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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

Yasutomo Asahi,[1] Ichiro Kubonishi,[1,4] Jun Imamura,[1] Mikio Kamioka,[1] Hiroshi Matsushita,[3] Mutsuo Furihata,[2] Yuji Ohtsuki[2] and Isao Miyoshi[1]

Departments of [1]Medicine and [2]Pathology, Kochi Medical School, Oko-cho, Nankoku, Kochi 783 and [3]Department of Pathology, Toranomon Hospital, 2-2-2 Toranomon, Minato-ku, Tokyo 107

Squamous cell lung carcinoma cells obtained from a patient who presented with leukocytosis and hypercalcemia were transplanted into nude mice and a serially transplantable cell line, OKa-N-1, was established. The nude mice transplanted with OKa-N-1 cells displayed leukocytosis and hypercalcemia. Serum levels of granulocyte colony-stimulating factor (G-CSF) and parathyroid hormone-related protein (PTHrP) were both elevated in these mice. *In vitro* cultivation of this tumor cell line gave rise to a clonal cell line, OKa-C-1. Nude mice transplanted with the OKa-C-1 cell line also showed leukocytosis and hypercalcemia with high serum G-CSF and PTHrP levels. The culture supernatant of OKa-C-1 contained high levels of G-CSF and PTHrP. Immunohistochemical studies showed the expression of PTHrP in OKa-C-1 cells. Reverse transcription polymerase chain reaction revealed the presence of G-CSF and PTHrP mRNA in this cell line. Dexamethasone treatment inhibited the transcription of G-CSF and PTHrP genes. This new human squamous carcinoma cell line, OKa-C-1, would be useful for studying the mechanism of simultaneous production of G-CSF and PTHrP and their control in cancer patients with leukocytosis and hypercalcemia.

Key words: Lung cancer — Squamous cell carcinoma — Cell line — G-CSF — PTHrP

Leukocytosis or hypercalcemia is a well-known complication associated with cancers.[1–9] The production of granulocyte colony-stimulating factor (G-CSF) or parathyroid hormone-related protein (PTHrP) by cancer cells is thought to be responsible for these paraneoplastic syndromes.[5, 10–15] Some cancer patients are known to manifest both leukocytosis and hypercalcemia.[1, 3–5, 9] The mechanism of simultaneous and unrestricted production of G-CSF and PTHrP by cancer cells, however, is not well delineated. Cell lines that produce G-CSF and/or PTHrP have provided useful tools to understand the unique features of certain cancer cells.[5, 11, 16–18] Recently, we have studied a patient with squamous cell lung carcinoma who exhibited both leukocytosis and hypercalcemia. We transplanted the patient's tumor cells into nude mice and established a serially transplantable *in vivo* cell line. Then, we established a clonal cell line *in vitro*. In this paper, we describe the establishment and characterization of this new lung carcinoma cell line continuously producing both G-CSF and PTHrP at the single cell level.

MATERIALS AND METHODS

Patient The patient was a 64-year-old male with poorly differentiated squamous cell carcinoma of the lung. 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 61,400/mm^3 with 98% mature neutrophils, and the serum calcium level increased to 17.3 mg/dl. The serum level of G-CSF was 100 pg/ml (normal <30) and PTHrP was 7.4 p*M* (normal <1.1) (Table I). The patient gradually deteriorated with cachexia, and died 8 months after diagnosis. At autopsy, the main tumor was found in the segment 1 plus 2 of the left lung with metastasis to the right adrenal gland. There was also a tumor involving the right anterior ribs. Immunohistochemical staining showed that the tumor cells were reactive with AE1/AE3 and MA903 antibodies to cytokeratin, EMA antibody to epithelial cell membrane antigen, and anti-G-CSF and anti-PTHrP antibodies (Table II). Bone marrow was hypercellular with marked proliferation of myeloid cells at various stages of maturation. In addition, calcium deposits were found in both kidneys.

Heterotransplantation A tumor sample obtained at autopsy from the anterior chest wall of the patient was finely minced with scissors and forceps. Tissue fragments were removed by passing the mince through a stainless steel mesh. One $\times 10^7$ cells in 1 ml of RPMI 1640 medium were transplanted subcutaneously at the back of 5 BALB/c nude mice at 3–4 weeks of age.

Culture Tumors produced on the back of the nude mice were excised, cut into small pieces, and cultured in 35-

[4] To whom correspondence should be addressed.

mm culture dishes using RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) at 37°C in an incubator, under a 100% humidified 5% CO_2 atmosphere. Cell cloning was performed by the limiting dilution method.

Morphology The nude mice were killed by ether inhalation. 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. Immunohistochemical staining of tumor cells was performed using the following monoclonal antibodies: CAM5.2 (Becton Dickinson, Mountain View, CA), AE1/AE3 (Becton Dickinson), MA902 (Enzo Diagnostics, New York, NY) and MA903 (Enzo Diagnostics) for cytokeratin, EMA (Dakopatts, Glostrup, Denmark) for epithelial cell membrane antigen, and CEA (Bio-Science, Emmenbrücke, Switzerland) for carcinoembryonic antigen. Cytospin preparations of trypsinized cultured cells were stained with May-Grünwald-Giemsa and also immunocytochemically after formalin fixation.

Detection of G-CSF and PTHrP The serum and culture supernatant G-CSF level was examined by enzyme-linked immunosorbent assay and PTHrP was tested by immunoradiometric assay. Formalin-fixed, paraffin-embedded patient's and nude mouse tumors, and formalin-fixed cultured cells were stained with anti-G-CSF (Oncogene Science, Uniondale, NY) and anti-PTHrP (Oncogene Science) antibodies.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR) Total RNA was extracted from the OKa-N-1 and OKa-C-1 cell lines by the standard procedure. One micro gram of the RNA was converted to cDNA with Moloney murine leukemia virus reverse transcriptase in 20 μl of reaction mixture. For quantification, 0.1 to 1.0 μl aliquots of the cDNA samples were subjected to PCR in 50 μl of reaction solution containing 0.5 μM of each specific primer, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTP, and 1 unit *Taq* polymerase. Primers used for G-CSF were 5′-TAGAGCAAGTGAGGAAGATCCAGG-3′ (G-CSF3) for sense and 5′-AGTTCTTCCATCTGCTGCCAGATG-3′ (G-CSF4) for antisense, giving a 328 bp fragment. Primers used for PTHrP were 5′-GCGACGATTCTTCCTTCACC-3′ (PLP1) for sense and 5′-AGAGTCTAACCAGGCAGAGC-3′ (PLP2) for antisense, yielding a 285 bp fragment. Primers used for β-actin were 5′-ACCTTCAACACCCCAGCCATG-3′ (BA3) for sense and 5′-GGCCATCTCTTGCTCGAAGTC-3′ (BA4) for antisense, giving a 309 bp fragment. Reaction was performed for 20 cycles for β-actin and 32 cycles for G-CSF and PTHrP in a DNA thermal cycler (Takara, Ohtsu). The PCR steps included denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and polymerization at 72°C for 1 min.

Table I. WBC, G-CSF, Ca, and PTHrP in Patient and Nude Mice Transplanted with OKa-N-1 and OKa-C-1 Cells

	Patient[a]	Nude mice transplanted with OKa-N-1	Nude mice transplanted with OKa-C-1
WBC (/mm^3)	61,400	316,000[b] (7,000–11,000)	85,500[f]
G-CSF (pg/ml)	100	754[c] (<30)	385[g]
Ca (mg/dl)	17.3	16.6[d] (8.5–9.3)	14.1[h]
PTHrP (pM)	7.4	6.0[e] (<1.1)	4.3[h]

a) The highest values are shown.
b) Mean value of 42 nude mice.
c) Mean value of 4 nude mice.
d) Mean value of 2 nude mice.
e) Mean value of 3 nude mice.
f) Mean value of 6 nude mice.
g) Mean value of 4 nude mice.
h) Mean value of 2 mice.
Numbers in parentheses indicate normal values of BALB/c mice.

Table II. Immunohistochemical Staining of Patient's Tumor Cells and Cell Lines

Antibody	Specificity	Patient's tumor cells	OKa-N-1	OKa-C-1
CAM5.2	Epithelial cells	−	−	−
AE1/AE3	Epithelial cells	+	+	+
MA902	Epithelial cells	−	−	−
MA903	Epithelial cells	+	+	+
EMA	Epithelial cells, plasma cells and some lymphoma cells	+	+	+
CEA	CEA-positive tumor cells	−	−	−
anti-PTHrP	PTHrP-producing cells	+	+	+
anti-G-CSF	G-CSF-producing cells	+	−	−

Amplification cycle numbers were optimized for each sequence using the serial dilution method to achieve a dose-dependent amplification.[19] One micro liter of PCR product was electrophoresed on 2% NuSieve GTG and Seakem GTG agarose gel (FMC BioProducts, Rockland, ME) and stained with ethidium bromide. The intensity of the bands was evaluated using a UV-light box imaging system (Atto, Tokyo).

Chromosome analysis Chromosome analyses of the tumor cells at the 13th nude mouse passage and OKa-C-1 cells at the 8th passage were done using the Giemsa-banding technique.

Effect of dexamethasone on G-CSF and PTHrP production When OKa-C-1 cells reached confluence in culture flasks, 0.001 to 0.1 m*M* dexamethasone was added for 2 days to examine the effect on G-CSF and PTHrP mRNA expression by quantitative RT-PCR. At these concentrations the drugs exerted no deleterious effect, but dexamethasone at more than 1 m*M* induced cell degeneration.

RESULTS

***In vivo* cell line establishment** The patient's cancer cells formed tumors, 2 to 3 cm in diameter, at the site of inoculation in all 5 nude mice after 4 months. These tumor cells were serially transplanted up to the 15th passage over a period of 20 months. Tumor takes occurred in all of 79 nude mice transplanted, and this *in*

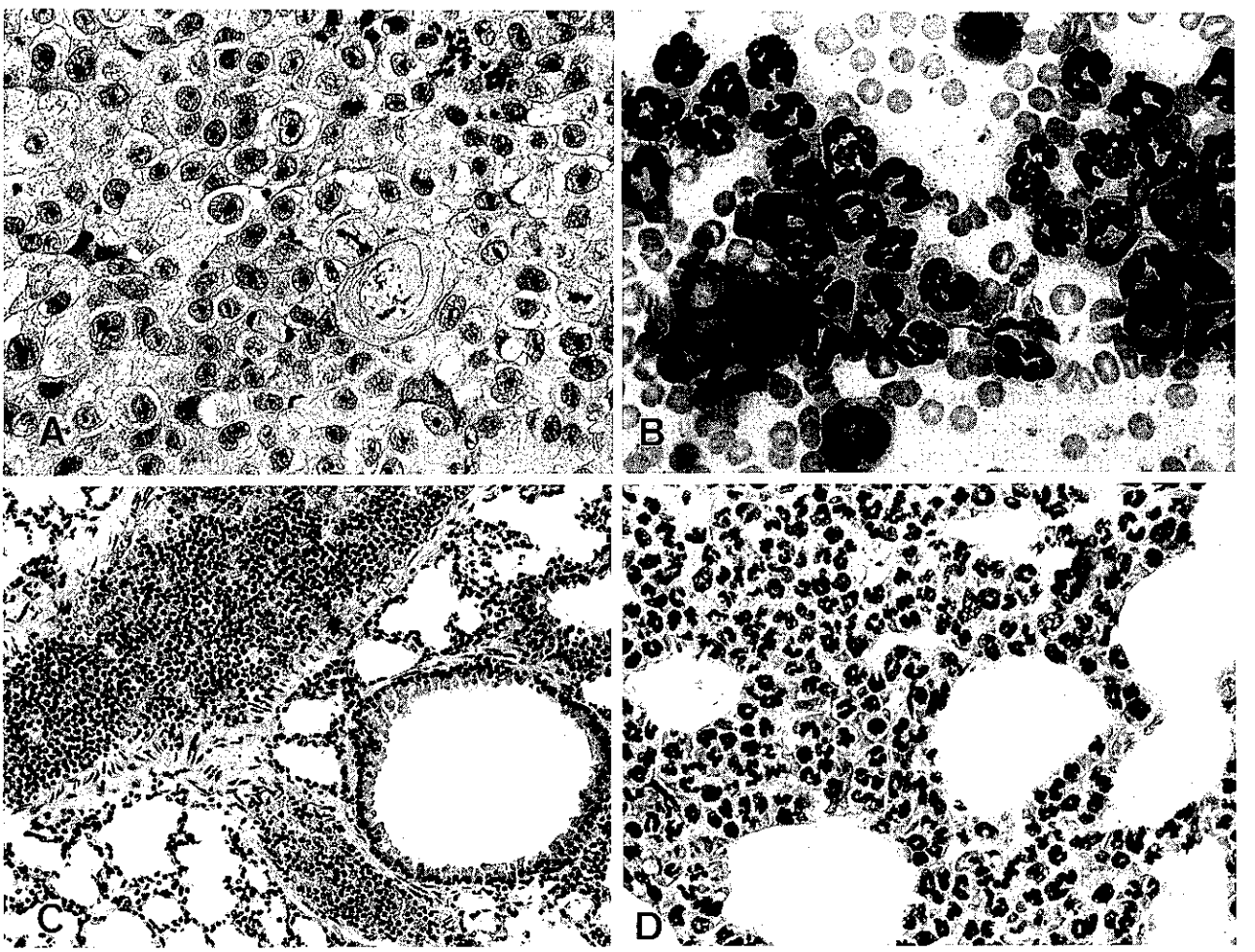

Fig. 1. Nude mouse tumor (OKa-N-1) produced by transplantation of patient's tumor cells, showing neoplastic cells with prominent nucleoli and relatively abundant cytoplasm (A). Hematoxylin and eosin stain (×160). Peripheral blood smear of nude mouse transplanted with OKa-N-1 tumor showing marked increase of mature neutrophils (B). May-Grünwald-Giemsa stain (×250). Lung of nude mouse transplanted with OKa-N-1 tumor showing capillary leukostasis (C) (×50) and alveolar septae packed with mature neutrophils (D). Hematoxylin and eosin stain (×160).

vivo cell line was designated OKa-N-1. Forty-three of 79 nude mice were examined histologically. The histological picture was that of primitive squamous cell carcinoma with little keratinization (Fig. 1A). Formation of a cystic cavity secondary to necrosis was observed. The mean WBC count of tumor-bearing mice of the 1st to 15th passages was 316,000/mm^3 (Table I) and the cells were mature neutrophils (Fig. 1B). Sections of the bone marrow showed marked proliferation of immature and mature neutrophils. The spleen was enlarged and germinal centers became atrophic due to massive proliferation of the myeloid cell series with many megakaryocytes and a few erythroblasts. Focal extramedullary hematopoiesis of immature and mature myeloid cells was found in the liver. The lungs showed marked capillary leukostasis of mature neutrophils in 35 (81%) of 43 mice (Fig. 1, C and D). In two mice, calcification was observed within the tumors and kidneys. The mean serum G-CSF level of tumor-bearing mice of the 9th and 13th passages was 754 pg/ml (Table I). The cystic fluid contained a mean G-CSF level of 383,000 pg/ml. The mean serum calcium level of tumor-bearing mice from passages 11 and 14 was 16.6 mg/dl. The mean PTHrP level examined at the 9th, 11th and 13th passages was 6.0 p*M*. Most of the tumor-bearing mice appeared cachectic. Immunohistochemical staining showed that OKa-N-1 cells were reactive with anti-AE1/AE3, MA 903, EMA and PTHrP antibodies (Table II). The expression of G-CSF and PTHrP mRNA was detected in nude mouse tumor of the 14th passage by quantitative RT-PCR (Fig. 3).

***In vitro* cell line establishment** The first nude mouse transplant was cultured *in vitro* and continuous growth of cells was obtained after 1 month. A clonal cell line, designated OKa-C-1, was established by repeating limiting dilution culture three times. OKa-C-1 grew adherent to the surface of culture flasks, having a cobblestone-like appearance (Fig. 2A), with a doubling time of 95 h. The cells looked anaplastic with prominent nucleoli (Fig. 2B). Immunohistochemical staining showed that OKa-C-1 cells were positive for AE1/AE3, MA 903, EMA,

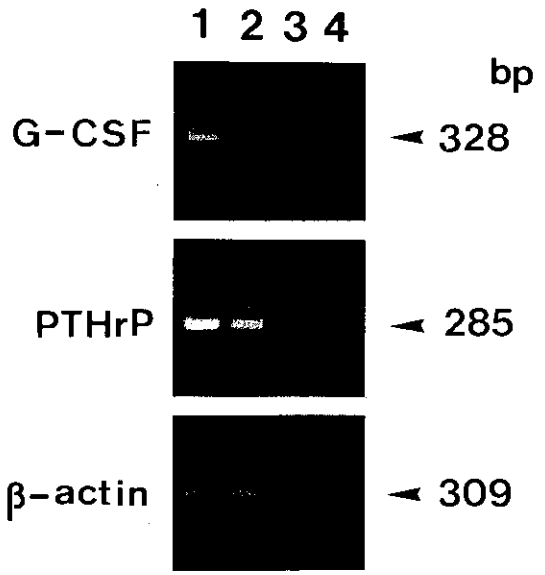

Fig. 3. Detection of G-CSF and PTHrP mRNA by RT-PCR. OKa-N-1 (lane 1), OKa-C-1 (lane 2), and OKa-C-1 treated with 0.1 m*M* dexamethasone (lane 3), and control BALL-2 cell line (lane 4).

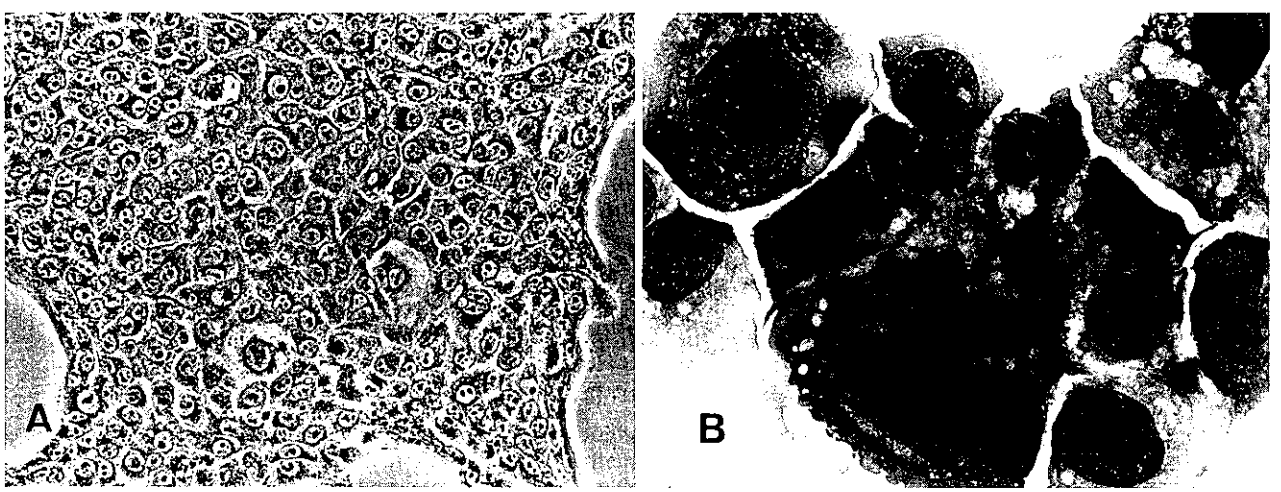

Fig. 2. OKa-C-1 cells growing in sheet-like arrangement (A). Phase contrast microscopy (×50). Cytospin smear of OKa-C-1 cells showing cancer cells of different sizes (B). May-Grünwald-Giemsa stain (×400).

and PTHrP (Table II). Inoculation of 10^7 OKa-C-1 cells from passages 12 and 13 into seven nude mice resulted in the growth of tumors after 2 months. These tumors had histological features identical to those of OKa-N-1 tumors. The mean WBC count of the tumor-bearing mice was 85,500/mm^3 with 96% mature neutrophils, as well as massive proliferation of mature and immature neutrophils in the bone marrow and spleen. Focal extramedullary hematopoiesis was also found in the liver. The mean serum level of G-CSF was 385 pg/ml, calcium 14.1 mg/dl, and PTHrP 4.3 p*M* in tumor-bearing mice (Table I). The culture supernatant of the OKa-C-1 cell line of the 8th passage contained 165 pg/ml of G-CSF and 161 p*M* PTHrP. The supernatant of cells from the 15th passage contained 266 pg/ml of G-CSF and 256 p*M* PTHrP. The G-CSF and PTHrP transcripts were detected by quantitative RT-PCR in this cell line from the 15th passage (Fig. 3).

Chromosome analysis Although the patient's original tumor cells were not examined, OKa-N-1 from the 13th passage and OKa-C-1 of the 8th passage were shown to have modal chromosome numbers of 75 and 78 with complex structural abnormalities, respectively. No abnormality was found in chromosomes 12p and 17q, where the PTHrP and G-CSF genes are located.

Dexamethasone effect Dexamethasone at various concentrations (0.001–0.1 m*M*) was added to the OKa-C-1 cell cultures of the 15th and 16th passages. The expression of G-CSF and PTHrP mRNA was found to be inhibited by 0.1 m*M* dexamethasone (Fig. 3).

DISCUSSION

Leukocytosis and hypercalcemia caused by G-CSF and PTHrP production are observed simultaneously in patients with squamous cell carcinoma and an aggressive clinical course.[1, 3–5, 9] G-CSF is one of the hematopoietic growth factors that plays an important role in the proliferation and differentiation of granulocytes.[20–22] PTHrP was originally characterized as a peptide responsible for the humoral hypercalcemia of malignancy syndrome.[12, 13, 23, 24] Thus, these two substances are distinct in their biological activities. Simultaneous and unrestricted production of G-CSF and PTHrP in a lung cancer patient posed an interesting question as to whether a single cancer cell produces both or not.

We transplanted the patient's tumor cells into nude mice and maintained them by serial transplantation. The tumor-bearing mice manifested leukocytosis and hypercalcemia along with high serum levels of G-CSF and PTHrP. Although bone metastases were found in our patient, the xenografts grew only locally, excluding the possibility of osteolytic origin of hypercalcemia. We then attempted to grow the nude mouse tumor cells *in vitro* and a clonal culture line, OKa-C-1, was successfully established by the limiting dilution method. Supernatant of the OKa-C-1 cell line was shown to contain high levels of both G-CSF and PTHrP. Immunohistochemical study showed that OKa-C-1 cells were positive for PTHrP. Quantitative RT-PCR analysis confirmed the G-CSF and PTHrP mRNA expression in this cell line. Furthermore, nude mice transplanted with OKa-C-1 cells likewise showed marked leukocytosis and hypercalcemia, as well as high serum levels of G-CSF and PTHrP. Although several cell lines have been established from patients with high leukocyte count and hypercalcemia,[3, 5, 25] an *in vitro* clonal cell line that produces both G-CSF and PTHrP simultaneously is unusual.[5] Our OKa-C-1 cell line should be useful in studies of the mechanism of the simultaneous production of G-CSF and PTHrP.

Using the quantitative RT-PCR method, we demonstrated the expression of G-CSF and PTHrP mRNA in both OKa-N-1 and OKa-C-1 cell lines. Ikeda *et al.*[26] reported that a corticosteroid hormone inhibited the expression of mRNA of PTHrP. On the other hand, Merryman *et al.*[27] reported that it stimulated the production of PTHrP by a squamous cell carcinoma cell line. In our study, the expression of both G-CSF and PTHrP mRNA in the OKa-C-1 cell line was suppressed by dexamethasone treatment at a dose that did not inhibit the growth of the cell line. Glucocorticoids are used for the treatment of patients with hypercalcemia.[28] They are considered to decrease serum calcium levels by inhibiting bone resorption. Our data suggest that, in addition to this action, glucocorticoids lower the calcium level by suppressing PTHrP mRNA expression of tumor cells.

Most of the tumors associated with leukocytosis and hypercalcemia are squamous cell carcinoma. The mechanisms underlying the increased production of G-CSF and PTHrP from cancer cells are not clear. PTHrP is produced by normal keratinocytes.[29, 30] Granulocyte-macrophage colony-stimulating factor is also produced by keratinocytes.[31] G-CSF is known to be produced by nonhematopoietic cells such as vascular endothelial cells, fibroblasts, certain epithelial cells and mesothelial cells.[22, 32, 33] Keratinocytes may also have a potential to produce G-CSF as well. Normal human fibroblasts transfected with *ras* oncogene were shown to express a high level of G-CSF mRNA.[33] Recently, increased PTHrP gene expression was also demonstrated in cells transformed by *ras* oncogene.[34] Overproduction of G-CSF and PTHrP in our case is postulated to have been the result of multiple oncogene mutations, including *ras*. The gene encoding G-CSF is located in the long arm of chromosome 17[35] and PTHrP in the short arm of chromosome 12.[13] There were no gross abnormalities in these chromosomes in our cell line. It is likely, therefore, that a common, *trans*-acting regulatory mechanism of G-CSF

and PTHrP genes was perturbed during oncogenesis, resulting in coordinate expression. The results of the dexamethasone study support this hypothesis.

In addition to extramedullary hematopoiesis, marked leukostasis of mature neutrophils was present in the lungs of our tumor-bearing mice. Similar findings were observed in mice transplanted with G-CSF-producing tumors or inoculated with G-CSF.[15, 36, 37] Clinically, pulmonary leukostasis was reported in association with leukocytosis in leukemias, excessive G-CSF administration, and retinoic acid syndrome.[38-44] Whether this is due to abnormal numbers and/or functions of neutrophils remains to be studied.

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