi, T. and Amano, Y. Autocrine growth of transitional cell carcinoma of the bladder induced by granulocyte-colony stimulating factor. *Cancer Res.*, **55**, 3438–3443 (1995).
35) Kyo, S., Kanaya, T., Takakura, M. and Inoue, M. A case of cervical cancer with aggressive tumor growth: possible autocrine growth stimulation by G-CSF and Il-6. *Gynecol. Oncol.*, **78**, 383–387 (2000).
36) Kikuchi, T., Nakahara, S. and Abe, T. Granulocyte colony-stimulating factor (G-CSF) production by astrocytoma cells and its effect on tumor growth. *J. Neurooncol.*, **27**, 31–38 (1996).
37) Pietsch, T., Mempel, K., Menzel, T., Ockler, R. and Welte, K. Medulloblastoma cells constitutively produce granulocyte colony-stimulating factor. *Klin. Padiatr.*, **202**, 235–239 (1990).
38) Caldwell, J. and Emerson, S. G. IL-1 alpha and TNF alpha act synergistically to stimulate production of myeloid colony-stimulating factors by cultured human bone marrow stromal cells and cloned stromal cell strains. *J. Cell. Physiol.*, **159**, 221–228 (1994).
39) Uemura, Y., Nakata, H., Kobayashi, M., Harada, R., Asahi, Y. and Taguchi, H. Regulation of granulocyte colony-stimulating factor and parathyroid hormone-related protein production in lung carcinoma cell line OKa-C-1. *Jpn. J. Cancer Res.*, **91**, 911–917 (2000).

40) Gerharz, C. D., Reinecke, P., Schneider, E. M., Schmits, M. and Gabbert, H. E. Secretion of GM-CSF and M-CSF by human renal cell carcinomas of different histologic types. *Urology*, **58**, 821–827 (2001).
41) Kimura, H., Yamaguchi, Y., Sun, L., Iwagami, S. and Sugita, K. Establishment of large cell lung cancer cell lines secreting hematopoietic factors inducing leukocytosis and thrombocytosis. *Jpn. J. Clin. Oncol.*, **22**, 313–319 (1992).
42) Quentmeier, H., Zaborski, M. and Drexler, H. G. The human bladder carcinoma cell line 5637 constitutively secretes functional cytokines. *Leuk. Res.*, **21**, 343–350 (1997).
43) Nakamura, M., Oshika, Y., Abe, Y., Ozeki, Y., Katoh, Y., Yamazaki, H., Kijima, H., Ueyama, Y., Ogata, T. and Tamaoki, N. Gene expression of granulocyte colony stimulating factor (G-CSF) in non-small cell lung cancer. *Anticancer Res.*, **17**, 573–576 (1997).